Cerebrospinal fluid biomarkers for stage determination and treatment outcome evaluation in patients affected by Human African trypanosomiasis

Tiberti, Natalia

UNIVERSITÉ DE GENÈVE
Section des Sciences Pharmaceutiques

FACULTÉ DES SCIENCES
Prof. Leonardo Scapozza

Département des Sciences des Protéines Humaines

FACULTÉ DE MÉDECINE
Prof. Jean-Charles Sanchez

Cerebrospinal Fluid Biomarkers for Stage Determination and Treatment Outcome Evaluation in Patients Affected by Human African Trypanosomiasis

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Natalia Tiberti
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SUMMARY

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease affecting rural communities in sub-Saharan Africa. The disease is caused by *Trypanosoma brucei* parasites, which are transmitted to humans through the bite of a tsetse fly. The two subspecies *T. b. gambiense* and *T. b. rhodesiense* are responsible for two clinically different forms of HAT. *T. b. gambiense*, widespread in West and central Africa, causes a chronic infection and accounts for 90% of all HAT reported cases, while *T. b. rhodesiense* causes an acute disease in East Africa and it is responsible for approximately 5% of reported cases.

In both cases the disease progresses from a first heamolymphatic stage (stage 1, S1), characterized by the presence of parasites in patients' blood and lymph, to a second meningo-encephalitic stage when the parasites are assumed to invade the central nervous system (CNS). Late stage patients can present extensive neurological disorders and need to be treated with drugs able to cross the blood-brain barrier (BBB). These drugs can be highly toxic (i.e. melarsoprol) or associated to logistic constrains (i.e. eflornithine), therefore the exposure of S1 patients to these treatments should be avoided, since they can be safely treated with pentamidine or suramin.

A patient is classified and treated as S2 after demonstration of the presence of parasites in the cerebrospinal fluid (CSF) and/or a counting of white blood cells (WBC) in CSF > 5/μL. However, current staging methods are unsatisfactory due to limited sensitivity, specificity or reproducibility, and not considered as a gold standard. Furthermore, due to a reduced efficacy of some S2 drugs observed during the last years, patients cannot be considered immediately cured but need to be followed after treatment to detect relapses. HAT follow-up consists in the finding of parasites in blood and CSF and in counting of CSF WBC each 6 months for two years. However, specific WHO guidelines to diagnose relapses are missing and the applied tools suffer from the same limitations highlighted for the staging. The need of alternative tools to WBC counting for both stage determination and treatment outcome evaluation is thus urgent and necessary to achieve a more reliable management of patients suffering from HAT.

The aim of the present study was to identify and validate staging markers for the stratification of HAT patients before the treatment and to predict the outcome during the follow-up. By using complementary discovery approaches and integrating data published in the literature, we highlighted 8 molecules, IgM, MMP-9, ICAM-1, VCAM-1, CXCL10, CXCL13, neopterin and B2MG, as the most promising *T. b. gambiense* staging markers. Through validation on a large multicenter cohort, we showed the high staging accuracy of neopterin (84% specificity and 100% sensitivity) for *T. b. gambiense* HAT and its potential for replacing the counting of WBC. To extend the utility of these markers, they were also evaluated for the treatment outcome prediction in patients suffering from *T. b. gambiense* and for staging *T. b. rhodesiense*. Interestingly, neopterin showed high accuracy in detecting relapses after treatment in *T. b. gambiense* patients and in shortening their follow-up as soon as 6 months (87% specificity and 92% sensitivity) or 12 months after treatment (97% specificity and 94% sensitivity). However, this marker did not show the same staging power when assessed on *T. b. rhodesiense* patients. For this form of HAT we highlighted IgM, MMP9 and CXCL13 as new promising staging markers, individually or combined into panels of three molecules with CXCL10.

In conclusion, we propose neopterin as a new alternative to the counting of the WBC to ameliorate the stage determination and the evaluation of the outcome after treatment for patients suffering from *T. b. gambiense* sleeping sickness. Its introduction on the field as a rapid test might have a major impact on both patients' management and disease control. Furthermore, our preliminary study on *T. b. rhodesiense* HAT suggests further investigations on this disease form to find new staging markers and to increase the knowledge on the physiopathology of HAT.

RÉSUMÉ

La trypanosomiase humaine Africaine (THA), ou maladie du sommeil, est une maladie tropicale négligée qui atteint les communautés rurales de l'Afrique sub-saharienne. La maladie est provoquée par un parasite, le *Trypanosoma brucei*, qui est transmit à l'homme par la mouche tsétsé. En Afrique il existe deux sous-espèces de ce parasite, *T. b. gambiense* et *T. b. rhodesiense,* qui sont responsables des deux formes différentes de la maladie. La première, *T. b. gambiense,* induit la majorité des cas reportés (> 90%) et provoque une maladie chronique en Afrique centrale et de l'ouest. Le parasite *T. b. rhodesiense*, quant à lui, est responsable d'une maladie aigüe répandue en Afrique de l'est qui correspond approximativement à 5% des cas reportés.

Dans les deux cas, la maladie évolue en deux stades. Le premier, le stade 1 (S1), se caractérise par la présence de parasites dans les compartiments sanguin et lymphatique. Le deuxième, le stade neuro-encéphalique ou stade 2 (S2), se caractérise par le passage du parasite au travers de la barrière hémato-encéphalique et par l'invasion du système nerveux central. Les patients au stade 2 peuvent présenter des signes et symptômes neurologiques étendus et doivent obligatoirement être soignés avec des médicaments capables de traverser la barrière hémato-encéphalique. Toutefois, ces médicaments peuvent être très toxiques, comme le melarsoprol, ou très compliqués à administrer, notamment l'eflornithine, et l'exposition de patients S1 à ces molécules doit donc être évitée.

Le classement des patients en stade 2 se fait sur la base de la détection des parasites dans le liquide céphalorachidien (LCR) et/ou d'un comptage des globules blancs (GB) dans le LCR supérieur à 5 cellules/µL. Ces méthodes de détermination du stade ne sont pas satisfaisantes puisqu'elles ont une sensibilité, spécificité ou reproductibilité limitée. De plus, les patients souffrants de la THA ne peuvent pas être considérés guéris immédiatement après le traitement, puisqu'ils peuvent subir une rechute de la maladie. Un suivi post-thérapeutique est donc nécessaire. Ce suivi est réalisé par des visites qui s'effectuent tous le 6 mois pendant deux ans. Les patients sont alors testés pour la présence de parasites dans le sang et dans le LCR et les globules blancs sont comptés dans le LCR. Toutefois, dans l'état actuel des choses, il n'existe pas de recommandations spécifiques de l'OMS pour diagnostiquer des rechutes, et les méthodes actuellement utilisées souffrent des mêmes

limitations que celles précédemment décrites pour la détermination du stade. Des alternatives au comptage des GB sont donc réellement nécessaires pour aboutir à une meilleure gestion des patients souffrants de la maladie du sommeil.

L'étude présentée ici a pour objectif d'identifier et de valider de nouveaux marqueurs pour la détermination du stade et l'évaluation du résultat post-thérapeutique pour la THA. Grâce à une approche de découverte multiple et à l'intégration des données bibliographiques, 8 molécules (IgM, MMP-9, ICAM-1, VCAM-1, CXCL10, CXCL13, néoptérine and B2MG) ont été sélectionnées comme les marqueurs les plus prometteurs pour la détermination du stade des patients souffrant de THA à *T. b. gambiense.* Lors de la validation de ces marqueurs sur une plus grande population, la néoptérine était la molécule la plus exacte pour la détermination du stade (84% spécificité, 100% sensitivité) et la plus prometteuse pour le remplacement du comptage des GB. Afin d'élargir leur utilité, ces marqueurs ont aussi été évalués pour le résultat du suivi post-thérapeutique des patients souffrant de la maladie à *T. b. gambiense*, ainsi que pour la détermination du stade des patients affectés par la forme *rhodesiense*. Dans le premier de ces deux cas, la néoptérine a montré une exactitude élevée pour la détection des rechutes et la réduction du suivi à 6 mois (87% spécificité et 92% sensibilité) ou à 12 mois (97% spécificité et 94% sensitivité) après le traitement. Toutefois, ce même marqueur n'était pas aussi efficace pour la détermination du stade pour la maladie à *T. b. rhodesiense*. Pour cette forme de THA, les molécules IgM, MMP-9 et CXCL13, individuellement ou en combinaison avec CXCL10, étaient les meilleurs marqueurs du stade.

Dans l'étude présentée dans ce manuscrit, nous proposons la néoptérine comme une nouvelle alternative au comptage des GB pour deux applications : la détermination du stade et du résultat après le traitement pour les patients souffrant de la THA à *T. b. gambiense*. L'introduction de ce marqueur sur le terrain sous forme d'un test rapide pourrait améliorer significativement la gestion des patients et le contrôle de la maladie. De plus, les résultats préliminaires obtenus pour la THA à *T. b. rhodesiense* peuvent représenter une base pour des recherches ultérieures sur la détermination du stade ainsi que pour la compréhension de la physiopathologie de la maladie du sommeil.

ABBREVIATIONS

HAT: human African Trypanosomiasis
T. b.: *Trypanosoma brucei*
SRA: serum resistance associated
WHO: World Health Organization
NGO: non-governmental organization
DALY: disability adjusted life year
S1: stage 1
S2: stage 2
CNS: central nervous system
SOREMP: sleep onset REM periods
CATT: card agglutination test for trypanosomiasis
CSF: cerebrospinal fluid
mHCT: microhematocrit centrifugation
QBC: quantitative buffy coat
mAECT: mini-anion exchange centrifugation
LOD: limit of detection
SE: sensitivity
LAMP: loop-mediated isothermal amplification
WBC: white blood cells
BBB: blood-brain barrier
PTRE: post-treatment reactive encephalopathy
NECT: nifurtimox-eflornithine combination therapy
VSG: variant surface glycoprotein
GPI: glycosylphosphatidyl-inositol
MHC II: major histocompatibility complex II
IFN-γ: interferon gamma
TNF-α: tumor necrosis factor alpha
NO: nitric oxide
IgM: immunoglobulin M
IL-: interleukin-
MΦ: macrophage
NIH: National Institutes of Health
LDL-C: low density lipoprotein cholesterol
PSA: prostate specific antigen
TP: true positive
TN: true negative
FP: false positive
FN: false negative
ACC: accuracy
SP: specificity
SE: sensitivity
PPV: positive predictive value
NPV: negative predictive value
LR+: positive likelihood ratio
LR-: negative likelihood ratio
Q_{IgG}: quotient immunoglobulin G
Q_{Alb}: quotient albumin
IgG_{IF}: intrathecal fraction of immunoglobulin G
TMT: tandem mass tag
ELISA: enzyme-linked immunosorbent assay
2-DE: two dimensional gel electrophoresis
DRC: Democratic Republic of the Congo
ROC curve: receiver operating characteristic curve
AUC: Area under the curve
CI: confidence interval
POCT: point of care test
TOC: test of cure
FIND: foundation for innovative new diagnostics
ITM: Institute of Tropical Medicine (Antwerp)
SRM: selected reaction monitoring

CHAPTER 1

Introduction

1. Human African trypanosomiasis (HAT)

1.1 Overview on HAT

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a disease endemic in sub-Saharan Africa affecting mainly rural communities.[1] The first reports on this disease date to the 18th century, when the naval surgeon John Atkins described the illness in West Africa.[2] During the 20th century, the discoveries made by a number of investigators led to the characterization of the disease, its causing agent and its vector (Table 1).

PRINCIPAL INVESTIGATORS	FINDING
T.M. Winterbottom, 1792	Described a human illness associated with a cervical lymphoadenopathy and lethargy
Livingstone, 1847	Identified the tsetse fly as the vector of an illness affecting domestic animals called *Nagana*
Bruce, 1894	Identified trypanosome parasites as the cause of *Nagana*
Dutton and Todd, 1902	Described trypanosomes as the causing agent of the illness described by Winterbottom in West Africa. They named the disease "Trypanosome fever" and the parasite "*Trypanosoma gambiense*"
Castellani, beginning of 20th century	Detected the presence of trypanosomes in patients' cerebrospinal fluid
Bruce, beginning of 20th century	Demonstrated that the tsetse fly is responsible for the transmission of the disease to humans
Stephens and Fantham, 1910	Discovered the *Trypanosoma rhodesiense* parasite in East Africa
Kinghorn and Yorke, 1911	Demonstrated the transmission of *Trypanosoma rhodesiense* to humans

Table 1 Summary of the most important findings leading to the characterization of human African trypanosomiasis.[2,3]

Thanks to the improvements in medical and parasitological tools, we know today that sleeping sickness is a focal disease caused by an extracellular protozoa belonging to the genus *Trypanosoma* that proliferates in body fluids.[4]

Two subspecies of this parasite are responsible for the human form of sleeping sickness: *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense.* The letter parasite is also responsible for the animal form of the disease called *Nagana.*[5] Animals represent reservoirs for the disease and play an important role in its transmission. These parasites are specifically transmitted to mammal hosts by tsetse flies of the genus *Glossina,* widespread in sub-Saharan

Africa. The geographical distribution of the tsetse fly determines the geographical distribution of the disease within the so-called tsetse belt (Figure 1A).[4]

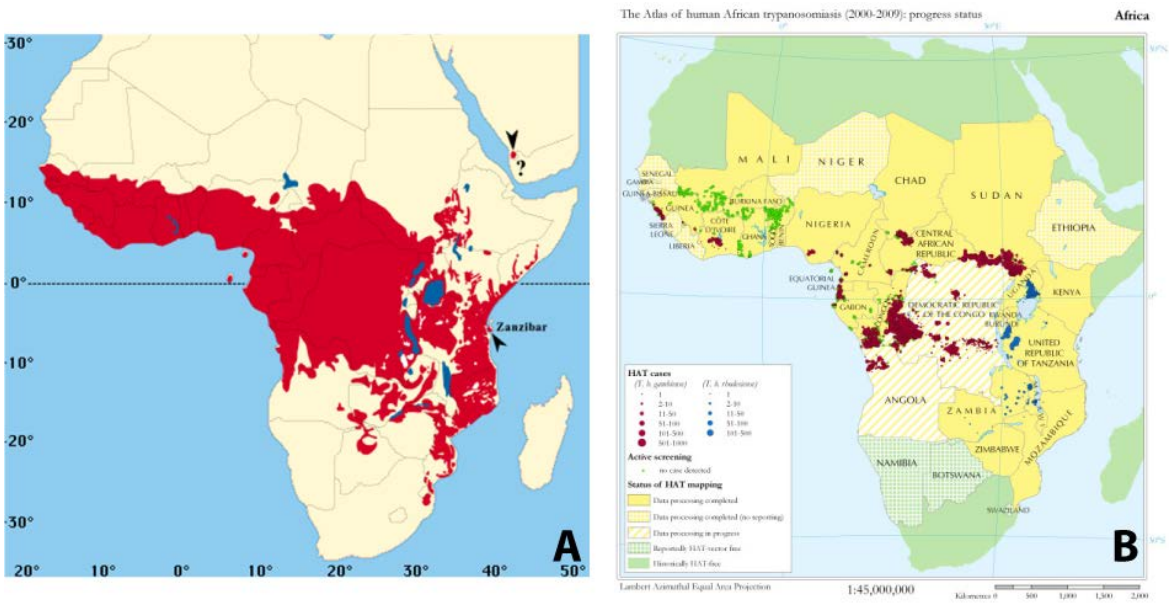

Figure 1 A) Geographical distribution of the tsetse fly within the tsetse belt; **B)** Representation of countries endemic for HAT, and the focal distribution of HAT; from Simarro *et al.*[6]

T. b. gambiense and *T. b. rhodesiense* are the only two sub-species of trypanosome able to cause the human illness.[4] The ability of these parasites in infecting humans has been attributed to their resistance to the trypanosome lytic factor present in human blood, to which the other species of parasite diffused in Africa, such as *T. b. brucei, T. vivax* and *T. congolense,* are instead sensitive.[7,8] The SRA gene, coding for the Serum Resistance Associated protein, has been identified as the resistance factor in *T. b. rhodesiense,*[9,10] while the mechanism of resistance of *T. b. gambiense* is still unknown.

The life cycle of *gambiense* and *rhodesiense* parasites is characterized by a cyclic transmission between the fly and the mammal host. A fly becomes infected when it takes a blood meal from a subject carrying the trypomastigote form of the parasite. Once in the fly gut, the parasite starts a development cycle which lasts for 3-4 weeks and ends with the generation of

metacyclic trypanosomes in the salivary gland. This metacyclic form is infective when transmitted to the host, where it starts multiplying by binary fission (Figure 2).[5] A fly carrying the parasite remains infective for its whole life, however, only 0.1% of the flies carry mature parasites infective for humans and cattle.[1]

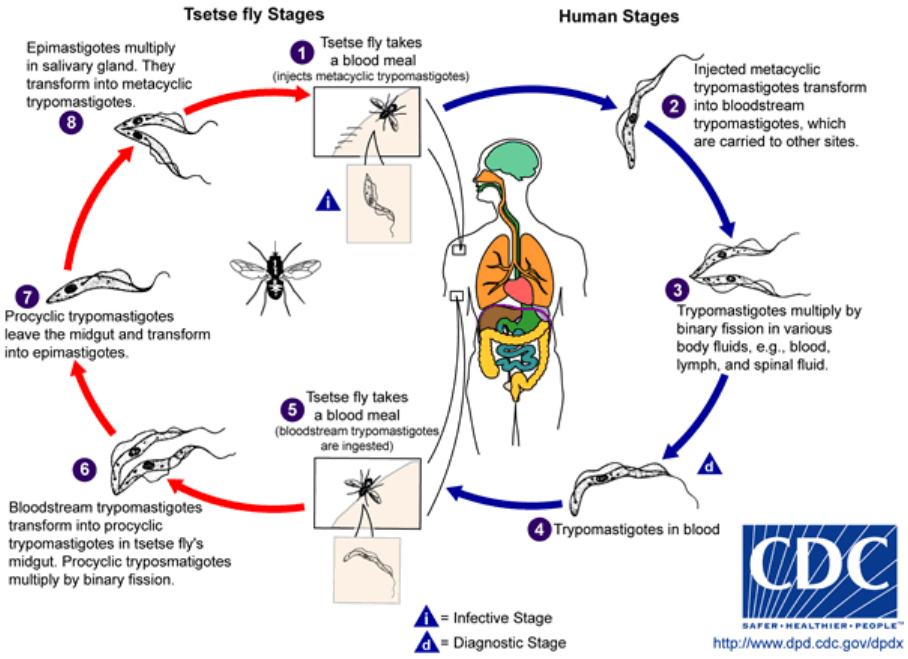

Figure 2 Life cycle of African trypanosomes in the tsetse fly and in the human host
From: http://www.dpd.cdc.gov/dpdx

1.2 Epidemiology

Sleeping sickness is endemic in 200 known foci in 36 countries of the sub-Saharan Africa[3] (Figure 1B). According to the last reports, less then 10'000 new cases have been reported in 2009.[4] *Trypanosoma brucei gambiense* is responsible for a chronic infection in 24 countries in Western and central Africa[11] accounting for more than the 90% of all HAT reported cases. On the other hand, *Trypanosoma brucei rhodesiense* causes an acute infection in Eastern Africa and it is responsible for approximately 5% of reported cases.[12] The two forms of parasite are

geographically separated by a thin line passing through Uganda, which is the only country where both forms are present.

Historically, the prevalence of the disease followed waves of epidemic according mainly to the socio-political stability of the affected countries. After the last epidemic during the 90's, WHO, together with NGOs, adopted a number of resolutions leading to a reduction of 69% and 21% of *gambiense* and *rhodesiense* reported cases during the period 1997-2006, respectively.[11]

Currently, the disease is considered to be under control, with 19 of the 36 endemic countries registering no new cases in 2009[12] and a total number of reported cases of 7'139 in 2010 (http://apps.who.int/neglected_diseases/ntddata/hat/hat.html). The real number of people suffering from sleeping sickness is, however, considered to be approximately three times higher.[13] Compared to other diseases endemic in Africa such as malaria, tuberculosis and HIV, the incidence of HAT is low and places it in a group of diseases called Neglected Tropical Diseases (NTD).[14] However, considering the DALY (Disability Adjusted Life Year) index, an estimator of the years of healthy life lost by premature mortality or disability due to a specific disease, HAT is one of the most disabling neglected tropical diseases. According to the WHO Global Burden of Disease (GBD) study, in 2004 the DALYs lost due to HAT were considered of 1'609'041.[15,16]

1.3 Clinical presentation and disease progression

Sleeping sickness typically progresses through two stages. The first stage (stage 1, S1), or heamolymphatic stage, occurs after an incubation period that can vary from 1 to 3 weeks after the bite of the tsetse fly. This stage is characterized by the proliferation of parasites in the bloodstream and the lymphatic system. If stage 1 patients are not properly treated, the disease progresses to the second stage (stage 2, S2) or meningo-encephalitic stage, due to the invasion of the central nervous system (CNS).[17] The disease is considered to be fatal if left untreated.[18]

The speed of progression from the first to the second stage varies according to the form of the infecting parasite: for *T. b. gambiense*, stage one can last for months or years before to evolve

to the second stage, while in the case of *T. b. rhodesiense* the evolution to the second stage occurs within few weeks after infection.[18]

The clinical manifestations of the two forms of HAT are different. A common feature is the unspecific clinical symptoms and signs presented by early stage patients, including general malaise, fever, headache, pruritus, oedema, splenomegaly, hepatomegaly and weight loss.[19] Often, stage 1 disease can mimic other illnesses endemic in the same regions, such as malaria and HIV, which can even coexist in patients affected by sleeping sickness.[5] Two signs can be indicative of stage 1 disease: the Winterbottom's sign (i.e. cervical lymphoadenopathy) for the *gambiense* form, and the inoculation chancre for the *rhodesiense* one.[4] However, both these clinical signs have limited utility, since they rapidly disappear after infection.

As the disease evolves into the second stage, the clinical symptoms and signs become more specific with appearance of neurological disorders of different entity as a consequence of the meningo-encephalitis.[18] These neurological disorders are more evident in patients affected by the *gambiense* form.[19] One of the most characteristic neurological complications of sleeping sickness, which gives the disease its name, is the dysfunction of the sleep-wake cycle, with daytime somnolence and an alteration of the normal sleep structures with the appearance of "SOREMPs" (sleep onset REM periods) events.[20] The most common clinical features of late stage sleeping sickness caused by *gambiense* and *rhodesiense* parasites are summarized in Table 2.

S2 clinical presentation	*T. b. gambiense*	*T. b. rhodesiense*
Entity of neurological involvement	Extensive	Mild
Onset of neurological manifestations	From several months to two years after infection	Few weeks after infection
Most important neurological manifestations	- Constant headache - Impaired motor functions - Psychiatric changes - Pruritus - Weight loss - Wasting - Splenomegaly and hepatomegaly - Adenopathy - Sleep disorders	- Tremors - Anaemia - Thrombocytopenia - Abnormal hepatic functions - Cardiac abnormalities - Sleep disorders
Main cause of death	Bacterial infections	Cardiac complications
Time interval between infection and death	From months to years	From 6 to 9 months

Table 2 Principal features of the clinical presentation of late stage HAT caused by *T. b. gambiense* and *T. b. rhodesiense*.[2,5,19]

1.4 Diagnosis and current clinical practice

The diagnostic workflow for *T. b. gambiense* HAT patients consists in three steps: i) serological screening, ii) parasitological confirmation and iii) staging. The diagnostic workflow for patients suffering from *T. b. rhodesiense* HAT consists only in parasitological examination and staging.

The serological screening of *gambiense* HAT is performed using the CATT (Card Agglutination Test for Trypanosomiasis), a test for the detection of antibodies against the parasite present in patients' blood. This rapid and chip test has been introduced in 1978[21], however it is not effective for the detection of *T. b. rhodesiense* HAT. Despite its high specificity (95%) and sensitivity (87-98%) in detecting patients carrying parasites in their blood, its accuracy is not sufficient to introduce it as a diagnostic gold standard[5]. Furthermore, it has been reported a decreased accuracy of the CATT in some foci where particular strains of trypanosomes are present.[22] Thus the diagnostic confirmation of both *T. b. gambiense* and *T. b. rhodesiense* is still based on the parasitological detection by microscopy of trypanosomes in blood, lymph or cerebrospinal fluid (CSF). A summary of the most used parasitological techniques and their limit of detection is reported in Table 3.

Technique	Principle	LOD (nb tryps/mL)	SE%	Notes
LYMPH NODE ASPIRATE	Detection by microscopy of motile trypanosomes in fresh preparation.	50-100	40-80	Performed only when enlarged cervical lymph nodes are present.
WET BLOOD FILM	Examination by microscopy of finger prick blood. Easy and cheap.	6000-10000	20	Not very useful for the detection of *T. b. gambiense* due to the low parasitemia.
THICK BLOOD FILM	Examination by microscopy of Geisma stained blood. Cheap and widely used on the field.	600-5000	40	The SE can be increased by repeating the examination. It allows visualizing also other parasites.
MICROHEMATOCRIT CENTRIFUGATION (mHCT)	Centrifugation of finger prick blood in capillaries containing anticoagulant. Parasites are visualized by microscopy at the buffy coat. For each patient 6-8 capillaries are examined. Cheap and used by mobile teams.	500-600	50	The presence of microfilaria interferes with the test.
QUANTITATIVE BUFFY COAT (QBC)	Centrifugation of blood in capillary containing acridine orange and heparin to easily visualize trypanosomes under UV light.	15-300	80	Requires relatively sophisticated material, it can detect *plasmodium.* The capillaries are not produced anymore.
MINI-ANION EXCHANGE CENTRIFUGATION (mAECT)	Separation and concentration of trypanosomes from venous blood by anion exchange chromatography. Parasites are then visualized by microscopy.	15-100	80-90	Time consuming and expensive. Specific for trypanosomes.

Table 3 Description of the methods currently used for the parasitological detection of trypanosomes in blood and/or lymph.[5] LOD: limit of detection; SE%: sensitivity%.

Current diagnostic tools have, however, some limitations, and it has been reported that 20-30% of *T. b. gambiense* patients could be missed at diagnosis as a consequence of a low parasitemia.[5,23,24]

Alternatives to the current serological and parasitological tools have been proposed, such as the Latex/Tbg,[25,26] immunoassays,[27] PCR for the amplification of specific DNA or RNA sequences of *gambiense* or *rhodesiense* parasites[22,28,29] or proteomic fingerprint.[30,31] However, the applicability of these approaches was hampered either by a lower accuracy compared to actual tools, or by a limited possibility of being used as field test. Recently a new isothermal PCR, called LAMP, has been developed and seems to be the most promising alternative to the classical parasitological examination.[32,33]

All patients having a positive parasitological examination need to undergo a lumbar puncture for stage determination in order to determine the treatment to be administered. As recommended by WHO,[34] stage determination is based on the counting of leukocytes, the finding of parasites and the measurement of the protein concentration in CSF. Due to the low reproducibility and the controversy concerning the recommended cut-off, protein concentration is no more performed.[19]

Late stage HAT is defined by CSF white blood cells (WBC) > 5 cells/µL and/or parasites detected in patients' CSF. Both examinations should be performed soon after the lumbar puncture due to the low stability and rapid lyses of leukocytes and trypanosomes in CSF. In case of high CSF parasitemia, trypanosomes can be visualized directly in the counting chamber during the WBC counting by microscopy. However, more frequently, parasites need to be concentrated using the double centrifugation[35] or the modified single centrifugation techniques[36] to increase the sensitivity.

Despite WHO recommendations, a gold standard for stage determination is still missing as current staging tools present a number of limitations.[37] The detection of parasites in CSF is not sensitive enough to be used as a unique staging method, while the counting of the leukocytes has limited specificity and reproducibility.[11,38,39] Furthermore, the cut-off of 5 WBC/µL of CSF is not universally accepted and some countries apply a cut-off for staging at 10 or 20 cells/µL.[38] This led to the introduction of a third "intermediate stage" or "early-late stage", represented by patients having WBC between 5 and 20/µL of CSF and no parasite detected. These patients have, in fact, been reported to be potentially cured with stage 1 drugs.[40,41]

1.5 Treatment and post-therapeutic follow-up

The choice of the treatment for HAT patients strictly depends on the form of the infecting parasite and on the stage of the disease. This aspect underlines the importance of a correct stratification: stage 2 patients need to be treated with drugs able to cross the blood-brain barrier

(BBB) and to diffuse in the central nervous system, but, since these drugs can be highly toxic, the exposure of S1 patients to them should be limited. Currently, stage 1 patients can be treated with pentamidine (*T. b. gambiense*) or with suramin (*T. b. rhodesiense*); both these drugs are relatively safe and cause only reversible side effects.[42]

Interestingly, low levels of pentamidine have been detected in patients' CSF, so that it has been proposed for the treatment of intermediate patients.[40] However this is not recommended as a routine clinical practice.

Till few years ago, the treatment of late stage patients was, based on melarsoprol, an arsenic derivate compound effective in treating both *gambiense* and *rhodesiense* diseases. This drug is associated to severe, and sometimes fatal, side effects. The most important one is represented by a post-treatment reactive encephalopathy (PTRE) that occurs in 4.7% of *gambiense* patients and in 8% of *rhodesiense* patients, and causes the death in 44% and 57% of them, respectively.[42] Furthermore, an increased relapse rate after melarsoprol treatment has been observed during the last years in some foci,[42,43] probably due to the development of resistant parasite strains.[44]

Unlikely, melarsoprol is the only treatment choice available for *T. b. rhodesiense* late stage patients; while *T. b. gambiense* S2 patients can be treated with eflornithine or NECT (nufurtimox-eflornithine combination therapy).[12,45]

Eflornithine has been introduced as first line treatment for S2 *gambiense* HAT after the demonstration of its efficacy and its lower toxicity compared to melarsoprol.[46] However, this drug presents some limitations, mainly associated to a complicated administration, high costs and logistic constrains. Furthermore, adverse drug reactions are common after treatment with eflornithine, and a high risk of development of resistance to this drug exists.[42] The combination of eflornithine with the oral drug nifurtimox as NECT should be preferred. NECT has the advantage of reducing the amount of eflornithine to be administered, thus reducing costs, and of decreasing the risk of development of resistant strains.[42,47]

Even so, none of these drugs represents an optimal treatment choice, and investigations to find new therapies able to safely treat both stage 1 and stage 2 patients or to ameliorate melarsoprol therapy for *rhodesiense* patients are ongoing.[48-50]

After treatment, patients cannot be considered immediately cured as relapses can occur (mainly for late stage cases). To early detect treatment failures or to confirm cure, HAT patients need to be followed for two years after treatment. The follow-up consists in the examination of blood and CSF for the presence of parasites, and in the counting of CSF WBC. These investigations are performed at the end of the treatment and then they are repeated each 6 months till the accomplishment of a follow-up of two years.[51]

According to WHO,[51] a relapse is diagnosed following the detection of trypanosomes in any body fluid at any time of follow-up. Patients without detected parasites, but having WBC ≥ 20/µL in CSF at any time of follow-up are classified as probable relapse. Both relapses and probable relapses are considered as treatment failures and should be re-treated. However, this does not reflect the current clinical practice and different criteria have been proposed and applied on the field.[51,52] A major drawback in the current practice for the detection of treatment failures is represented by the lack of a generally accepted cut-off for the WBC counting. Two important studies have recently been published proposing new algorithms for the detection of cases of treatment failure and for the reduction of the follow-up at 6 and 12 months after the end of the treatment.[53,54] The lack of a cut-off for the counting of the WBC is not the only limitation of current tools. As already described for the staging, cell counting and parasite finding suffer in specificity and sensitivity, respectively.

1.6 Pathogenesis

Thanks to a variety of studies in experimental models (mainly mouse, rat and primate) and a limited number of studies on human post-mortem samples, many aspects of HAT pathogenesis

in both blood and CNS have been clarified. Even if many mechanisms still need to be understood, it is now clear that the host immune response plays a central role in HAT pathogenesis.[17]

HAT in the blood compartment

One of the most interesting mechanisms characterizing the pathogenesis of sleeping sickness is the antigenic variation of trypanosomes, which allows parasites to escape the host immune response. In hosts' body fluids, the surface of the parasite is covered with variant surface glycoproteins (VSG) and the 10% of the whole parasite genome encodes for different VSG.[55] One VSG type is expressed at one time, and the trypanosome switches to a different one as soon as the host immune response becomes effective. This mechanism has two main consequences: the development of waves of parasitemia in patients' blood, and the inefficiency of the host immune response in achieving a complete clearance of parasites.[56] This process of antigenic variation has so far hampered the development of vaccines against HAT[57] and, despite the efforts in trying to target non- or less-variable parasite antigens, for the moment the production of anti-HAT vaccines does not seem to be a short term reality.[58]

The host immune response is, however, effective in killing most of the parasites, leading to the release in body fluids of soluble VSG (sVSG) linked to glycosylphosphatidyl-inositol (GPI) anchors. The GPI-sVSG complexes are highly immunogenic and further stimulate the immune response.[17,59] Four central inflammatory mechanisms are elicited by the parasites. Briefly, parasites can directly activate macrophages and B cells (i.e. innate immunity activation); activated B cells will in turn produce antibodies able to clear circulating parasites expressing the specific VSGs. Peptides derived from GPI-sVSG can then be expressed on the surface of antigen presenting cells in association with MHC II molecules leading to the activation of Th lymphocytes and the type 1 cytokine immune response. Th lymphocytes participate in the further activation of B lymphocytes (Th dependent activation) to achieve an increased production of antibodies. Th cells are also involved in the production of IFN-γ, which further activates macrophages and induces the release of trypanocidal factors, such as TNF-α and NO.[56,60,61]

HAT in the central nervous system

The neuroinflammatory response in late stage HAT presents some peculiar characteristics including the early activation of macrophages and astrocytes, the presence of perivascular infiltrates of inflammatory cells (perivascular cuffing) and of Mott cells (plasma cells containing IgM), and the up-regulation of inflammatory cytokines.[61-63]

The activation of astrocytes is one of the earliest events in the neuro-pathogenesis of HAT.[64] Through studies in mouse models, it has been proposed that this activation could occur prior to the development of the inflammatory response in the brain.[18]

Activated astrocytes and macrophages are two important sources of cytokines and chemokines in the brain. These include IFN-γ, IL-10, TNF-α, IL-8 and IL-6.[17,18] It has been proposed that the balance between pro- and counter-inflammatory cytokines can determine the outcome of the disease and the clinical manifestations.[17] Cytokines and chemokines are also involved in the process of leukocytes transmigration across the BBB.[65,66] This mechanism, in fact, requires a chemotactic gradient, for the recruitment of leukocytes to the site of inflammation, and a number of interactions between surface molecules of leukocytes and endothelial cells (integrins and adhesion molecules), which mediate the passage of leukocytes through the basement membrane.[63,66] Leukocytes are then sequestrated in the perivascular space and matrix metalloproteinases are needed for the further passage through the basement membrane and the glial limitans to reach the brain parenchyma.[67,68]

An important role in the neuroinflammation is also played by IFN-γ, which seems to be involved in the penetration of the parasites into the brain.[69] Although this mechanism of penetration is not completely understood, the most widely accepted theory indicates that trypanosomes penetrate in the CNS where the barrier is more permissive, such as at the choroid plexus, through a temporary opening of the tight junctions, but without the destruction of the integrity of the BBB.[70] Further investigations are needed to better explain the spatio-temporal

relation of leukocytes and parasites penetration in the brain and the development of the immune response. A graphical representation of these events and their relation is reported in Figure 3.

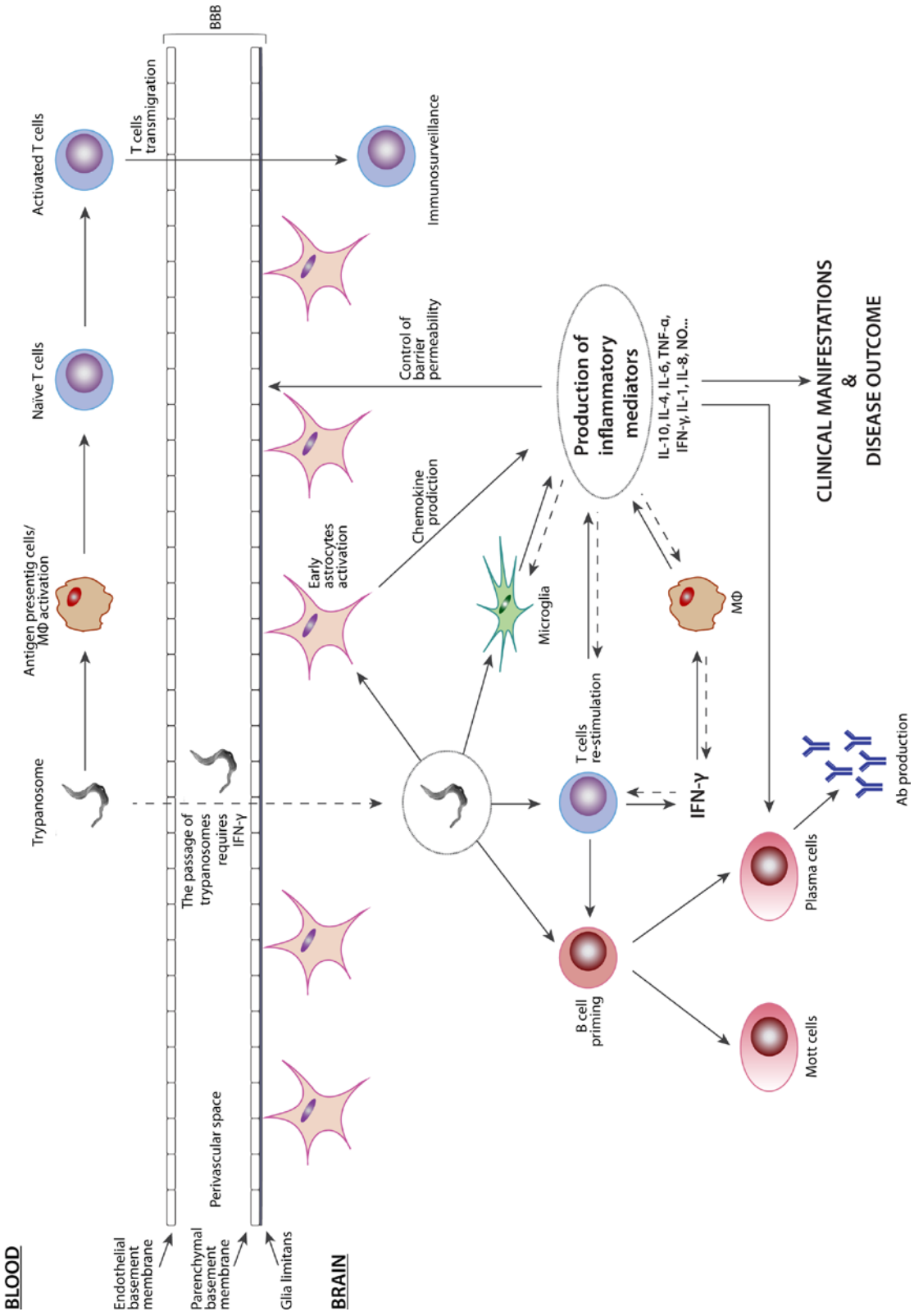

Figure 3 Principal mechanisms involved in the neuro-inflammation of HAT. The sequelae of events leading to the invasion of the CNS and the development of a neuro-inflammatory response has been derived from studies in animal models and human samples. Figure adapted from *Kennedy PG, 2004*[18] and *Rodgers J, 2010*[63]. MΦ=macrophage.

2. The quest for new HAT biomarkers

2.1 Disease biomarkers

According to a definition given in 2001 by a Working Group of the National Institutes of Health (NIH), a biomarker, or biological marker, is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[71] Examples of clinically useful biomarkers are the measurement of low density lipoprotein cholesterol (LDL-C) as indicator of risk for cardio-vascular diseases,[72] C-reactive protein as indicator of inflammation[73] or serum prostate specific antigen (PSA) as a marker for prostate cancer.[74]

The development of new disease biomarkers for clinical use has been recently described as a 5 steps process consisting in: i) discovery and verification of potential candidates; ii) validation through the development of pre-clinical assays; iii) test of biomarkers utility in prospective longitudinal studies; iv) prospective screening and v) determination of the impact of the biomarker on disease control and management.[75] Although proposed for cancer biomarkers, this workflow can easily be applied to other pathologies.

Disease biomarkers are currently a widely studied topic of research. Different approaches can be used to discover new molecules able to diagnose or prognosticate specific disease states or to indicate the effects of a therapeutic intervention. Thanks to the recent technological improvements, some popular approaches today consist in the systematic evaluation of variations in gene expression (i.e. transcriptomics), protein expression (i.e. proteomics) and metabolite production (i.e. metabolomics).[76-78] Furthermore, the introduction of new bioinformatics analyses and tools allows integrating the information obtained with the different “omics” techniques to obtain a more complete evaluation of the mechanisms leading to the pathological condition,[79] as well as the combination of markers into panels to achieve an increased accuracy.[80] However, despite the high number of studies proposing new potential biomarkers, only few of them subsequently enter clinical validation studies for the assessment of their potential application.

Most of them, in fact, show limited accuracy, usually expressed in term of specificity and sensitivity, when validated on larger cohorts. This aspect highlights the importance of the validation studies to determine the clinical utility of a newly discovered marker. The most commonly used indicators of the diagnostic or prognostic accuracy of a marker are listed in Table 4.

Indicator	Abbreviation	Formula	Definition
TRUE POSITIVE	TP		Patients correctly classified as positive
TRUE NEGATIVE	TN		Patients correctly classified as negative
FALSE POSITIVE	FP		Patients incorrectly classified as positive
FALSE NEGATIVES	FN		Patients incorrectly classified as negative
ACCURACY	ACC	(TP+TN)/(P+N)	Proportion of correctly classified patients in the population
SPECIFICITY	SP	TN/(TN+FP)	Proportion of negative patients correctly rejected by the test
SENSITIVITY	SE	TP/(TP+FN)	Proportion of positive patients correctly detected by the test
POSITIVE PREDICTIVE VALUE	PPV	TP/(TP+FP)	Proportion of positive tests that correctly indicates positive patients
NEGATIVE PREDICTIVE VALUE	NPV	TN/(TN+FN)	Proportion of negative tests that correctly indicates negative patients
POSITIVE LIKELIHOOD RATIO	LR+	SE/(1-SP)	Likelihood of the test being positive in positive patients compared to the likelihood of the test being positive in negative patients
NEGATIVE LIKELIHOOD RATIO	LR-	(1-SE)/SP	Likelihood of the test being negative in positive patients compared to the likelihood of the test being negative in negative patients

Table 4 Definition of the most important clinical indicators of marker accuracy. Adapted from[80,81]. P = positive results (TP+FP), N = negative results (TN+FN).

2.2 Biomarkers & HAT

The reduction in the number of reported cases of sleeping sickness observed during the last years[12] indicates that the disease could potentially be eliminated.[11] However, many aspects in patients' management need further improvements. The weakest points are currently represented by the stage determination, the evaluation of the outcome after treatment, for which diagnostic gold standards are still missing, and the therapeutic intervention. Despite the efforts to try to find new drugs able to safely treat both stages of disease, in some cases, treatment is still based on the use of obsolete and toxic drugs, such as melarsoprol, or drugs difficult to administer and whose distribution in remote rural areas represents an important issue. These aspects underline the central need of a correct stage determination. On the other hand, the early detection of cases

of treatment failures is essentially to promptly re-treat relapsing patients. Improved tools in both these areas are also essential to keep the disease under control. Current methods for both stage determination and treatment outcome evaluation, i.e. counting of WBC and detection of parasites, are unsatisfactory (see paragraph 1.4 and 1.5) and alternative tools are strongly needed. New HAT markers should, however, present some characteristics associated to the nature of sleeping sickness as a tropical neglected disease. To be clinically useful for HAT, a biomarker should in fact not only be highly accurate, but should also have the potential of being translated into a cheap and easy to use rapid field test for mass population applications.

A number of studies have been led to try to identify new markers for stage determination and/or treatment outcome evaluation. Late stage HAT and relapses of late stage disease are considered to be characterized by a meningo-encephalitis. Thus, it is not surprising that most of the studies performed so far focused on the finding of CSF biomarkers. The choice of focusing on CSF can find two main explanations: i) both stage 1 and stage 2 patients have parasites in their blood, making the probability of finding a plasma marker able to discriminate those patients having parasites in the brain from those who have not, very difficult and challenging, ii) CSF examination is part of the current diagnostic workflow, thus a CSF biomarker would not represent a worsening of the current clinical practice. Only few examples can be found in the literature focusing on HAT plasma markers, however the most interesting published results concerned mainly the observation of decreased plasma levels of molecules such as NO , IFN-γ[82] or IL-10[83] after treatment rather than the comparison of pre-treatment plasma markers between stages.

2.3 CSF as a source of disease biomarkers

Cerebrospinal fluid is the body fluid that best reflects the physiological or pathological state of the central nervous system due to its proximity with CNS structures.[84,85] It is mainly produced at the choroid plexus, were it is separated by the blood circulation through the blood-CSF barrier. CSF is produced at a flow-rate of approximately 500mL/24h[86] and its composition is strictly

regulated by the selectivity of the BBB.[85] The CSF proteome is for its most part composed by proteins derived from blood and, as it happens in plasma, albumin and immunoglobulins represent approximately 70% of the total CSF protein amount.[87] The 20% of CSF proteins are synthesized in the brain, although they are rarely considered brain specific.[86,88]

CSF protein content is of particular clinical utility since increased protein concentration can be indicative of neurological disorders. This increase can be the result of either a BBB dysfunction, thus an increased passage of proteins from the blood to the CSF, or of an increased intrathecal synthesis.[86] These conditions can be evaluated considering that: i) the albumin present in the CSF exclusively originate from blood; ii) in normal conditions the ratio between albumin concentration in blood and CSF (Q_{Alb}) is constant and iii) in normal conditions, for those proteins that can be synthesized in the brain such as IgG, the relation between Q_{IgG} and Q_{Alb} is constant. An increased Q_{Alb} is thus considered to be indicative of BBB brain barrier dysfunction, while the evaluation of Q_{IgG} and Q_{Alb} through Reibergrams can highlight the intrathecal origin of proteins (e.g. IgG_{IF}).[85,88]

The assessment of the intrathecal synthesis of immunoglobulins can be particularly helpful as it can indicate an inflammatory process occurring in the brain.[88,89] Furthermore, the humoral immune response in the brain has some important and peculiar characteristics such as the absence of the switch between IgM and IgG, a low decay of CSF antibodies and an increased cell count,[88] which together considered can further help in evaluating pathological conditions.

These characteristics make CSF an important source of biomarkers for the diagnosis, prognosis or for therapeutic decision for many malignant conditions affecting the CNS. Classical examples of biochemical markers of clinical utility are represented by the detection of oligoclonal bands in the CSF for the diagnosis of multiple sclerosis,[90] Aβ42 protein, total and phosphorylated tau protein for Alzheimer's disease[91] and 14-3-3 protein for Creutzfeldt-Jakob disease.[92]

2.4 New CSF tools for HAT stage determination and treatment outcome evaluation

Cerebrospinal fluid has been extensively investigated for the finding of new staging markers and new tools for the detection of treatment failure in HAT.

The increased concentration of immunoglobulins in the CSF of HAT late stage patients is known since long time.[93] More recently it has been shown that IgM is the predominant class found in the CSF of S2 patients and their intrathecal production was demonstrated,[41,94] indicating the presence of a brain inflammatory process not associated to damages to the BBB. IgM, and in particular those of intrathecal origin, are actually considered the best alternative to the counting of the WBC for staging *T. b. gambiense* HAT. A rapid agglutination test for the evaluation of IgM concentration in the CSF was developed (Latex/IgM).[25,95,96] High correlation between the Latex/IgM end titre and the intathecal IgM production was shown,[96] however, there are still not enough evidences to introduce it into clinical practice for stage determination. The same approach has also been tested for the evaluation of the outcome after treatment during the 24 months post-therapeutic follow-up. Latex/IgM, combined to the counting of the WBC, was able to accurately detect relapses at 18 months, but CSF IgM levels showed a slow normalization during time in cured patients.[97]

Another alternative proposed for both applications is the amplification of specific parasite DNA sequences by PCR. This approach is highly specific, however its global accuracy for stage determination and treatment outcome evaluation under field conditions has still not be determined yet.[98,99]

Other approaches that have been proposed include the detection of auto-antibodies against brain proteins (galactocerebroside and neurofilament),[100,101] the detection of B-cells expressing CD19 through the formation of rosettes[102,103] and the recording of the sleep patterns characteristic of HAT sleep disturbances through polysomnography.[20,104] This latter approach represents the only non-invasive alternative proposed so far. All these methods need further investigations to determine their accuracy for stage determination and/or treatment outcome evaluation on large populations, and to assess the possibility of their use under field conditions.

As explained in the paragraph 1.6, it is now well recognized that the inflammatory response within the brain is responsible for the neuro-pathogenesis of HAT and that the balance between pro- and anti-inflammatory cytokines determines the outcome and the clinical manifestations of the disease.[18,105] Therefore, it is not surprising that a number of studies focused on the evaluation of immune mediators as indicators of the CNS involvement, even if the temporal relation between the penetration of the parasites in the CNS, the development of the neuro-inflammation and the onset of clinical manifestations of late stage HAT is still not understood.

This approach led to the identification of molecules over-expressed in the CSF of late stage patients that could be potentially used as biomarkers. A summary of the most important findings is reported in Table 5.

Despite their potential as diagnostic markers of the meningo-encephalitic stage of *T. b. gambiense* or *T. b. rhodesiense* HAT, they further verification in prospective longitudinal studies is strongly required.

Marker	Form of HAT	Reference
IL-1β	*Tbg*	106
CXCL8	*Tbg*	106
CCL-2	*Tbg*	106
CCL-3	*Tbg*	106
IL-10*	*Tbr & Tbg*	83,97,107,108
IL-6	*Tbr & Tbg*	107,108
Neopterin	*Tbr*	107
IL-8	*Tbg*	108
Lipocalin-2	*Tbr & Tbg*	109
SLPI	*Tbr & Tbg*	109
CXCL10	*Tbr & Tbg*	109,110
IFN-γ	*Tbg*	110
CXCL13	*Tbg*	111

Table 5 List of immune-related factors proposed for the staging and/or follow-up of HAT patients. *Tbg*: *T. b. gambiense*; *Tbr*: *T. b. rhodesiense.* *IL-10 was evaluated for both staging and follow-up applications.

3. Aim of the project

The lack of a generally accepted gold standard for stage determination and treatment outcome evaluation represents a major limitation in the management of patients suffering from HAT. Based on the need of new alternatives to current methods, the aim of the project here presented, was to identify CSF markers able to accurately diagnose the meningo-encephalitic involvement characterizing HAT late stage and relapsing patients. Based on the integration of different discovery approaches, the ultimate goal was to characterize cerebrospinal fluid biomarkers for three different applications: i) staging *T. b. gambiense* patients; ii) treatment outcome evaluation for *T. b. gambiense* patients; iii) staging *T. b. rhodesiense* patients.

A classical workflow for biomarker discovery and validation was first applied for the investigation of *T. b. gambiense* HAT. This form of disease was preferred to *T. b. rhodesiense* due its higher prevalence (>90% of cases) and, as a consequence, the availability of larger study cohorts. The CSF proteome of patients affected by *T. b. gambiense* HAT was thus first investigated using complementary proteomics approaches, i.e. 2D gels and quantitative mass spectrometry, to assess whether differentially expressed proteins could distinguish between stage 1 and stage 2 patients (Chapter 2).

The technical limitations of proteomics tools, however, do not allow detecting all proteins, and those less abundant are often missed. Based on the hypothesis that the inflammatory response is central in the pathogenesis of CNS disease, a number of immune-related factors as well as 3 brain damage markers, H-FABP, GSTπ-1 and S100β,[112-114] were tested using immunoassays on a small number of CSF samples (Chapter 3 and 4), to highlight molecules able to stratify patients.

Altogether considered, and with the addition of some potential markers taken from the literature or tested by the Institute of Tropical Medicine (ITM - Antwerp, Belgium), a list comprising 36 candidate biomarkers for the staging of *gambiense* sleeping sickness was obtained. A statistical selection was performed to highlight the 8 best molecules (IgM, B2MG, neopterin,

ICAM-1, VCAM-1, MMP-9, CXCL10 and CXCL13), which showed the highest accuracy as staging tools. These 8 molecules were tested on a multicenter cohort encompassing 512 *T. b. gambiense* patients collected in three different countries (Chapter 5). To try to extend the potential utility of these markers, they were also assessed as test-of-cure on a large population of *T. b. gambiense* patients followed after treatment, comprising S2 patients cured and S2 patients experiencing a relapse, to evaluate their ability in detecting treatment failures and their potential in shortening the follow-up (Chapter 6).

Finally, a small population of patients suffering from the acute form of sleeping sickness, i.e. *T. b. rhodesiense* HAT, was investigated to determine whether the same markers could be used for the stratification before treatment of both forms of HAT (Chapter 7).

A global view of all the results reported in the different chapters will be given in chapter 8, together with the discussion of the most important implications of the present study and the future perspectives.

REFERENCES

1. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet 2010;375:148-59.

2. Maudlin I. African trypanosomiasis. Annals of tropical medicine and parasitology 2006;100:679-701.

3. Burri C, Brun R. Human African trypanosomiasis. In: Cook G, Zumula A, Manson's Tropical Diseases 22nd edn London, WB Saunders 2009:1307-25.

4. Malvy D, Chappuis F. Sleeping sickness. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2011;17:986-95.

5. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.

6. Simarro PP, Cecchi G, Paone M, et al. The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. International journal of health geographics 2010;9:57.

7. Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, et al. Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature 2003;422:83-7.

8. Vanhollebeke B, Pays E. The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill. Molecular microbiology 2010;76:806-14.

9. Vanhamme L, Pays E. The trypanosome lytic factor of human serum and the molecular basis of sleeping sickness. International journal for parasitology 2004;34:887-98.

10. Gibson WC. The SRA gene: the key to understanding the nature of Trypanosoma brucei rhodesiense. Parasitology 2005;131:143-50.

11. Simarro PP, Jannin J, Cattand P. Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS medicine 2008;5:e55.

12. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS neglected tropical diseases 2011;5:e1007.

13. WHO. Human African trypanosomiasis (sleeping sickness): epidemiological update. Releve epidemiologique hebdomadaire / Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record / Health Section of the Secretariat of the League of Nations 2006;81:71-80.

14. Hotez PJ, Molyneux DH, Fenwick A, et al. Control of neglected tropical diseases. The New England journal of medicine 2007;357:1018-27.

15. WHO. The global burden of disease: 2004 update. Available at: http://wwwwhoint/healthinfo/global_burden_disease/2004_report_update/en/indexhtml 2004.

16. Fevre EM, Wissmann BV, Welburn SC, Lutumba P. The burden of human African trypanosomiasis. PLoS neglected tropical diseases 2008;2:e333.

17. Sternberg JM. Human African trypanosomiasis: clinical presentation and immune response. Parasite immunology 2004;26:469-76.

18. Kennedy PG. Human African trypanosomiasis of the CNS: current issues and challenges. The Journal of clinical investigation 2004;113:496-504.

19. Lejon V, Buscher P. Review Article: cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. Tropical medicine & international health : TM & IH 2005;10:395-403.

20. Buguet A, Bisser S, Josenando T, Chapotot F, Cespuglio R. Sleep structure: a new diagnostic tool for stage determination in sleeping sickness. Acta tropica 2005;93:107-17.

21. Magnus E, Vervoort T, Van Meirvenne N. A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of T. B. gambiense trypanosomiasis. Annales de la Societe belge de medecine tropicale 1978;58:169-76.

22. Deborggraeve S, Buscher P. Molecular diagnostics for sleeping sickness: what is the benefit for the patient? The Lancet infectious diseases 2010;10:433-9.

23. Wastling SL, Welburn SC. Diagnosis of human sleeping sickness: sense and sensitivity. Trends in parasitology 2011;27:394-402.

24. Robays J, Bilengue MM, Van der Stuyft P, Boelaert M. The effectiveness of active population screening and treatment for sleeping sickness control in the Democratic Republic of Congo. Tropical medicine & international health : TM & IH 2004;9:542-50.

25. Buscher P, Lejon V, Magnus E, Van Meirvenne N. Improved latex agglutination test for detection of antibodies in serum and cerebrospinal fluid of Trypanosoma brucei gambiense infected patients. Acta tropica 1999;73:11-20.
26. Penchenier L, Grebaut P, Njokou F, Eboo Eyenga V, Buscher P. Evaluation of LATEX/T.b.gambiense for mass screening of Trypanosoma brucei gambiense sleeping sickness in Central Africa. Acta tropica 2003;85:31-7.
27. Lejon V, Buscher P, Magnus E, Moons A, Wouters I, Van Meirvenne N. A semi-quantitative ELISA for detection of Trypanosoma brucei gambiense specific antibodies in serum and cerebrospinal fluid of sleeping sickness patients. Acta tropica 1998;69:151-64.
28. Kabiri M, Franco JR, Simarro PP, Ruiz JA, Sarsa M, Steverding D. Detection of Trypanosoma brucei gambiense in sleeping sickness suspects by PCR amplification of expression-site-associated genes 6 and 7. Tropical medicine & international health : TM & IH 1999;4:658-61.
29. Radwanska M, Chamekh M, Vanhamme L, et al. The serum resistance-associated gene as a diagnostic tool for the detection of Trypanosoma brucei rhodesiense. The American journal of tropical medicine and hygiene 2002;67:684-90.
30. Papadopoulos MC, Abel PM, Agranoff D, et al. A novel and accurate diagnostic test for human African trypanosomiasis. Lancet 2004;363:1358-63.
31. Agranoff D, Stich A, Abel P, Krishna S. Proteomic fingerprinting for the diagnosis of human African trypanosomiasis. Trends in parasitology 2005;21:154-7.
32. Njiru ZK, Mikosza AS, Matovu E, et al. African trypanosomiasis: sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. International journal for parasitology 2008;38:589-99.
33. Matovu E, Kazibwe AJ, Mugasa CM, Ndungu JM, Njiru ZK. Towards Point-of-Care Diagnostic and Staging Tools for Human African Trypanosomiaisis. Journal of tropical medicine 2012;2012:340538.
34. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.
35. Cattand P, Miezan BT, de Raadt P. Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes. Bulletin of the World Health Organization 1988;66:83-6.
36. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:293.
37. Kennedy PG. Difficulties in diagnostic staging of human African trypanosomiasis. J Neuroparasitol 2011;2.
38. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.
39. Lejon V, Buscher P. Stage determination and follow-up in sleeping sickness. Medecine tropicale : revue du Corps de sante colonial 2001;61:355-60.
40. Doua F, Miezan TW, Sanon Singaro JR, Boa Yapo F, Baltz T. The efficacy of pentamidine in the treatment of early-late stage Trypanosoma brucei gambiense trypanosomiasis. The American journal of tropical medicine and hygiene 1996;55:586-8.
41. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.
42. Burri C. Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitology 2010;137:1987-94.
43. Moore AC. Prospects for improving African trypanosomiasis chemotherapy. The Journal of infectious diseases 2005;191:1793-5.
44. Barrett MP, Boykin DW, Brun R, Tidwell RR. Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. British journal of pharmacology 2007;152:1155-71.
45. Simarro PP, Franco J, Diarra A, Postigo JA, Jannin J. Update on field use of the available drugs for the chemotherapy of human African trypanosomiasis. Parasitology 2012;139:842-6.
46. Chappuis F, Udayraj N, Stietenroth K, Meussen A, Bovier PA. Eflornithine is safer than melarsoprol for the treatment of second-stage Trypanosoma brucei gambiense human African trypanosomiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2005;41:748-51.

47. Priotto G, Kasparian S, Mutombo W, et al. Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet 2009;374:56-64.
48. Kuepfer I, Schmid C, Allan M, et al. Safety and efficacy of the 10-day melarsoprol schedule for the treatment of second stage rhodesiense sleeping sickness. PLoS neglected tropical diseases 2012;6:e1695.
49. Kennedy PG. An alternative form of melarsoprol in sleeping sickness. Trends in parasitology 2012;28:307-10.
50. Thuita JK, Wang MZ, Kagira JM, et al. Pharmacology of DB844, an Orally Active aza Analogue of Pafuramidine, in a Monkey Model of Second Stage Human African Trypanosomiasis. PLoS neglected tropical diseases 2012;6:e1734.
51. WHO. Recommendations of the informal counsultation on issues for clinical product development for human African trypanosomiasis. WHO/CDS/NTD/IDM/20071 2007.
52. Mumba Ngoyi D, Lejon V, N'Siesi FX, Boelaert M, Buscher P. Comparison of operational criteria for treatment outcome in gambiense human African trypanosomiasis. Tropical medicine & international health : TM & IH 2009;14:438-44.
53. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. The Journal of infectious diseases 2010;201:453-63.
54. Priotto G, Chappuis F, Bastard M, Flevaud L, Etard JF. Early prediction of treatment efficacy in second-stage gambiense human African trypanosomiasis. PLoS neglected tropical diseases 2012;6:e1662.
55. Donelson JE. Antigenic variation and the African trypanosome genome. Acta tropica 2003;85:391-404.
56. Field MC, Lumb JH, Adung'a VO, Jones NG, Engstler M. Macromolecular trafficking and immune evasion in african trypanosomes. International review of cell and molecular biology 2009;278:1-67.
57. La Greca F, Magez S. Vaccination against trypanosomiasis: can it be done or is the trypanosome truly the ultimate immune destroyer and escape artist? Human vaccines 2011;7:1225-33.
58. Magez S, Caljon G, Tran T, Stijlemans B, Radwanska M. Current status of vaccination against African trypanosomiasis. Parasitology 2010;137:2017-27.
59. Paulnock DM, Coller SP. Analysis of macrophage activation in African trypanosomiasis. Journal of leukocyte biology 2001;69:685-90.
60. Bisser S, Ouwe-Missi-Oukem-Boyer ON, Toure FS, et al. Harbouring in the brain: A focus on immune evasion mechanisms and their deleterious effects in malaria and human African trypanosomiasis. International journal for parasitology 2006;36:529-40.
61. Mansfield JM, Paulnock DM. Regulation of innate and acquired immunity in African trypanosomiasis. Parasite immunology 2005;27:361-71.
62. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.
63. Rodgers J. Trypanosomiasis and the brain. Parasitology 2010;137:1995-2006.
64. Kennedy PG. Human African trypanosomiasis-neurological aspects. Journal of neurology 2006;253:411-6.
65. Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature reviews Immunology 2003;3:569-81.
66. Engelhardt B, Ransohoff RM. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. Trends in immunology 2005;26:485-95.
67. Owens T, Bechmann I, Engelhardt B. Perivascular spaces and the two steps to neuroinflammation. Journal of neuropathology and experimental neurology 2008;67:1113-21.
68. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Progress in neurobiology 2010;91:152-71.
69. Masocha W, Rottenberg ME, Kristensson K. Migration of African trypanosomes across the blood-brain barrier. Physiology & behavior 2007;92:110-4.
70. Mulenga C, Mhlanga JD, Kristensson K, Robertson B. Trypanosoma brucei brucei crosses the blood-brain barrier while tight junction proteins are preserved in a rat chronic disease

model. Neuropathology and applied neurobiology 2001;27:77-85.
71. Biomarkers Definitions Working G. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clinical pharmacology and therapeutics 2001;69:89-95.
72. Tardif JC, Heinonen T, Orloff D, Libby P. Vascular biomarkers and surrogates in cardiovascular disease. Circulation 2006;113:2936-42.
73. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. The Journal of clinical investigation 2003;111:1805-12.
74. Brawley OW. Prostate cancer epidemiology in the United States. World journal of urology 2012;30:195-200.
75. Pepe MS, Etzioni R, Feng Z, et al. Phases of biomarker development for early detection of cancer. Journal of the National Cancer Institute 2001;93:1054-61.
76. Gerszten RE, Wang TJ. The search for new cardiovascular biomarkers. Nature 2008;451:949-52.
77. Gerszten RE, Asnani A, Carr SA. Status and prospects for discovery and verification of new biomarkers of cardiovascular disease by proteomics. Circulation research 2011;109:463-74.
78. Serkova NJ, Standiford TJ, Stringer KA. The emerging field of quantitative blood metabolomics for biomarker discovery in critical illnesses. American journal of respiratory and critical care medicine 2011;184:647-55.
79. Villoslada P, Baranzini S. Data integration and systems biology approaches for biomarker discovery: challenges and opportunities for multiple sclerosis. Journal of neuroimmunology 2012;248:58-65.
80. Robin X, Turck N, Hainard A, Lisacek F, Sanchez JC, Muller M. Bioinformatics for protein biomarker panel classification: what is needed to bring biomarker panels into in vitro diagnostics? Expert review of proteomics 2009;6:675-89.
81. Puntmann VO. How-to guide on biomarkers: biomarker definitions, validation and applications with examples from cardiovascular disease. Postgraduate medical journal 2009;85:538-45.
82. MacLean L, Odiit M, Okitoi D, Sternberg JM. Plasma nitrate and interferon-gamma in Trypanosoma brucei rhodesiense infections: evidence that nitric oxide production is induced during both early blood-stage and late meningoencephalitic-stage infections. Transactions of the Royal Society of Tropical Medicine and Hygiene 1999;93:169-70.
83. MacLean L, Odiit M, Sternberg JM. Nitric oxide and cytokine synthesis in human African trypanosomiasis. The Journal of infectious diseases 2001;184:1086-90.
84. Pendyala G, Trauger SA, Kalisiak E, Ellis RJ, Siuzdak G, Fox HS. Cerebrospinal fluid proteomics reveals potential pathogenic changes in the brains of SIV-infected monkeys. Journal of proteome research 2009;8:2253-60.
85. Reiber H. Cerebrospinal fluid--physiology, analysis and interpretation of protein patterns for diagnosis of neurological diseases. Mult Scler 1998;4:99-107.
86. Thompson EJ. Cerebrospinal fluid. Journal of neurology, neurosurgery, and psychiatry 1995;59:349-57.
87. Yuan X, Desiderio DM. Proteomics analysis of human cerebrospinal fluid. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 2005;815:179-89.
88. Reiber H, Peter JB. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. Journal of the neurological sciences 2001;184:101-22.
89. Reiber H. Dynamics of brain-derived proteins in cerebrospinal fluid. Clinica chimica acta; international journal of clinical chemistry 2001;310:173-86.
90. Bourahoui A, De Seze J, Guttierez R, et al. CSF isoelectrofocusing in a large cohort of MS and other neurological diseases. European journal of neurology : the official journal of the European Federation of Neurological Societies 2004;11:525-9.
91. Hampel H, Frank R, Broich K, et al. Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. Nature reviews Drug discovery 2010;9:560-74.
92. Van Everbroeck B, Boons J, Cras P. Cerebrospinal fluid biomarkers in Creutzfeldt-Jakob disease. Clinical neurology and neurosurgery 2005;107:355-60.
93. Lambert PH, Berney M, Kazyumba G. Immune complexes in serum and in cerebrospinal fluid in African trypanosomiasis. Correlation with polyclonal B cell activation and with intracerebral immunoglobulin synthesis. The Journal of clinical investigation 1981;67:77-85.

94. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. Journal of the neurological sciences 2002;193:127-35.
95. Lejon V, Buscher P, Sema NH, Magnus E, Van Meirvenne N. Human African trypanosomiasis: a latex agglutination field test for quantifying IgM in cerebrospinal fluid. Bulletin of the World Health Organization 1998;76:553-8.
96. Lejon V, Legros D, Richer M, et al. IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Tropical medicine & international health : TM & IH 2002;7:685-92.
97. Lejon V, Roger I, Mumba Ngoyi D, et al. Novel markers for treatment outcome in late-stage Trypanosoma brucei gambiense trypanosomiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2008;47:15-22.
98. Deborggraeve S, Lejon V, Ekangu RA, et al. Diagnostic accuracy of PCR in gambiense sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS neglected tropical diseases 2011;5:e972.
99. Mugasa CM, Adams ER, Boer KR, et al. Diagnostic accuracy of molecular amplification tests for human African trypanosomiasis--systematic review. PLoS neglected tropical diseases 2012;6:e1438.
100. Ayed Z, Brindel I, Bouteille B, et al. Detection and characterization of autoantibodies directed against neurofilament proteins in human African trypanosomiasis. The American journal of tropical medicine and hygiene 1997;57:1-6.
101. Bisser S, Ayed Z, Bouteille B, et al. Central nervous system involvement in African trypanosomiasis: presence of anti-galactocerebroside antibodies in patients' cerebrospinal fluid. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:225-6.
102. Boda C, Courtioux B, Roques P, et al. Immunophenotypic lymphocyte profiles in human african trypanosomiasis. PloS one 2009;4:e6184.
103. Bouteille B, Mpandzou G, Cespuglio R, et al. Cerebrospinal fluid B lymphocyte identification for diagnosis and follow-up in human African trypanosomiasis in the field. Tropical medicine & international health : TM & IH 2010;15:454-61.
104. Mpandzou G, Cespuglio R, Ngampo S, et al. Polysomnography as a diagnosis and post-treatment follow-up tool in human African trypanosomiasis: a case study in an infant. Journal of the neurological sciences 2011;305:112-5.
105. Kennedy PG. Cytokines in central nervous system trypanosomiasis: cause, effect or both? Transactions of the Royal Society of Tropical Medicine and Hygiene 2009;103:213-4.
106. Courtioux B, Boda C, Vatunga G, et al. A link between chemokine levels and disease severity in human African trypanosomiasis. International journal for parasitology 2006;36:1057-65.
107. Maclean L, Odiit M, Sternberg JM. Intrathecal cytokine responses in Trypanosoma brucei rhodesiense sleeping sickness patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006;100:270-5.
108. Lejon V, Lardon J, Kenis G, et al. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of Trypanosoma brucei gambiense sleeping sickness patients before and after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 2002;96:329-33.
109. Amin DN, Ngoyi DM, Nhkwachi GM, et al. Identification of stage biomarkers for human African trypanosomiasis. The American journal of tropical medicine and hygiene 2010;82:983-90.
110. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.
111. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Tropical medicine & international health : TM & IH 2009;14:529-34.
112. Zimmermann-Ivol CG, Burkhard PR, Le Floch-Rohr J, Allard L, Hochstrasser DF, Sanchez JC. Fatty acid binding protein as a serum marker for the early diagnosis of stroke: a pilot study. Molecular & cellular proteomics : MCP 2004;3:66-72.
113. Burgess JA, Lescuyer P, Hainard A, et al. Identification of brain cell death associated proteins in human post-mortem cerebrospinal fluid. Journal of proteome research 2006;5:1674-81.

114. Bloomfield SM, McKinney J, Smith L, Brisman J. Reliability of S100B in predicting severity of central nervous system injury. Neurocritical care 2007;6:121-38.

CHAPTER 2

Discovery and validation of osteopontin and beta-2-microglobulin as promising markers for staging human African trypanosomiasis

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The first step towards the identification of biomarkers for stage determination in patients affected by sleeping sickness consisted in the comparison of CSF samples taken from early and late stage patients affected by *T. b. gambiense* HAT, using proteomics strategies.

Two complementary approaches have been applied: two-dimensional gel electrophoresis, for the investigation of CSF samples from 9 HAT patients, and quantitative mass spectrometry based on tandem mass tag (TMT) labelling on pooled CSF samples. By combining the results of the two approaches we obtained a list of proteins over-expressed in the CSF of HAT late stage patients.

Based on protein functions, the potential involvement on disease progression and on the novelty for HAT staging, we selected beta-2-microglobulin, osteopontin and complement factor H for further verifications using immunoassays.

This work was published in 2010 in Molecular and Cellular Proteomics. My contribution consisted in performing 2-DE experiments, data analyses and markers verification and in writing the paper.

Discovery and validation of osteopontin and beta-2-microglobulin as promising markers for staging human African trypanosomiasis

Natalia Tiberti[1], Alexandre Hainard[1], Veerle Lejon[2], Xavier Robin[1], Dieudonné Mumba Ngoyi[3], Natacha Turck[1], Enock Matovu[4], John Enyaru[5], Joseph Mathu Ndung'u[6], Alexander Scherl[1], Loïc Dayon[1], Jean-Charles Sanchez[1]

1 *Biomedical Proteomics Research Group, Medical University Centre, Geneva, Switzerland;* ***2*** *Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium;* ***3*** *Institut National de Recherche Biomedicale, Kinshasa, D.R. Congo;* ***4*** *Department of Veterinary Parasitology and Microbiology, Faculty of Veterinary Medicine, Makerere University, Kampala, Uganda;* ***5*** *Department of Biochemistry, Faculty of Science, Makerere University, Kampala, Uganda;* ***6*** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland.*

SUMMARY

Human African trypanosomiasis (HAT), or sleeping sickness, is a parasitic disease endemic in sub-Saharan Africa, transmitted to humans through the bite of a tsetse fly. The first or haemolymphatic stage of the disease (S1) is associated with presence of parasites in the bloodstream, lymphatic system and body tissues. If patients are left untreated, parasites cross the blood-brain barrier (BBB) and invade the cerebrospinal fluid (CSF) and the brain parenchyma, giving rise to the second or meningoencephalitic stage (S2). Stage determination is a crucial step in guiding the choice of treatment, as drugs used for S2 are potentially dangerous. Current staging methods, based on counting white blood cells (WBC) and demonstrating trypanosomes in CSF, lack specificity and/or sensitivity. In the present study, we used a number of proteomic strategies to discover new markers with potential for staging HAT.

CSF samples were collected from patients infected with *Trypanosoma brucei gambiense* in the Democratic Republic of Congo. The stage was determined following the guidelines of the national control program. The proteome of the samples was analysed by two-dimensional gel electrophoresis (n=9), and by sixplex tandem mass tag (TMT) isobaric labeling (n=6) quantitative mass spectrometry. Overall, 73 proteins were over-expressed in patients presenting the second stage of disease. Two of these, osteopontin and beta-2-microglobulin, were confirmed to be potential markers for staging HAT by Western blot and ELISA. The two proteins significantly discriminated between S1 and S2 patients with high sensitivity (68% and 78%, respectively) for 100% specificity, and a combination of both improved the sensitivity to 91%. The levels of osteopontin and beta-2-microglobulin in CSF of S2 patients (µg/ml range), as well as the fold increased concentration in S2 compared to S1 (3.8 and 5.5 respectively) make the two markers good candidates for the development of a test for staging HAT patients.

INTRODUCTION

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by an extracellular protozoan parasite of the genus *Trypanosoma,* which is transmitted through the bite of a tsetse fly (genus *Glossina*). Two morphologically identical subspecies of the parasite, are responsible for the two geographically and clinically different forms of HAT: a chronic form, widespread in West and Central Africa, caused by *T. b. gambiense*, and an acute form, endemic in eastern Africa, caused by *T. b. rhodesiense*.[1] In both forms of the disease, parasites are initially localized in the blood stream, lymph, and peripheral tissues; this is the first or haemolymphatic

stage (S1). During this stage, patients present generic clinical features that are common to other infectious diseases such as HIV, malaria and tuberculosis (TB), which can co-exist with HAT, thus making its early diagnosis difficult.[2] If treatment is not carried out, the disease progresses to the second or meningoencephalitic stage (S2) after trypanosomes cross the blood-brain barrier (BBB) and invade the central nervous system (CNS). This phase is characterized by a broad range of neurological signs that are indicative of CNS involvement.[1] Diagnosis of HAT is based on parasitological demonstration of parasites in blood or lymph-node aspirate.[3] All positive or suspect patients have to undergo a lumbar puncture and cerebrospinal fluid (CSF) examination, to determine whether they have second stage disease.[4] According to the World Health Organization (WHO) guidelines, the meningoencephalitic stage is defined by the presence of parasites in CSF and/or a white blood cell (WBC) count of more than 5 cells per µl.[5] Other parameters, such as intrathecal IgM production could also provide additional information to determine whether the CNS is involved.[6,7]

Treatment of HAT patients varies depending on the infecting parasite and the stage of disease.[5,8] S2 drugs in current use, including melarsoprol, eflornithine, and a combination of nifurtimox and eflornithine have a number of limitations, such as a high rate of toxicity (melarsoprol causes death to 5% of treated patients),[9] complex logistics and mode of administration.[6,10] Consequently, staging is a vital step in the diagnosis and treatment of HAT. However, the poor specificity or sensitivity of WBC counting and of parasitological techniques for demonstration of parasites in CSF, highlight the need for discovery of better tools for staging the disease.

Several attempts have been made during the last decade to identify potential biomarkers able to discriminate between the two stages of sleeping sickness. Most of the efforts focused on cytokines and chemokines, since the patient's immune system plays a crucial role in the brain pathology.[11,12,13,14]

Proteomic approaches are increasingly being applied in biomedical research and clinical medicine to investigate body fluids as a source of biomarkers,[15] including the diagnosis of neurological disorders such as Alzheimer's disease,[16] Parkinson's disease[17] and multiple sclerosis.[18,19] The protein composition of CSF is strictly regulated and can reflect the physiological or pathological state of the CNS.[15] Thus in the present study, we addressed the challenge of staging HAT by analysing CSF from *T. b. gambiense* patients using two complementary proteomic strategies: a classical approach based on two-dimensional gel electrophoresis (2-DE), and quantitative mass spectrometry (MS) using isobaric tandem mass tag (TMT) technology (sixplex TMT® MS/MS).[20]

EXPERIMENTAL PROCEDURES

Samples - The CSF samples used in the present study were collected at Dipumba hospital in Mbuji-Mayi (East Kasai province, Democratic Republic of Congo) as part of a longitudinal study monitoring the outcome of treated HAT patients, whose results are described elsewhere.[21] The patients were enrolled prospectively using the following inclusion criteria: presence of trypanosomes in lymph node aspirate, blood or CSF, age ≥ 12 years, and living within a 100 km radius around Mbuji-Mayi. The exclusion criteria were pregnancy, follow-up not guaranteed, moribund condition, haemorrhagic CSF, and serious concurrent illness such as tuberculosis, bacterial or cryptococcal meningitis. No information on their HIV status was available at the moment of inclusion, but HIV prevalence was retrospectively found to be 3.1%.[21] No systematic testing for malaria was done, but since the prevalence of the disease in the region is high, anti-malarial drugs were administered to all patients prior to treatment for HAT. A lumbar puncture was performed on each patient and the CSF examined within 30 minutes to determine the stage of disease before treatment.[22] This was done counting CSF WBC in disposable counting chambers (Uriglass, Menarini, Vienna, Austria) under a microscope. When the number of WBC was less than 20/µl, a second count was carried out. The modified single centrifugation method[23] was used to determine whether parasites were present in the CSF. The stage of disease was established in accordance with the guidelines of national sleeping sickness control program (PNLTHA). Patients with a WBC count of ≤ 5/µl and no trypanosomes were classified as S1, and those with > 5 WBC/µl and/or trypanosomes in the CSF as S2. The patients were further classified based on three categories of neurological signs as described by Hainard *et al.*[14]: absent (no neurological signs), moderate (at least one major neurological sign but no generalised tremors) or severe (at least two major neurological signs, including generalised tremors).

The CSF used in the present study was taken from the supernatant after the MSC, frozen in liquid nitrogen, shipped in dry ice, and stored at -80°C until use. The samples were handled at room temperature (30-35°C) for not more than 30 minutes between collection and freezing. They were then thawed and aliquoted, such

that a different aliquot was used for each subsequent test.
The ethical committees of The Ministry of Health, DRC and of the University of Antwerp, Belgium approved the study. Patients, or their relatives, were informed about the objectives and modalities of the study, and provided written consent prior to inclusion.

Analytical 2-DE gels - Five S1 and 4 S2 CSF samples (without significant difference in age and sex) were analysed by 2-DE. For each sample, 250 µl were precipitated with cold acetone prior to protein separation. Two-DE experiments were performed as described by Sanchez *et al.*,[24] except for the second dimension separation, which was performed on 12.5% polyacrylamide gels.

Preparative 2-DE gels - Duplicate preparative 2-DE gels were obtained by separating a pool of S2 CSF samples (n = 5, final volume 250 µl), in order to generate protein spots for identification by MS. The protocol was similar to that used for the analytical gels, apart from the staining procedure. In order to enable subsequent identification, preparative gels were stained following the protocol for MS-compatible silver staining,[25] and the protein spots cut from the gels manually.

Image analysis - Gel images were analysed by ImageMaster™ 2D Platinum 6.0 software (GE Healthcare, Uppsala, Sweden). Selection of differentially expressed protein spots was done by carrying out inter-class statistical analysis using the Kolmogorov-Smirnov test comparing the percentage volume of all matched spots. All protein spots whose percent volume was significantly different between the two groups (p value < 0.05) were considered. Additionally, S2/S1 ratios were calculated based on the corresponding mean spot % Vol, and finally only spots with a ratio higher than 2 were selected for identification by MS.

In gel tryptic protein digestion - Excised protein spots were in-gel digested as described by Burgess *et al.*[26] for identification by MALDI TOF-TOF MS (37 spots) and LTQ-OT MS (52 spots). After peptide extraction, samples were completely dried under speed vacuum.
MALDI TOF-TOF MS – Samples were desalted and then spotted in duplicate onto a 384-well MALDI plate. Matrix (α-cyano-4-hydroxycinnamic acid in H_2O/ACN 50:50, 10 mM $NH_4H_2PO_4$) was then added and mass spectra were acquired with a MALDI TOF-TOF 4800 analyzer (Applied Biosystems, Foster City, CA) using the positive ionization mode and an *m/z* scan window of 800-4000 Th. The 20 most intense precursors were then subjected to MS/MS analysis. Argon was used as collision gas with the medium collision energy mode.

LTQ-OT MS - Electrospray ionization (ESI) LTQ-OT MS was performed on a LTQ Orbitrap XL from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters (Milford, MA, USA). Separation was run on a home-made analytical column using a gradient of H_2O and CH_3CN. Mass spectra were acquired in the positive mode with an *m/z* window of 400-2000 Th. A maximum of 4 precursors were selected for collision-induced dissociation with analysis in the LTQ (isolation width of 2 *m/z*). The normalized collision energy was set to 35%.

Protein identification – Peak lists were generated using either the 4000 Series Explorer software from Applied Biosystem (MALDI TOF/TOF) or the embedded software (extract MSN.exe) from Thermo Electron (LTQ-OT). This was followed by searching for peak lists individually against UniProt-Swiss-Prot database (57.4 of 16-Jun-2009, 565634 protein entries) using Phenyx 2.6 (GeneBio, Geneva, Switzerland). *Homo sapiens* taxonomy (40335 protein entries) was specified for database searching. Variable amino acid modification was oxidized methionine, while carbamidomethylation of cysteines was set as fixed modification. Trypsin was selected as the enzyme. The peptide p value was 1 E-6. Protein accession number and peptide scores were set up at 7.0 for both instruments. The minimum peptide length was six amino acids. The parent ion tolerance was 1.0 Da for MALDI TOF-TOF and 10 ppm for LTQ-OT. The scores were set to have a false discovery rate below 1%. For all subsequent analyses, only proteins identified with two different peptide sequences were kept.

Quantitative mass spectrometry with sixplex TMT - Eighteen age and sex matched CSF samples, comprising 9 S1 and 9 S2, were pooled in groups of three to obtain 6 different pools (i.e. 3 S1 and 3 S2 pools).

Depletion by immunoaffinity – Each pool was spiked with 1 µg of bovine β-lactoglobulin (Sigma, St Louis, MO, USA) and then subjected to depletion of fourteen abundant proteins using MARS Hu-14 column (Agilent Technologies, Wilmington, DE, USA). After collection of the flow-through fractions (containing unbound proteins), buffer was exchanged with H_2O using AMICON ultra-15 centrifugal filter units (Millipore,

Billerica, MA, USA) and samples dried completely under speed vacuum.

Table 1 Characteristics of the population assessed with ELISA for verification experiments were mainly performed as described by Dayon *et al*.[20]

		Stage 1 (S1)	Stage 2 (S2)
Population	n	21	37
Gender	Male	8	24
	Female	13	13
Age	Median (range)	32 (14-60)	35 (16-65)
WBC/µL	Median (range)	2 (0-5)	91 (6-2064)
Patients with parasites in CSF	n	0	25
Neurological signs*	Absence	11	10
	Moderate	10	21
	Severe	0	5

*Neurological signs were not reported for one patient

Reduction, alkylation, digestion, and TMT labelling – Reduction, alkylation, digestion, and TMT labelling were mainly performed as described by Dayon *et al*.[20] Briefly, reduction was carried out for 1 hour at 60°C after addition of TCEP 50 mM. Alkylation was done with IAA 400 mM (30 minutes in the dark) and overnight digestion was performed at 37°C with freshly prepared trypsin (0.2 µg/µl). Each sample was then labelled with one of the 6 TMT reagents (Proteome Sciences, Frankfurt, Germany) according to manufacturer's instructions. The three pools corresponding to S1 patients were labelled with TMTs 126.1, 128.1 and 130.1 respectively. Pools corresponding to S2 patients were labelled with the 3 other TMTs (i.e. 127.1, 129.1 and 131.1). All the samples were finally pooled and evaporated under speed-vacuum.

Off-gel electrophoresis – Off-gel electrophoresis was performed according to manufacturer's instructions (Agilent). Briefly, desalted and dehydrated samples were reconstituted in OFFGEL solution. Focusing was done on an IPG dry strip (13 cm, pH 3-10, linear; GE Healthcare) set up with a 12-well frame, for 20 kVh with a maximum current of 50 µA and power of 200 mW. The collected fractions were desalted, evaporated under speed-vacuum and stored at -20°C.

Liquid chromatography (LC) MALDI TOF-TOF MS – LC-MS/MS was performed as described by Dayon *et al.*[20] Each sample was subjected to reverse-phase chromatography using an Alliance LC system (Waters) and deposited directly on a MALDI plate using a home-made spotter. After matrix addition, mass spectra were acquired with a MALDI TOF-TOF 4800 analyzer as described before.

LC ESI LTQ-OT MS - ESI LTQ-OT MS was performed as described elsewhere.[27] Mass spectra were acquired in the positive mode with an *m/z* window of 400-2000 Th. A maximum of 3 precursors were selected for high-energy C-trap dissociation with analysis in the OT. The normalized collision energy was set to 40% for HCD.

Protein identification – Generation of peak lists was done in the same way as for 2-DE identifications, using either the 4000 Series Explorer software from Applied Biosystems (MALDI TOF/TOF) or the embedded software (extract_MSN.exe) from Thermo Electron (LTQ-OT). The peak lists generated from the 12 off-gel fractions were analysed as described for 2-DE, with the following modifications. TMT-sixplex amino terminus and TMT-sixplex lysine (+229.1629 Da) were additionally set as fixed modifications. The AC and peptide scores were set up at 7.7 for the analysis with MALDI TOF-TOF and 12.5 for the analysis with LTQ-OT with a false peptide discovery rate evaluated at 0.95% and 0.99% respectively. The parent ion tolerance was set to 1.1 Da for MALDI TOF-TOF and to 6 ppm for LTQ-OT. *Bos taurus* taxonomy (8168 entries) was separately specified to search for the spiked LACB. For all analyses, only proteins identified with two different peptide sequences were selected. To search for parasite proteins, a database restricted to *Homo sapiens* and *Kinetoplastida* taxonomies (92136 protein entries) was created using the FASTA files available from Uniprot (www.uniprot.org) and the same parameters described earlier were applied.

Protein quantification - Quantification of proteins was mainly done following the procedure described by Dayon *et al*.,[20] who demonstrated the accuracy of relative quantitation with TMT method using both protein mixture model and CSF samples and estimated a technical variation between 7% and 20%. The following procedure was used in the present study. Based on the data obtained with Phenyx, TMT reporter peak intensities (or area under peak for MALDI TOF-TOF results) of each identified peptide were extracted (Supporting MS information). An isotopic purity correction was done for each reporter based on the isotopic distribution of sixplex-TMT provided by the manufacturer. Isotopic purity equations were calculated using Maple software (Maple 11, Maple Inc., Waterloo, Canada). Peptides with missing reporter intensities were removed from the quantification. Spiked LACB was used to minimize experimental biases, and a normalization of the reporter intensities by the sum of all the reporter intensities was performed. A S2/S1 ratio was

calculated for each peptide as the sum of S2 channels (127.1, 129.1 and 131.1) divided by the sum of S1 channels (126.1, 128.1 and 130.1). Peptide ratios were further subjected to outlier removal using the Outlier software(http://www.sediment.unigoettingen.de/staff/dunkl/software/outlier.html).

This web-free software computes 4 different outlier tests: the Grubbs test, the Dixon test, the IQR test, and the Gauss g-test. A peptide was considered an outlier and the protein removed from the quantification if one of these tests was positive. The protein ratio S2/S1 was calculated as the geometric mean of its corresponding peptide ratios. For each protein ratio, the geometric standard deviation (SD) was calculated as described by Tan *et al*. [28] and the coefficient of variation (CV) determined as the SD divided by the protein ratio, and multiplied by 100. To determine the cut-off threshold for considering a protein differentially expressed between the two stages, a method based on random ratios was applied. For each identified peptide, the normalized intensities of S1 reporters were duplicated in order to mimic a sixplex experiment (126.1, 128.1, 130.1, 126.1*, 128.1* and 130.1*). All these intensities were then randomly mixed and ratios calculated as described above: sum (126.1*, 128.1*, 130.1*) / sum (126.1, 128.1, 130.1). The SD was evaluated among the random ratios obtained. In an ideal case, all the ratios calculated should be equal to one. Thus, the significant cut-off threshold to consider a protein up-regulated in S2 patients was determined as 1 + 2 SD.[28] The corresponding cut-off for down-regulated proteins was calculated as the reciprocal value. The results of these calculations are presented in Supporting MS information. Quantified proteins presenting a CV > 50% were removed. Proteins quantified with both instruments were only excluded if in both cases the CV was higher than 50%.

Western blot - The expression of complement factor H (CFH), osteopontin (OPN), and beta-2-microglobulin (B2MG) was evaluated by Western blot in 4 S1 and 4 S2 CSF samples. Goat anti-complement factor H polyclonal antibody (Calbiochem – Merck, Darmstadt, Germany) was used at a concentration of 8.4 µg/ml. Mouse anti-osteopontin and mouse anti-beta-2-microglobulin monoclonal antibodies (Abcam, Cambridge, UK) were used at a final concentration of 1 µg/ml and 5 µg/ml respectively. All HRP-conjugated secondary antibodies were purchased from Dako (Glostrup, Denmark) and applied at 1:1000 (anti-mouse secondary antibody) and 1:2000 (anti-goat secondary antibody) dilutions. The images obtained were analysed with ImageQuant™ TL 7.0 (GE Healthcare) and band volume data analysed with GraphPad Prism software (version 4.03, GraphPad software Inc., San Diego, CA, USA) to determine significant differences.

ELISA - The concentrations of CFH, OPN and B2MG were measured in CSF of HAT patients using commercially available sandwich ELISA kits (B2MG, Calbiotech, CA, USA; OPN, R&D Systems, MN, USA; CFH, Hycult Biotech, NL, USA), following manufacturer's instructions. Detailed description of patients whose CSF was analysed are reported in Table 1. CSF samples were diluted 1:50 for CFH and 1:101 for OPN and B2MG. After colour development, absorbance was read on a Vmax Kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 450 nm. The concentration of the 3 proteins in the CSF samples was back-calculated using either 4-PL or 5-PL curves (SoftMax Pro software, Molecular Devices, CA, USA) based on the measured respective standard values.

Data and statistical analysis - Descriptive statistics were performed using GraphPad Prism 4.03 software. As none of the proteins presented a normal distribution (Kolmogorov-Smirnov test), differences between groups were tested with the non-parametric Mann-Whitney U test (comparison between 2 groups) and Kruskal-Wallis test followed by Dunn's post-hoc test (comparison between 3 groups). Statistical significance for the tests was set at 0.05 (2-tailed test). The concentrations of the different molecules were considered as independent variables. Bivariate non-parametric correlations using the Spearman rho coefficient were carried out with statistical significance set at 0.01 (2-tailed test). To calculate sensitivity and specificity of individual predictors with respect to staging, the specific receiver operating characteristic curve of each analyte was determined. The cut-off value was selected as the threshold predicting stage 2 patients with 100% specificity. Aabel software (version 2.4.2, Gigawiz Ltd. Co., Tulsa, OK, USA) was used for box plots.

Protein combination and panel selection - To evaluate the possibility of improving the potential of the 3 molecules in staging HAT patients, they were combined in a panel as described by Hainard *et al.* [14]. Briefly, the optimized cut-off values were obtained by modified iterative permutation-response calculations (rule-induction-like) using the 3 analytes. Each cut-off value was changed iteratively by quantiles of 2%

increment and sensitivity determined after each iteration, until a maximum sensitivity for 100% specificity was achieved.

RESULTS

Two-dimensional gel electrophoresis – The protein expression patterns of the 9 CSF samples separated by 2-DE, just before image analysis, showed macroscopic differences between the two stages of disease, principally in expression of immunoglobulin. IgM and IgG heavy and light chains were particularly increased in samples from second stage compared to first stage patients, as previously shown by nephelometry.[7] The data on percent spot volume provided by the

Table 2A Protein spots over-expressed in stage 1 CSF samples

Spot #	MW	pI	DB entry	Description	S1/S2Ratio
1	23'010.01	6.15	P02753	Plasma retinol-binding protein (1-176)	3
	69'366.70	5.98	P02768	Serum Albumin	
2	75'181.46	6.84	P02787	Serotransferrin (Transferrin)	2.29
	49'306.61	6.55	P01871	Ig mu chain C region	
3	45'265.84	5.40	P01011	Alpha-1-antichymotrypsin His-Pro-less	8.48
	53'154.22	5.96	P01019	Angiotensin-3 (Ang III)	
	53'863.55	5.13	Q96KN2	Beta-Ala-His dipeptidase	
4	46’342.31	6.12	P36955	Pigment epithelium-derived factor (PEDF)	2.43
5	77'049.89	6.97	P02787	Serotransferrin (Transferrin)	2.25
6	18'698.03	8.68	P41222	Prostaglandin-H2 D-isomerase	3.19
7	18'698.03	8.68	P41222	Prostaglandin-H2 D-isomerase	2.89
	11'608.85	6.04	P01834	Ig kappa chain C region	
	11.236.69	5.56	P01842	Ig lambda chain C region	
8	50'062.57	5.97	P10909	Clusterin alpha chain (Apo J)	3.38
9	34'236.69	5.56	P02649	Apolipoprotein E (Apo E)	3.03
10	46'342.31	31	P36955	Pigment epithelium-derived factor (PEDF)	3.58
11	18'698.03	8.68	P41222	Prostaglandin-H2 D-isomerase	4.07
	11'236.52	7.90	P01842	Ig lambda chain C region	
	11'608.85	6.04	P01834	Ig kappa chain C region	
12	23'511.57	5.02	P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (OMD 1)	51.99
	23'602.63	5.13	P19652	Alpha-1-acid glycoprotein 2 (AGP 2) (OMD 2)	
13	20'168.93	6.22	P05452	Tetranectin (TN)	5.99
14	69'366.70	5.98	P02768	Serum Albumin	11.16
	80'640.64	5.64	P06396	Gelsolin (ADF) [ISOFORM 2]	
	49'306.61	6.55	P01871	Ig mu chain C region	
15	69'366.70	5.98	P02768	Serum Albumin	3.24

Table 2B Protein spots over-expressed in stage 2 CSF samples

Spot #	MW	pI	DB entry	Description	S2/S1Ratio
16	15'998.41	7.13	P68871	LVV-hemorphin-7	2.64
	11'731.17	6.46	P61769	Beta-2-microglobulin form pI 5.3	
	69'366.70	5.98	P02768	Serum Albumin	
17	11’608.65	6.04	P01834	Ig kappa chain C region	2.00
	11'236.52	7.90	P01842	Ig lambda chain C region	
18	11'608.85	6.04	P01834	Ig kappa chain C region	4.68
	11'236.52	7.90	P01842	Ig lambda chain C region	
	69'366.70	5.98	P02768	Serum Albumin	
19	11'731.17	6.46	P61769	Beta-2-microglobulin form pI 5.3	2.02
20	75'181.46	6.84	P02787	Serotransferrin (Trnasferrin)	5.25
	51'790.42		P01871	Ig mu chain C region [ISOFORM 2]	
	11'608.85	6.04	P01834	Ig kappa chain C region	
21	13'714.57	6.41	P61769	Beta-2-microglobulin form pI 5.3	1.77
22	11'608.85	6.04	P01834	Ig kappa chain C region	6.17
23	11'608.85	6.04	P01834	Ig kappa chain C region	5.02
24	11'608.85	6.04	P01834	Ig kappa chain C region	12.06
	69'366.70	5.98	P02768	Serum Albumin	
25	11'236.52	7.90	P01842	Ig lambda chain C regions	9.43
26	26'855.73	7.57	Q92876	Kallikrein-6	9.07
	11'608.85	6.04	P01834	Ig kappa chain C region	
	31'105.92	8.60	P01857	Ig gamma-1 chain C region	

Table 2C Protein spots expressed only in stage 2 CSF samples

Spot #	MW	pI	DB entry	Description
27	49'306.61	6.55	P01871	Ig mu chain C region [ISOFORM 2]
28	11'139.59	7.91	P04080	Cystatin-B
	69'366.70	5.98	P02768	Serum Albumin
29	11'236.52	7.90	P01842	Ig lambda chain C region
30	85'532.90	6.81	P00751	Complement factor B Bb fragment
	49'306.61	6.55	P01871	Ig mu chain C region
31	11'608.85	6.04	P01834	Ig kappa chain C region
32	49'761.12	5.68	P01019	Angiotensin-3 (Ang III)
33	69'366.70	5.98	P02768	Serum Albumin
34	69'366.70	5.98	P02768	Serum Albumin
	36'105.92	8.60	P01857	Ig gamma-1 chain C region
	11'608.85	6.04	P01834	Ig kappa chain C region
35	69'366.70	5.98	P02768	Serum Albumin
36	75'181.46	6.84	P02787	Serotransferrin (Transferrin)
	69'366.70	5.98	P02768	Serum Albumin
	37'654.65	6.26	P01876	Ig alpha-1 chain C region
	11'608.85	6.04	P01834	Ig kappa chain C region
37	75'181.46	6.84	P02787	Serotansferrin (Transferrin)
	69'366.70	5.98	P02768	Serum Albumin
38	113'715.57	4.92	Q02413	Desmoglein-1 (DG1) (DGI)

software were used to evaluate protein spots expression. This resulted in 754 spots matched between S1 and S2 master gels, of which 59 had a p value < 0.05. The 59 comprised 25 spots expressed in S2 gels only, 13 over-expressed in S2 gels compared to S1 with an average percent volume ratio S2/S1 larger than 2, and 21 over-expressed in S1 compared to S2 (average percent volume ratio S1/S2 > 2). Among the 59 spots, 52 were visualised and excised from the preparative S2 gels and subsequently analysed by LTQ-OT MS and/or with MALDI TOF-TOF MS. Out of the 52 spots, 38 (73.1%) were successfully identified as corresponding to 25 different proteins, as shown in Table 2a-c and in Figure 1.

Quantitative mass spectrometry with sixplex TMT - The six TMT-labelled pools were subjected to protein identification and relative quantitation using both MALDI TOF-TOF MS and LTQ-OT MS. With MALDI TOF-TOF MS, 128 proteins were identified from a total of 916 tryptic peptides. In the same way, LTQ-OT MS allowed the identification of 141 proteins from a total of 3334 tryptic peptides (Supporting MS information). In all, 172 proteins were identified, each with at least two unique peptides. Among these proteins, 97 were identified with both instruments, 44 with LTQ-OT only and 31 with MALDI TOF-TOF only. Interestingly, no parasite protein was identified after the simultaneous search against *Homo sapiens* and *Kinetoplastida* databases.

After removal of the unquantifiable and outlier peptides, the ratio and corresponding SD were calculated for each protein (Supporting MS information). The significant cut-off thresholds were then evaluated for each set of data as described before. With MALDI-TOF-TOF MS, the SD for random S1 ratio was 0.323, thus 1.65 (1 + 2 SD) was selected as the up-regulation significant threshold ratio (S2/S1) and reciprocally, 0.61 was identified as the down-regulation significant threshold. With ESI LTQ-OT MS, calculated SD was 0.370 and up-regulation and down-regulation thresholds were 1.74 and 0.57 respectively (Supporting MS information). According to these thresholds, one protein (i.e. C-reactive protein) was down-regulated while 59 were significantly up-regulated in second stage patients. Out of the 59 proteins, 29 were quantified with both MS instruments, 8 with the LTQ-OT only and 22 with the MALDI TOF-TOF only (Supporting Tables S1 a-c).

The two proteomic approaches used in the present study identified 85 proteins differentially expressed between S1 and S2 HAT. Among these, 73 were over-expressed in S2 CSF samples. The two discovery techniques were highly complementary as, among all proteins over-expressed in S2 patients, only immunoglobulin chains, beta-2-microglobulin and complement factor B were identified with the two approaches. Three over-expressed proteins, including complement factor H, osteopontin and beta-2-microglobulin were chosen for verification by immunoassay methods on a larger number of patients.

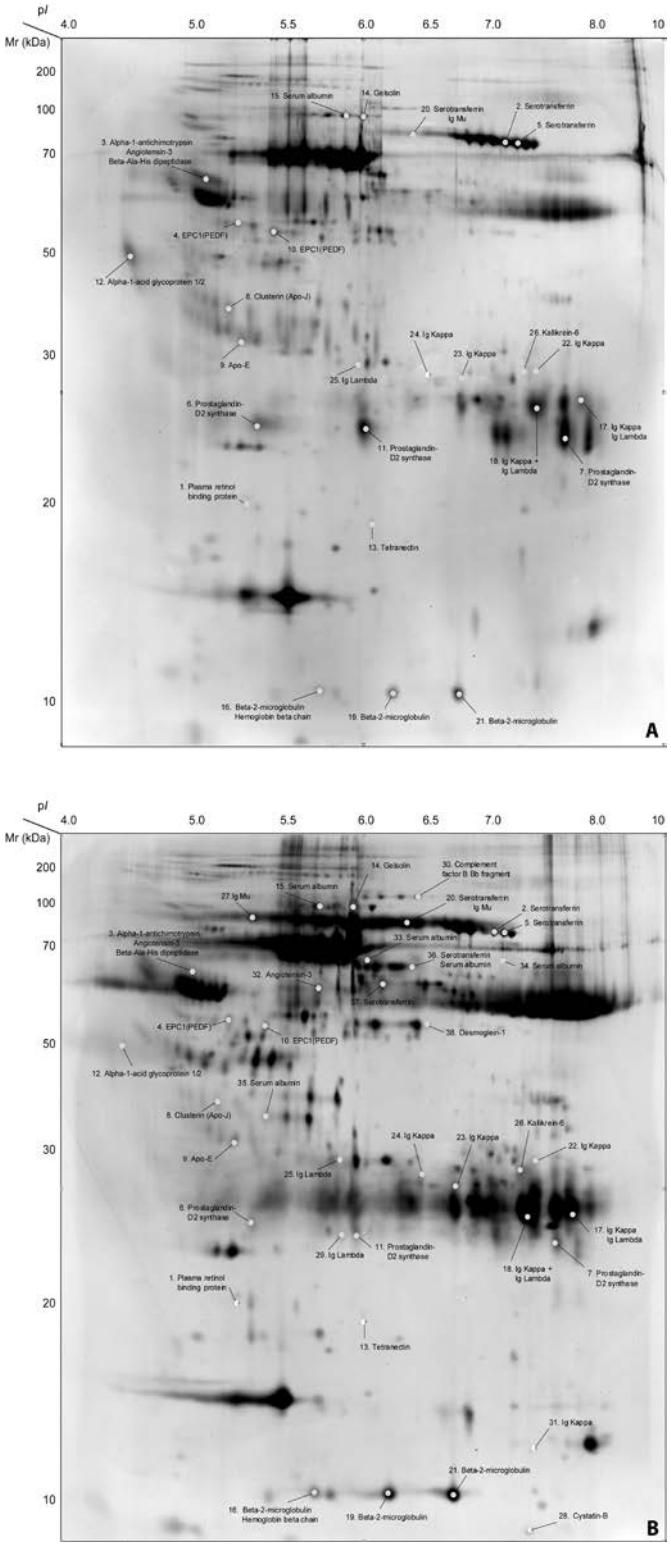

Figure 1 Representative 2-DE image of cerebrospinal fluid from stage 1 (A) and stage 2 (B) HAT patients. Master gels obtained separating 250 µl of CSF of a S1 (A) and a S2 patient (B). For each gel, proteins were separated on 18 cm pH 3-10 NL IPG-strips (GE Healthcare). The second dimension was performed on 12.5% polyacrylamide gels and proteins were finally visualized with silver staining. The 38 identified protein spots are reported on the gels. Spots 1-15: spots over-expressed in stage 1 patients (S1/S2 > 2.0, p value < 0.05); spots 16-26: spots over-expressed in stage 2 patients (S2/S1 > 2.0, p value < 0.05); spots 27-38: spots expressed only in stage 2 patients. The approximate pI and MW (kDa) have been assigned according to the CSF SWISS-2DPAGE map available on the ExPASy website (http://www.expasy.org/ch2d/).

Table 3 Staging performance of the 3 verified proteins according to the different techniques

	Quantitative MS (n=18)		Western blot (n=8)		ELISA (n=52-58)				
Protein name	**Ratio[a]**	**SD[a]**	**Ratio**	**p value[b]**	**Ratio**	**p value[b]**	**% AUC**	**% Sensitivity**	**% Specificity**
Beta-2-microglobulin (B2MG)	6.33 / 4.90	1.78 / 1.98	2.37	<0.05	5.51	<0.0001	92	78	100
Osteopontin (OPN)	3.64	0.28	6.00	<0.05	3.83	<0.0001	85	68	100
Complement factor H (CFH)	1.93 / 1.97	0.22 / 0.30	1.29	ns	1.51	<0.05	73	31	100

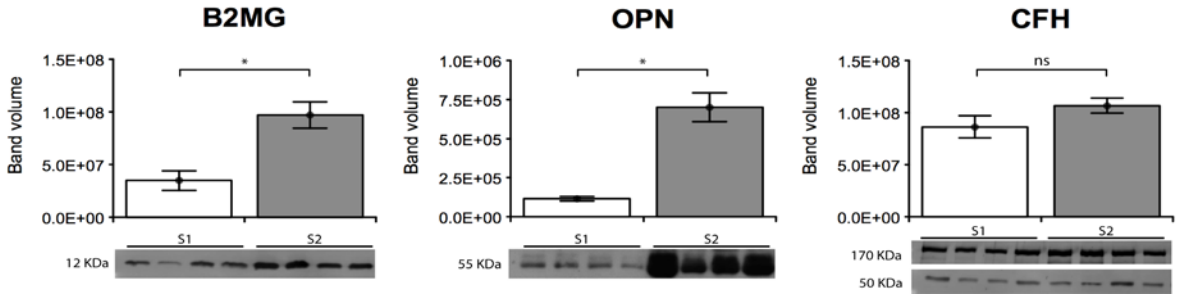

Figure 2 Western blot results for B2MG, OPN and CFH. Expression of B2MG, OPN and CFH in cerebrospinal fluid of early and late stage HAT patients (n = 8). The same volume for each sample (10 µl for B2MG, 20 µl for OPN and CFH) was analysed on a 12.5% (B2MG) or 10% (OPN and CFH) polyacrylamide gel. Bars represent the mean quantified band volume with the respective standard error. * corresponds to significant p value < 0.05; ns corresponds to non significant p value (Mann-Whitney U test). Images of the corresponding analysed bands are presented below each graph.

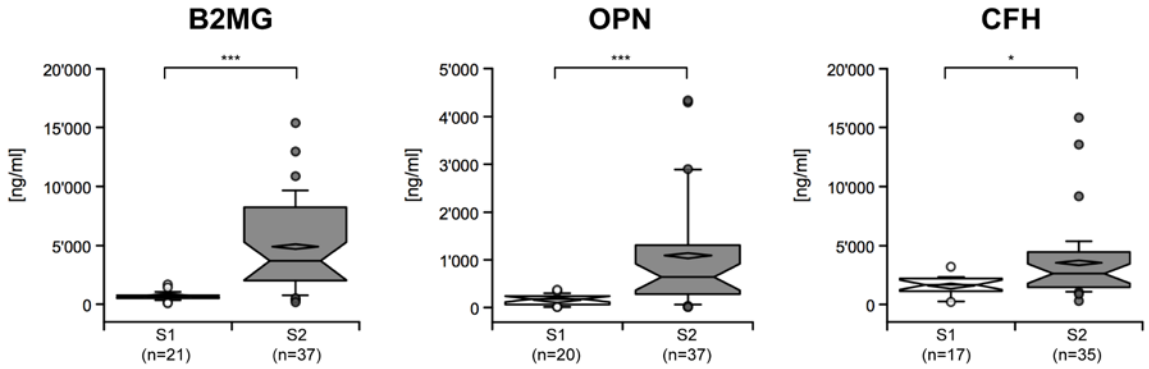

Figure 3 Box-plot of B2MG, OPN and CFH concentrations according to HAT staging**.** ELISA results representing the measured concentrations (ng/ml) in S1 and S2 CSF samples of B2MG (n = 58), OPN (n = 57), and CFH (n = 52). Median and mean are represented as a solid line in the box and a diamond, respectively. Whisks are defined as 5^{th}-95^{th} percentile with outliers. Half-width of the notch was calculated automatically by the software. *** and * correspond to a significant difference between the two groups, respectively < 0.0001 and < 0.05 (Mann-Whitney U test). S1: stage 1 samples; S2: stage 2 samples.

Verification by Western blot – CFH was identified as over-expressed using the TMT approach, with S2/S1 ratios of 1.93 and 1.97 for LTQ-OT and MALDI TOF-TOF instruments respectively. The detection of CFH with a polyclonal anti-CFH antibody on Western blot resulted in the visualization of two intense bands (50 and 170 kDa) among other weaker ones (data not shown). According to the molecular weight, the higher band

most likely corresponded to the complete form of CFH, while the lower one might correspond to the factor H-like protein 1 (FHL-1), obtained from alternative splicing.[29] However, when considered together or separately, the volumes of the two bands were not significantly different between the S1 and S2 groups (Table 3 and Figure 2). Western blot results confirmed the over-expression of OPN in the CSF of S2 patients, which was previously detected with the TMT approach (MALDI TOF/TOF MS ratio S2/S1 = 3.64). The acidity and characteristic behaviour of the protein during gel separation[30] is likely to hinder its visualization on 2-DE gels. Although the reported molecular weight is approximately 35 kDa, the observed 55 kDa band should correspond to the principal form of OPN as the protein undergoes extensive post-translational modifications, which make its molecular weight higher than the theoretical one.[30] In Western blot the quantified volume of this 55 kDa band was significantly increased in S2 compared to S1 patients (p value < 0.05, Mann-Whitney U test; median band volume S2/S1 ratio = 6.00) (Table 3 and Figure 2).

The third protein, B2MG, was identified with both discovery techniques. Three spots were identified as B2MG on 2-DE gels and the geometric mean of the percentage volume ratio S2/S1 of the three spots was of 2.11. With quantitative MS B2MG was identified with S2/S1 ratios of 6.33 (LTQ-OT MS) and 4.90 (MALDI TOF-TOF MS) (Table 3). These results were confirmed by Western blot, where a single 12 kDa band was visualized in all HAT samples, with a statistically significant increase in band volume in S2 samples (p value < 0.05, Mann-Whitney U test) (Figure 2) and a calculated ratio on the median band volume S2/S1 of 2.37 (Table 3).

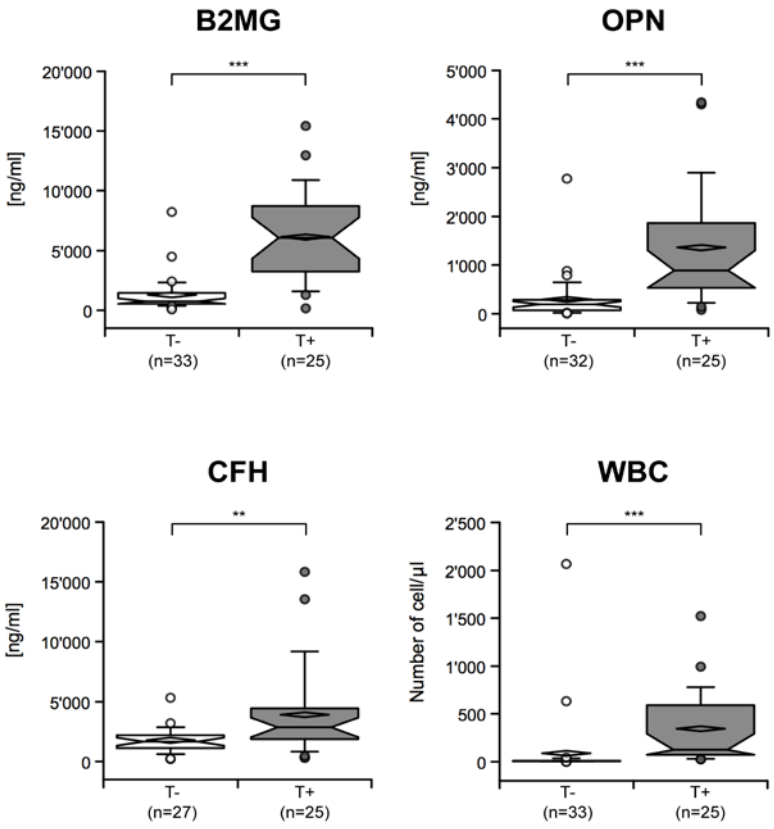

Figure 4 Box-plot of B2MG, OPN, CFH concentrations and WBC number, classified according to the presence of the parasite in CSF. Median and mean are represented as a solid line in the box and a diamond, respectively. Whisks are defined as 5th-95th percentile with outliers. Half-width of the notch was calculated automatically by the software. *** and ** correspond to a significant difference between the two groups, respectively < 0.0001 and < 0.01 (Mann-Whitney U test). T-: patients without parasite in CSF; T+: patients having parasites in CSF.

Verification by ELISA – All CSF samples analysed by ELISA were classified in 2 groups based on their stage (21 S1 and 37 S2). The concentrations of B2MG and OPN resulted significantly increased in the CSF of S2

patients ($p < 0.0001$, Mann-Whitney U test). CFH showed a significant, but less marked, increased concentration in the same group of patients ($p < 0.05$, Mann-Whitney U test) (Figure 3). In order to assess the sensitivity and specificity of the three molecules, ROC curves were built (Supporting Figure S1). The staging efficiency of the 3 molecules was evaluated using the AUC and the sensitivity for 100% specificity. Indeed, this configuration (maximum of specificity) avoided false negatives, such that a patient positive with the test was truly in the second stage. CFH had the lowest AUC (0.723) and a sensitivity of 31% (95% CI; 17-49%) for 100% specificity. OPN and B2MG showed higher performances with AUC of 0.848 and 0.915, and sensitivity of 68% (95% CI; 50-82%) and 78% (95% CI; 62-90%) respectively. The S2/S1 ratio was calculated for each molecule, based on the median value of each group of patients. This ratio corresponded to 1.51 for CFH, which was the lowest, and 3.83 and 5.51 for OPN and B2MG respectively (Table 3). To evaluate the staging potential of the three molecules further, we assessed the correlation between their concentration in CSF and the number of WBC, the reference staging method. B2MG and OPN showed a high correlation with the number of WBC, with Spearman rho coefficients at 0.725 and 0.723 respectively. There was also a significant correlation between CFH and the number of WBC but with a lower Spearman rho coefficient (0.562) compared to the others. Detailed results for the 3 molecules are reported in Supporting Table S2.

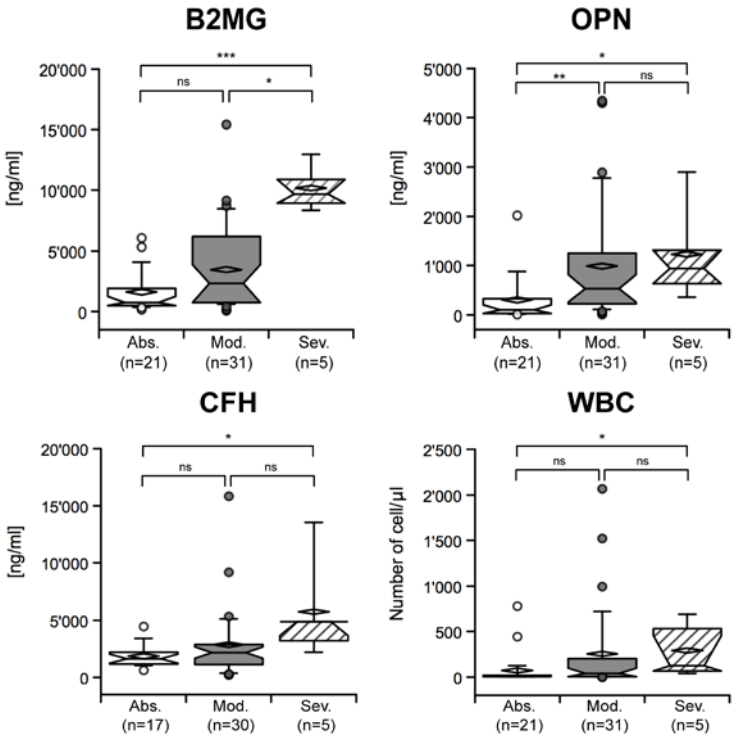

Figure 5 Box-plot of B2MG, OPN, CFH concentrations and WBC number, classified according to the severity of neurological signs. Median and mean are represented as a solid line in the box and a diamond, respectively. Whisks are defined as 5^{th}-95^{th} percentile with outliers. Half-width of the notch was calculated automatically by the software. *** corresponds to a significant difference between the two groups < 0.0001; ** corresponds to a significant difference < 0.01 and * < 0.05; ns indicates a non significant difference (Dunn's post-hoc test). Abs: absence of neurological signs; Mod: moderate neurological signs; Sev: severe neurological signs.

The concentration of the three molecules was then evaluated in relation to the presence or absence of parasites in CSF, and the neurological signs reported before treatment. The 3 proteins were significantly increased in the CSF of patients with parasites in CSF (Figure 4), with p values < 0.0001 for B2MG and OPN, and < 0.01 for CFH. When the severity of neurological signs was considered, CFH could only discriminate between the absence and the presence of severe neurological signs with a p value < 0.05 (Kruskal-Wallis test) (Figure 5). OPN showed a lower p value ($p < 0.001$, Kruskal-Wallis) and significantly discriminated between all the groups, except between the moderate and severe ones. B2MG had the lowest p value ($p < 0.0001$, Kruskal-Wallis) and significantly discriminated between absent and severe neurological signs, as well as between the moderate and severe ones (Figure 5).

When the three molecules were combined using the RIL process, a panel comprising B2MG (cutoff: 1802.5 ng/ml) and OPN (cutoff: 408.8 ng/ml) that discriminated between first and second stage disease with a sensitivity of 91% for 100% specificity was identified (Supporting Figure S1). This panel gave a positive test response (i.e. S2 patient identification) any time the concentration of one molecule was above its cut-off value.

DISCUSSION

In this study, we analysed CSF samples from *T. b. gambiense* infected patients with a combination of proteomic strategies to identify new biomarkers that could complement or replace current methods of staging HAT. A total of 73 host proteins whose expression was increased in patients presenting the second stage of disease were identified. No parasite proteins were identified with the applied approaches, probably because CSF samples were first centrifuged for the parasitological examination[23] leading to removal of all parasites and cells.

The application of two different proteomic strategies was particularly useful in obtaining complementary information, such that only few proteins were commonly found. This can be explained if the different workflows as well as the intrinsic limitations associated to both techniques are taken into account. All samples analysed by TMT mass spectrometry were first depleted of the 14 most abundant proteins while whole CSF samples were separated by 2-DE. Further, 2-DE is based on protein separation with potential identification of specific protein isoforms but also loss of hydrophobic proteins, while TMT quantitative mass spectrometry is based on peptide separation. Many steps in the workflow of the latter approach could lead to peptide loss, such as the use of TCEP for protein reduction,[31,32] peptide-gel interaction during the off-gel electrophoresis separation, and the peptide tagging. In addition, proteins identified with 1 unique peptide were excluded from subsequent analysis. The use of more optimized protocols, especially for the TMT MS approach, could therefore lead to the discovery of less abundant proteins or specific trypanosome antigens. These antigens are known to be present in host's CSF,[33] but probably they are not concentrated enough to be detected with the techniques applied in the present study.

A preliminary analysis of the functions of the proteins identified indicates that they are involved in the immune response, both cell-mediated and humoral, as well as in cell-cell adhesion and transport. The 3 proteins chosen for further verification, CFH, OPN and B2MG, were differentially expressed between the 2 stages (i.e. S2/S1 ratios higher than 2 for 2-DE, and higher than 1.65 or 1.74 for TMT MS), and to our knowledge, they have never been described in HAT patients. Furthermore, based on their known functions, these proteins could potentially be involved in disease progression.

It is well established that many pathogens can find mechanisms to escape the host immune response and one of the main targets of these evasion mechanisms is the complement cascade.[34] Trypanosomes are able to activate the alternative complement pathway in blood,[35] while decreased complement activation was reported during the 80's in infective cultures of *T. cruzi,* responsible for Chagas' disease.[36] Complement factor H is the principal inhibitor of the alternative pathway, and is also involved in the protection of epithelial, endothelial, and some cancer cells against complement action.[37] In our population of *T. b. gambiense* patients, over-expression of CFH during the second stage of disease was only confirmed with ELISA. Despite the high similarity between the results obtained by TMT and ELISA, a well established quantitative method widely applied in clinical research,[38] CFH did not come out as a promising marker for staging HAT, since the AUC was only 0.73, which was below the 0.8 arbitrary limit that we established for considering a test as having staging potential. Furthermore, the ratio calculated from the TMT results was close to the cut-off for considering a protein over-expressed in S2 patients.

The data for OPN and B2MG were very promising, with both discriminating S1 and S2 patients with high accuracy, as indicated by the elevated AUC values.

OPN, also known as early T lymphocyte activation 1 (Eta-1), is expressed by a variety of immune and non-immune cells, including brain cells, macrophages and activated Th1 cells,[30,39] and is believed to act as a pro-inflammatory cytokine.[40] The protein is a ligand for two classes of adhesion molecules: CD44, expressed on activated and memory T cells, and different types of integrins, including $\alpha_V\beta_3$ and $\alpha_4\beta_1$, expressed by T lymphocytes. It also binds VCAM-1, which, in turn, is expressed on cytokine-activated endothelial cells.[41] At the same time, OPN induces the production of interferon-γ (IFN-γ) and IL-12 by macrophages, and inhibits the production of IL-10,[42] participating in the polarization of the cellular immune response towards the Th1 type involved in phagocytosis and killing of microbes. Expression of OPN is highly increased during chronic inflammatory diseases or tissue injury, especially in proximity of activated T cells and monocytes/macrophages.[43] The protein has been extensively studied in multiple sclerosis, where it appears to be involved, through the $\alpha_4\beta_1$ integrin, in the entry of effector T-cells in the brain,[41] and through CD44 receptor, in the permanence of T cells at the site of inflammation. Recent information has suggested that the levels of OPN in CSF are not disease specific, but can point to involvement of the CNS or damage to the blood-brain barrier (BBB).[44,45] The levels of OPN in the CSF of S2 HAT patients analysed in the present study were significantly higher than in S1 patients, suggesting an association between CSF OPN and disease progression.

B2MG, found differentially expressed with both discovery approaches, was revealed to have the highest staging potential. This 11.8 kDa protein, expressed on the surface of all nucleated cells, is non-covalently associated to the MHC class I molecules and therefore involved in cellular immune response against invading pathogens mediated by cytotoxic T-lymphocytes $CD8^+$.[46] A number of studies have reported that the levels of free B2MG in body fluids are increased in many malignant conditions[47] and they can be an indicator of a high cellular turnover.[48] It has been suggested that the levels of B2MG could correlate with both the degree of CNS involvement and neuronal damage in children with symptomatic congenital CMV infection.[48] Furthermore, proteomic and non-proteomic approaches have associated B2MG with many neurological disorders, including Alzheimer's disease[16,19] and cancers.[47] In the present study, CSF B2MG was significantly elevated in second stage patients, enabling the molecule to distinguish S1 and S2 patients with the best sensitivity and specificity.

The potential of B2MG and OPN as new markers for staging HAT patients was further supported by a highly significant correlation between their levels and the number of WBC in CSF. Furthermore, when compared with the number of WBC, CSF B2MG and OPN were better indices of both the presence of parasites in patients' CSF and the severity of neurological signs. Finally, when the concentrations of both proteins were considered together as a panel, they identified S2 patients with a sensitivity of 91%.

The CSF concentration of OPN and B2MG in both S1 and S2 HAT patients was relatively high (µg/ml range). This finding could be particularly relevant in the development of an antibody-based field test such as a lateral-flow assay, resulting in major improvement in accuracy of staging and reduction in costs, although still limited by the necessity of a lumbar puncture. The lumbar puncture could be eliminated during staging of HAT patients only if markers could be found in patients' blood. However, preliminary tests for B2MG on 30 plasma samples (15 S1 and 15 S2) did not reveal any significant differences between S1 and S2 (data not shown), probably as a consequence of the presence of parasites in the blood of both groups of patients.

In order to further validate the potential of CSF OPN and B2MG in staging HAT, a larger multi-centric cohort, including *T. b. gambiense* and *T. b. rhodesiense* patients, as well as control CSF from patients with other infectious diseases (e.g. TB, and HIV), will be carried out.

In conclusion, the present study has revealed beta-2-microglobulin and osteopontin as good markers that could potentially replace WBC count in staging HAT patients. Treatment of HAT patients is hampered by lack of safe drugs effective for both stages of the disease. Erroneous determination of the stage could, in fact, have serious consequences on the safety and health of patients, with S1 patients being unnecessarily exposed to the toxicity of stage 2 drugs, and S2 patients not getting cured with stage 1 drugs, and thus exposed to the risk of relapses and death. The present discovery of biomarkers that increase the accuracy of staging HAT represents an important improvement for guiding treatment decision.

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SUPPORTING INFORMATION

Supporting Table S1a Proteins found over-expressed with both instruments (A: LTQ-OT MS, B: MALDI TOF-TOF).

Supporting Table S1b Proteins found over-expressed with LTQ-OT MS.

Supporting Table S1c Proteins found over-expressed with MALDI TOF-TOF MS.

Supporting Table S2 Detailed results for the three molecules tested in respect with the stage of the disease.

Supporting Figure S1 ROC curves for B2MG, OPN, CFH, and the panel composed of B2MG and OPN. Cut-off values for each molecule [ng/ml] and for the panel are reported as a point with the corresponding numeric value on each ROC curve. Percentage sensitivity set for 100% specificity is reported in brackets on each graph. Area under the ROC curve (AUC) is also given.

Supporting MS information (.xls file)

REFERENCES

1. Kennedy PG. Human African trypanosomiasis of the CNS: current issues and challenges. The Journal of clinical investigation 2004;113:496-504.

2. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.

3. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.

4. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.

5. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.

6. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. Journal of the neurological sciences 2002;193:127-35.

7. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.

8. Fairlamb AH. Chemotherapy of human African trypanosomiasis: current and future prospects. Trends in parasitology 2003;19:488-94.

9. Blum J, Nkunku S, Burri C. Clinical description of encephalopathic syndromes and risk factors for their occurrence and outcome during melarsoprol treatment of human African trypanosomiasis. Tropical medicine & international health : TM & IH 2001;6:390-400.

10. Priotto G, Kasparian S, Mutombo W, et al. Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet 2009;374:56-64.

11. Lejon V, Lardon J, Kenis G, et al. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of Trypanosoma brucei gambiense sleeping sickness patients before and after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 2002;96:329-33.

12. Maclean L, Odiit M, Macleod A, et al. Spatially and genetically distinct African Trypanosome virulence variants defined by host interferon-gamma response. The Journal of infectious diseases 2007;196:1620-8.

13. Courtioux B, Boda C, Vatunga G, et al. A link between chemokine levels and disease severity in human African trypanosomiasis. International journal for parasitology 2006;36:1057-65.

14. Hainard A, Tiberti N, Robin X, et al. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS neglected tropical diseases 2009;3:e459.

15. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics 2006;6:6326-53.

16. Puchades M, Hansson SF, Nilsson CL, Andreasen N, Blennow K, Davidsson P. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. Brain research Molecular brain research 2003;118:140-6.

17. Pienaar IS, Daniels WM, Gotz J. Neuroproteomics as a promising tool in Parkinson's disease research. J Neural Transm 2008;115:1413-30.

18. Rithidech KN, Honikel L, Milazzo M, Madigan D, Troxell R, Krupp LB. Protein expression profiles in pediatric multiple sclerosis: potential biomarkers. Mult Scler 2009;15:455-64.

19. Zhang J, Keene CD, Pan C, Montine KS, Montine TJ. Proteomics of human neurodegenerative diseases. Journal of neuropathology and experimental neurology 2008;67:923-32.

20. Dayon L, Hainard A, Licker V, et al. Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Analytical chemistry 2008;80:2921-31.

21. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. The Journal of infectious diseases 2010;201:453-63.

22. WHO. Guidelines for the collection of clinical specimens during field investigation of outbreaks. 2000;WHO/CDS/CSR/EDC/2000.4 Available: http://www.who.int/csr/resources/publications/surveillance/whocdscsredc2004.pdf.

23. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:293.
24. Sanchez JC, Converset V, Nolan A, et al. Effect of rosiglitazone on the differential expression of diabetes-associated proteins in pancreatic islets of C57Bl/6 lep/lep mice. Molecular & cellular proteomics : MCP 2002;1:509-16.
25. Mortz E, Krogh TN, Vorum H, Gorg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. Proteomics 2001;1:1359-63.
26. Burgess JA, Lescuyer P, Hainard A, et al. Identification of brain cell death associated proteins in human post-mortem cerebrospinal fluid. Journal of proteome research 2006;5:1674-81.
27. Dayon L, Pasquarello C, Hoogland C, Sanchez JC, Scherl A. Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. Journal of proteomics 2010;73:769-77.
28. Tan HT, Tan S, Lin Q, Lim TK, Hew CL, Chung MC. Quantitative and temporal proteome analysis of butyrate-treated colorectal cancer cells. Molecular & cellular proteomics : MCP 2008;7:1174-85.
29. Cheng ZZ, Corey MJ, Parepalo M, et al. Complement factor H as a marker for detection of bladder cancer. Clinical chemistry 2005;51:856-63.
30. Sodek J, Ganss B, McKee MD. Osteopontin. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 2000;11:279-303.
31. Wang Z, Rejtar T, Zhou ZS, Karger BL. Desulfurization of cysteine-containing peptides resulting from sample preparation for protein characterization by mass spectrometry. Rapid communications in mass spectrometry : RCM 2010;24:267-75.
32. Liu P, O'Mara BW, Warrack BM, et al. A tris (2-carboxyethyl) phosphine (TCEP) related cleavage on cysteine-containing proteins. Journal of the American Society for Mass Spectrometry 2010;21:837-44.
33. Lejon V, Buscher P. Review Article: cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. Tropical medicine & international health : TM & IH 2005;10:395-403.
34. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. Nature reviews Microbiology 2008;6:132-42.
35. Donelson JE, Hill KL, El-Sayed NM. Multiple mechanisms of immune evasion by African trypanosomes. Molecular and biochemical parasitology 1998;91:51-66.
36. Joiner K, Sher A, Gaither T, Hammer C. Evasion of alternative complement pathway by Trypanosoma cruzi results from inefficient binding of factor B. Proceedings of the National Academy of Sciences of the United States of America 1986;83:6593-7.
37. Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. J Immunol 2000;164:6075-81.
38. Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clinical chemistry 2005;51:2415-8.
39. Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. Nature reviews Immunology 2009;9:137-41.
40. Ashkar S, Weber GF, Panoutsakopoulou V, et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 2000;287:860-4.
41. Steinman L. A molecular trio in relapse and remission in multiple sclerosis. Nature reviews Immunology 2009;9:440-7.
42. Chabas D, Baranzini SE, Mitchell D, et al. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 2001;294:1731-5.
43. Denhardt DT, Giachelli CM, Rittling SR. Role of osteopontin in cellular signaling and toxicant injury. Annual review of pharmacology and toxicology 2001;41:723-49.
44. Chowdhury SA, Lin J, Sadiq SA. Specificity and correlation with disease activity of cerebrospinal fluid osteopontin levels in patients with multiple sclerosis. Archives of neurology 2008;65:232-5.
45. Iwanaga Y, Ueno M, Ueki M, et al. The expression of osteopontin is increased in vessels with blood-brain barrier impairment. Neuropathology and applied neurobiology 2008;34:145-54.
46. Cresswell P, Bangia N, Dick T, Diedrich G. The nature of the MHC class I peptide loading complex. Immunological reviews 1999;172:21-8.
47. Shi C, Zhu Y, Su Y, Chung LW, Cheng T. Beta2-microglobulin: emerging as a promising cancer therapeutic target. Drug discovery today 2009;14:25-30.
48. Alarcon A, Garcia-Alix A, Cabanas F, et al. Beta2-microglobulin concentrations in cerebrospinal fluid correlate with neuroimaging findings in newborns with symptomatic congenital cytomegalovirus infection. European journal of pediatrics 2006;165:636-45.

CHAPTER 3

A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients

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The discovery of new disease biomarkers can be achieved through the evaluation of molecules, potentially involved in disease progression, based on their molecular function and on bibliographic knowledge. Since it has been proposed that the inflammatory process may play a central role in the development of HAT brain disease, which characterizes second stage patients, we hypothesized that immune related factor could be differentially expressed in the CSF of early and late stage patients.

In the present study, we investigated the CSF levels of 13 immune-mediators and 3 brain damage markers using immunoassays (either bead suspension assays or ELISAs) on a population comprising 100 *T. b. gambiense* patients, 21 stage 1 and 79 stage 2.

This study, published in PLoS NTD in 2009, highlighted the high staging power of CXCL10, individually or combined into a panel of three molecules with CXCL8 and H-FABP. I partially contributed in analysing the data and writing the manuscript, mainly the discussion.

A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients

Alexandre Hainard[1], **Natalia Tiberti[1]**, Xavier Robin[1], Veerle Lejon[2], Dieudonné Mumba Ngoyi[3], Enock Matovu[4], John Charles Enyaru[5], Catherine Fouda[1], Joseph Mathu Ndung'u[6], Frédérique Lisacek[7], Markus Müller[7], Natacha Turck[1], Jean-Charles Sanchez[1]

1 *Biomedical Proteomics Research Group, Medical University Centre, Geneva, Switzerland;* ***2*** *Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium;* ***3*** *Institut National de Recherche Biomedicale, Kinshasa, D.R. Congo;* ***4*** *Department of Veterinary Parasitology and Microbiology, Faculty of Science, Makerere University, Kampala, Uganda;* ***5*** *Department of Biochemistry, Faculty of Science, Makerere University, Kampala, Uganda;* ***6*** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland;* ***7*** *Swiss Institute of Bioinformatics, Medical University Centre, Geneva, Switzerland.*

SUMMARY

Background Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic tropical disease. It progresses from the first, haemolymphatic stage to a neurological second stage due to invasion of parasites into the central nervous system (CNS). As treatment depends on the stage of disease, there is a critical need for tools that efficiently discriminate the two stages of HAT. We hypothesized that markers of brain damage discovered by proteomic strategies and inflammation-related proteins could individually or in combination indicate the CNS invasion by the parasite.

Methods Cerebrospinal fluid (CSF) originated from parasitologically confirmed *Trypanosoma brucei gambiense* patients. Patients were staged on the basis of CSF white blood cell (WBC) count and presence of parasites in CSF. One hundred samples were analysed: 21 from stage 1 (no trypanosomes in CSF and ≤5 WBC/µL) and 79 from stage 2 (trypanosomes in CSF and/or >5 WBC/µL) patients. The concentration of H-FABP, GSTP-1 and S100β in CSF was measured by ELISA. The levels of thirteen inflammation-related proteins (IL-1ra, IL-1β, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 and CXCL10) were determined by bead suspension arrays.

Results CXCL10 most accurately distinguished stage 1 and stage 2 patients, with a sensitivity of 84% and specificity of 100%. Rule Induction Like (RIL) analysis defined a panel characterized by CXCL10, CXCL8 and H-FABP that improved the detection of stage 2 patients to 97% sensitivity and 100% specificity.

Conclusion This study highlights the value of CXCL10 as a single biomarker for staging *T. b. gambiense*-infected HAT patients. Further combination of CXCL10 with H-FABP and CXCL8 results in a panel that efficiently rules-in stage 2 HAT patients. As these molecules could potentially be markers of others CNS infections and disorders, these results should be validated on a larger multi-centric cohort including other inflammatory diseases such as cerebral malaria and active tuberculosis.

INTRODUCTION

Human African trypanosomiasis (HAT), also called sleeping sickness, is a parasitic disease that occurs in sub-Saharan Africa. More than sixty million people are at risk of being infected. The World Health Organization (WHO) has reported impressive progress since 1995 in the control of HAT, leading to a substantial reduction of new cases detected yearly to 10'800 in 2007. The total number of cases is now estimated to be between 50'000 and 70'000 per year.[1]

AUTHOR SUMMARY

The actual serological and parasitological tests used for the diagnosis of human African trypanosomiasis (HAT), also known as sleeping sickness, are not sensitive and specific enough. The card agglutination test for trypanosomiasis (CATT) assay, widely used for the diagnosis, is restricted to the *gambiense* form of the disease and parasitological detection in the blood and cerebrospinal fluid (CSF) is often very difficult. Another very important problem is the difficulty to stage the disease; a crucial step in the decision of the treatment to be given. While eflornithine is difficult to administer, melarsoprol is highly toxic with incidences of reactive encephalopathy as high as 20%. Staging, which could be diagnosed as early (stage 1) or late (stage 2), relies on the examination of CSF for the presence of parasite and/or white blood cell (WBC) counting. However, the parasite is rarely found in CSF and WBC count is not standardised (cutoff set between 5 and 20 WBC per µL). In the present study, we hypothesized that an early detection of stage 2 patients with one or several proteins in association with clinical evaluation and WBC count would improve staging accuracy, and allow more appropriate therapeutic interventions.

The parasite that causes HAT belongs to the *Trypanosoma brucei* family with two subspecies, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, responsible for the human disease. Trypanosomes are transmitted to humans by the bite of a tsetse fly and are initially confined to the blood, lymph nodes and peripheral tissues. This corresponds to the first stage (early stage; or haemolymphatic stage) of the disease. After an unknown period that varies from weeks to months, the parasites invade the central nervous system (CNS). This is called the second stage (late stage; or neurologic; or meningo-encephalitic stage) of HAT.

Clinical symptoms of HAT are not specific for the disease, and definite diagnosis is always based on parasitological examination of body fluids. The card agglutination test for trypanosomiasis (CATT), an assay that is based on trypanosome-specific antibody detection, is widely used for mass screening. However, it suffers from limited sensitivity and restricted to the *T. b. gambiense* form of the disease.[2] A positive parasitological diagnosis must always be followed by stage determination, which is performed by examination of the cerebrospinal fluid (CSF). This is a vital step in the diagnostic process, as the treatment differs depending on the stage of the disease. If HAT patients are not treated, they always die.[3-5] Early stage drugs are inefficient for late stage patients, and additionally, melarsoprol (MelB or Arsobal), which has been the most widely used drug to treat late stage patient, has itself an overall mortality rate of 5% due to its toxicity.[6] As a consequence, melarsoprol has in many countries been replaced by eflornithine as the first line treatment for *T. b. gambiense* infections but the latter drug suffers from important logistic constraints.

WHO defined late-stage HAT by the following criteria: presence of trypanosomes in CSF and/or an elevated WBC count above 5/µL of CSF.[7] However, presence of WBC in the CSF is not specific for the disease and parasite detection methods are not sensitive enough [8]. Furthermore, recent studies suggest the need to increase the cutoff between the first and second stages to 10 or 20 WBC/µL.[2,8,9] This has contributed to the concept of a potential intermediate stage of HAT with CSF WBC count >5 and ≤20 WBC/µL.[10] There is therefore a critical need for a reliable and efficient staging tool that would replace or complement trypanosome detection and WBC count.

Parasite migration and invasion of the CNS causes a neuroinflammatory process, associated with activation of microglial cells and astrocytes,[11,12] and infiltration of the CNS with leukocytes (predominantly mononuclear cells).[13] Cytokines and chemokines are known to be actively involved in this process. Thus, TNF-α, IL-6, CXCL8 and IL-10 concentrations have been demonstrated to be elevated in the CSF of late-stage patients[11,14] and the IFN-γ level has been reported as associated with the severity of the late stage disease.[15] The levels of CCL2, IL-1β and CXCL8 have also been correlated with presence of parasites in the CSF and neurological signs in HAT patients.[16] Additionally, levels of IL-1ra, G-CSF, VEGF, CCL4 and CXCL10 were found modulated in either the CSF or plasma of patients suffering from cerebral malaria,[17-19] and could potentially be also modulated in HAT patients.

Proteomic analysis of human body fluids has become an important approach for biomarkers discovery.[20] In this context, we recently explored the concept of *post-mortem* CSF as a model of massive and global brain insult,[21] which allowed the identification of potential brain damage biomarkers by proteomics strategies. Indeed, heart-fatty acid binding protein (H-FABP), identified from *post-mortem* CSF, has been validated as a marker of stroke[22] and Creutzfeldt-Jakob disease,[23] respectively. Similarly, GSTP-1 was also found over-expressed in *post-mortem* CSF[24] compared to *ante-mortem*, and was recently validated as an early diagnostic marker of stroke and traumatic brain

injury (Turck *et al*. Personal communication). Additionally, S100β protein has already been demonstrated to be a marker of blood-brain barrier (BBB) and neuronal damage[25] as well as a useful serum biomarker of CNS injury and a potential tool for predicting clinical outcome after brain damage.[26]
In this context, we hypothesized that markers of brain damage discovered by proteomic strategies as well as inflammation-related proteins could individually or in combination indicate the CNS invasion by the trypanosome parasite. We measured the CSF concentrations of H-FABP, GSTP-1, S100β and thirteen inflammation-related proteins (IL-1ra, IL-1β, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 and CXCL10) and evaluated their potential for staging the disease.

MATERIAL AND METHODS

Samples

Samples originated from a prospective observational study on shortening of post treatment follow-up in *gambiense* human African trypanosomiasis (THARSAT), conducted between 2005 and 2008 at Dipumba hospital in Mbuji-Mayi (Kasai Oriental province, Democratic Republic of the Congo). Details of the THARSAT study design and results are reported elsewhere (D. Mumba Ngoyi, in preparation). The study protocol was approved by the Ministry of Health, Kinshasa, DRC and by the Ethical Committee of the University of Antwerp, Belgium. Briefly, 360 *T. b. gambiense* patients in total were enrolled into the THARSAT study. Inclusion criteria were 1° confirmed presence of trypanosomes in lymph nodes, blood or CSF; 2° ≥12 years old and; 3° living within a perimeter of 100 km around Mbuji-Mayi. Exclusion criteria were 1° pregnancy; 2° no guarantee for follow-up; 3° moribund; 4° haemorrhagic CSF before treatment and; 5° presence of another serious illness (active tuberculosis - treated or not, bacterial or cryptococcal meningitis). HIV and malaria were not considered as exclusion criteria. Each patient underwent a clinical examination. Staging of disease was based on CSF examination. WBC count was performed in disposable cell counting chambers (Uriglass, Menarini) and was performed in duplicate when the first count was <20 cells/µL. Trypanosomes were searched for in CSF by direct examination prior or during the cell counting procedure, followed by the modified single centrifugation method.[27] Second stage patients were defined as having >5 WBC/µL and/or trypanosomes in the CSF. First stage patients were defined as having 0-5 WBC/µL and no trypanosomes in the CSF. Patients having >5 and ≤20 WBC/µL and no trypanosomes in CSF were defined and treated as stage 2 patients, but highlighted as being in the potential intermediate stage. Patients or their responsible were informed about the study objectives and modalities and were asked to provide written consent. Treatment was provided according to the guidelines of the national control program for HAT (PNLTHA).
CSF samples were centrifuged immediately after collection. The supernatant remaining after the diagnostic procedure was aliquoted, stored and shipped frozen at -20°C or below. For the study reported here, a total of 100 CSF samples, taken before treatment, were tested. These samples originated from 21 stage 1 (S1) and 79 stage 2 patients (S2). S1 patients were age and sex matched with 21 S2 patients. Remainder S2 patients were chosen in order to obtain homogenous median age values. Patients were classified into three categories of neurological signs; absent (no neurological signs), moderate (at least one major neurological sign but no generalised tremors) or severe (at least two major neurological signs including generalised tremors). Major neurological signs were defined as: daytime somnolence, sensory and gait disturbances, presence of primitive reflexes (Babinski's sign, palmo-mental reflex, perioral reflex), modified tendon reflexes (exaggeration or abolition), abnormal movements such as tremor (fine, diffuse and generalised). Neurological signs were not reported for two patients.

S100β, H-FABP and GSTP-1 measurements

The concentration of S100β was measured using a commercially available sandwich ELISA assay kit (Abnova, Taiwan) following the manufacturer's instructions. Briefly, calibrators, Quality control (QC) and CSF samples diluted 1:4 were incubated 2 hours on microtiter strips pre-coated with polyclonal anti-cow S100β antibodies. After 3 washes, horseradish peroxidase (HRP) labelled anti-human S100β antibodies were added, incubated for 90 minutes and washed again before addition of the substrate solution (tetramethylbenzidine). Color development was stopped with sulphuric acid and absorbance was read on a Vmax Kinetic microplate reader, (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) at a wavelength of 450 nm.
H-FABP concentration was also determined using a commercially available ELISA kit (Hycult Biotechnology, Uden, Netherlands) according to the manufacturer's instructions. CSF samples (non-diluted) and standards were incubated (1 hour) together with peroxidase conjugated secondary antibodies in microtiter wells coated with antibodies recognizing human H-FABP.

After 3 washes, tetramethylbenzidine was added and color development was stopped by adding citric acid.
The concentration of GSTP-1 was determined using a homemade ELISA as described by Allard *et al*.[28] Briefly, biotinylated anti-GSTP-1 antibodies (2 µg/mL) (Biosite, California, USA) were coated onto a 96-well Reacti-Bind NeutrAvidin coated Black Plates (Pierce, Rockford, IL) for 1 hour at 37°C. After 3 washes, CSF samples (diluted 1:4), quality controls and standards (recombinant GSTP-1 at concentrations ranging from 0 to 100 ng/mL) were incubated for 1 hour at 37°C, and followed by a washing step. Alkaline phosphatase conjugated antibodies against human GSTP-1 (Biosite, California, USA) at 2µg/mL were added and incubated for 1 hour at 37°C. After 3 washes, Attophos AP fluorescent substrate (Promega, Madison, WI) was added and plates were read immediately on a SpectraMax GEMINI-XS (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) plate reader, using the kinetic mode. Vmax values were automatically calculated by the instruments based on relative fluorescence units (RFU) ($\lambda_{excitation}$=444 nm and $\lambda_{emission}$=555 nm).
Concentrations of S100β, H-FABP and GSTP-1 in the CSF samples were back-calculated using a linear calibration curve based on measured standards values.

Bead suspension array

The levels of thirteen cytokines and chemokines (IL-1ra, IL-1β, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 and CXCL10) were determined using the Bioplex bead suspension arrays according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Briefly, thirteen sets of color-coded polystyrene beads were conjugated separately with one of the thirteen different antibodies against the molecule of interest. All the sets were then mixed together by the supplier and delivered ready-to-use. An equal amount of beads was added to each well of a 96-well filter plate. After a series of washes, standards and samples (diluted 1:4) were added and incubated for 30 minutes at room temperature. After washing, a mix of the corresponding thirteen biotinylated detection antibodies was added and incubated 30 minutes at room temperature. After washing, streptavidin-phycoerythrin (streptavidin-PE) was added for 10 minutes. After a last series of washes, beads were re-suspended in the provided assay buffer and each well was aspirated using the Bio-Plex system. Each bead was identified and the corresponding target simultaneously quantified based respectively on bead color and fluorescence. The concentration of each target was automatically calculated by the Bio-Plex Manager software using corresponding standard curve (5-PL regression) obtained from recombinant protein standards.

Data and statistical analysis

Descriptive statistics were performed using the SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 4.03, GraphPad software Inc., San Diego, CA, USA) software. Because none of the markers presented a normal distribution in concentrations (Kolmogorov-Smirnov test), differences between groups were tested with non-parametric Mann-Whitney U test (comparison between two groups) and Kruskal-Wallis test followed by Dunn's post-hoc test (comparison between three groups). Statistical significance for these tests was set at 0.05 (2-tailed tests). The stage, the presence of the parasite in CSF and the severity of neurological signs were successively considered as the dependent variables. The different marker concentrations were considered as independent variables. Bivariate non-parametric correlations using the Spearman correlation coefficient were carried out with statistical significance set at 0.01 (2-tailed tests).
To calculate the sensitivity and specificity of each individual predictor with respect to staging, the specific receiver operator characteristic (ROC) curve of each analyte was determined and the cutoff value was selected as the threshold predicting stage 2 patients with 100% of specificity (Figure S1).
Aabel (version 2.4.2, Gigawiz Ltd. Co., Tulsa, OK, USA) was used for box plots, SPSS for scatter plots and R (version 2.8.0) [29] was used for plotting ROC curves.

Panel development

Panel selection was mainly performed as described by Reynolds *et al*.[30] Briefly, the optimized cutoff values were obtained by modified iterative permutation-response calculations (rule-induction-like, RIL) using only the molecules that presented a *p* value <0.0001 (Mann-Whitney U test), an AUC above 75% and a significant Spearman correlation with WBC above 0.4 (Table 2). Each cutoff value was changed iteratively by quantile of 2% increment and sensitivity was determined after each iteration until a maximum sensitivity was achieved for 100% specificity. The permutation–response calculations were conducted using a PERL program (ActivePerl version 5.10.0.1004, ActiveState Software Inc.) and data were coded in CSV format.

Table 1 Characteristics of the studied population

		Stage 1	Stage 2
Population	n	21	79
Gender	Male	8	51
	Female	13	28
Age	Median (range)	32.0 (14-60)	33.0 (13-65)
WBC/µL	Median (range)	2 (0-5)	126 (6-6304)
Parasite in CSF	n	0	64
Neurological signs*	Absence	11	11
	Moderate	10	51
	Severe	0	15
>5 and ≤20 WBCµL No trypanosomes in CSF**	n	0	8

*Neurological signs were not reported for two patients
** Correspond to the number of patients highlighted as being in the potential intermediate stage

RESULTS

Biomarker concentration as a function of disease stage

The main characteristics of the 100 patients evaluated in this study are presented in table 1. The analytes were classified into three groups, based on the results presented in table 2. Criteria for the classification were the significance (Mann-Whitney U test), the AUC and the correlation with WBC. In the first group (GR1) comprising IL-1ra, G-CSF, CCL4, and VEGF, no significant difference in CSF concentrations between the two stages of HAT was observed. The second group (GR2) encompassed IFN-γ, IL-9, CCL2 and S100β, for which concentrations in the CSF were significantly different between stage 1 and stage 2 patients ($0.001<p<0.01$, Mann-Whitney U test). The third group (GR3) included GSTP-1, H-FABP, TNF-α, IL-1β, IL-6, IL-10, CXCL8 and CXCL10, for which the difference between stages was highly significant ($p<0.0001$, Mann-Whitney U test) (Figure S2).

To assess the sensitivity and specificity of these analytes for S2 HAT, ROC curves were built. GR1 and GR2 had a low to medium area under ROC curve (AUC) ranging from 54 to 70% and also displayed a low sensitivity in detecting S2 patients (4-13% for GR1 and 10-44% for GR2, see Table 2) at a predefined specificity of 100%. GR3 showed higher AUC (79-95%), and sensitivities for identification of S2 patient up to 84% (Table 2). CXCL10 appeared then as the most accurate predictor for staging, as, with a cutoff set at 2080 pg/mL, this molecule identified 66 out of 79 late stage patients and ruled-out all the early-stage patients.

Correlation between WBC and biomarker concentrations

As the white blood cell count was one of the two reference staging parameters, we investigated the correlation between the concentrations of the sixteen biomarkers and the number of WBC in CSF (Table 2). There was no significant correlation in the concentrations of the first and second group of analytes (GR1 and GR2) with WBC, except for S100β, which had a significant but low Spearman rho coefficient (0.269, $p<0.01$). Otherwise, strong correlations were observed between WBC and the concentrations of GR3 biomarkers (GSTP-1, IL-1β, IL-6, H-FABP, TNF-α, IL-10, CXCL8 and CXCL10), with Spearman rho ranging from 0.417 to 0.732 (Table 2 and Figure 1).

Table 2 Detailed results for all the molecules tested in respect with the stage of the disease

		Absence of parasite and ≤5 WBC/µL	Presence of parasite and/or >5 WBC/µL	Mann-Whitney *U* test	Correlation with WBC	ROC curve		
	Markers	Median (range)	Median (range)	p value	(spearman rho)	% AUC	Cutoff [pg/mL]	Sensitivity, % (95% CI)[a]
GR3	CXCL10	347.3 (24.3-2048.8)	14130.0 (24.3-128900.0)	<0.0001	0.625**	95	> 2080.0	84 (74-91)
	CXCL8	56.9 (1.3-96.5)	178.9 (1.6-1791.0)	<0.0001	0.557**	94	> 97.1	82 (72-90)
	IL-10	6.7 (0.9-19.6)	74.5 (2.1-573.1)	<0.0001	0.702**	89	> 20.0	80 (69-88)
	TNF-α	3.3 (0.5-8.4)	22.5 (1.0-295.4)	<0.0001	0.636**	93	> 8.5	78 (68-87)
	H-FABP	226.4 (19.8-564.0)	748.3 (0.0-16680.0)	<0.0001	0.417**	86	> 571.8	62 (50-73)
	IL-6	5.0 (0.2-57.7)	63.8 (0.8-3286.0)	<0.0001	0.732**	94	> 58.0	52 (40-63)
	IL-1β	0.1 (0.1-0.7)	0.6 (0.1-42.2)	<0.0001	0.445**	80	> 0.7	48 (37-60)
	GSTP-1	1272.9 (149.7-5026.9)	3014.0 (61.2-75810.0)	<0.0001	0.437**	79	> 5078.0	24 (15-35)
GR2	IFN-γ	68.7 (8.6-209.2)	100.4 (1.7-995.5)	0.0049	0.094	70	> 210.9	10 (4-19)
	IL-9	23.4 (3.6-44.5)	30.7 (3.6-209.6)	0.0051	0.041	70	> 45.0	23 (14-34)
	S100β	43.2 (4.9-113.0)	78.4 (0.0-353.0)	0.0053	0.269**	70	> 114.3	29 (19-40)
	CCL2	428.1 (58.6-632.9)	590.2 (15.8-5391.0)	0.0055	0.156	70	> 664.7	44 (33-56)
GR1	G-CSF	43.4 (2.4-209.8)	63.2 (2.0-785.9)	0.0866 (ns)	-0.029	62	> 281.7	4 (1-11)
	IL-1ra	817.3 (128.6-3087.6)	782.0 (34.0-11760.0)	0.5229 (ns)	-0.065	55	> 3092.0	13 (6-22)
	CCL4	94.2 (1.5-301.0)	91.9 (5.4-753.9)	0.5423 (ns)	-0.143	54	> 316.6	5 (1-12)
	VEGF	48.3 (20.0-215.7)	49.4 (3.5-1009.0)	0.9393 (ns)	-0.105	54	> 222.4	9 (4-17)

[a]Sensitivity was set for a specificity of 100% (95% CI, 84-100)
** Correlation is significant at the 0.01 level (2-tailed)

The levels of GR3 molecules in 8 potential intermediate stage patients (parasite not detected in CSF and having >5 and ≤20 WBC/µL) demonstrated the intermediate behaviour of this category with some patients appearing as S1 and others as S2 patients (Figure 1). Based on the above results, only the GR3 molecules (GSTP-1, IL-1β, IL-6, H-FABP, TNF-α, IL-10, CXCL8 and CXCL10) were selected for further analyses.

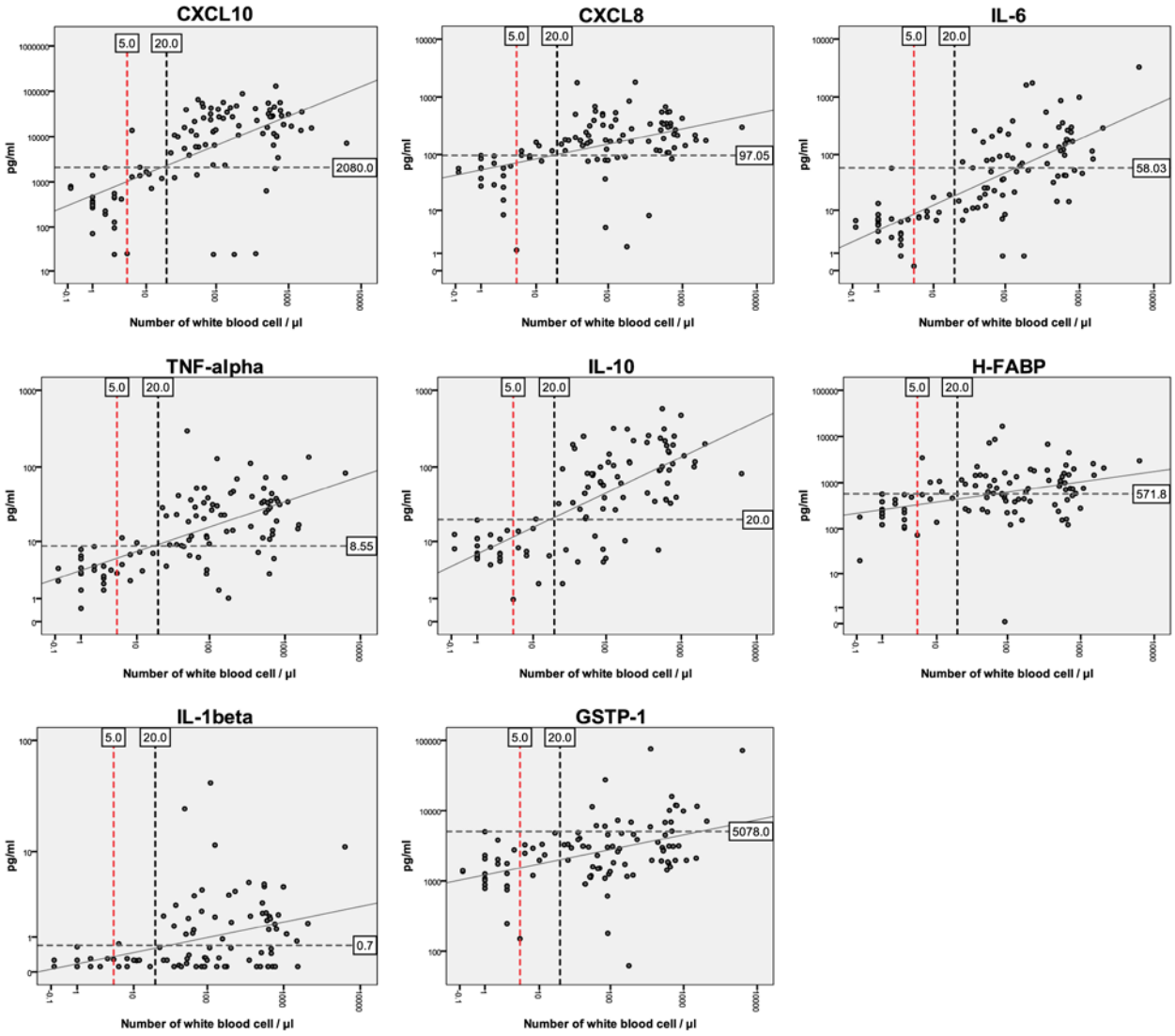

Figure 1 Scatter plots correlating the level of GR3 molecules with the WBC count. The horizontal dashed line corresponds to the cutoff value for the molecule that discriminates between S1 and S2 patients with a specificity of 100%. The left vertical dashed line corresponds to the WBC count cutoff value used for staging. The second vertical dashed line indicates the suggested cutoff value for staging. Patients between these lines (>5 and ≤20 WBC/µL) corresponded to potential intermediate stage patients. The diagonal line corresponds to the linear regression.

Parasites in CNS and biomarker concentrations

GR3 molecule concentrations were classified according to the absence/presence of trypanosomes in CSF. GSTP-1, IL-1β, IL-6, H-FABP, TNF-α, IL-10, CXCL8 and CXCL10 concentrations were significantly increased in patients with parasites in CSF (Figure 2, Table S1). The six biomarkers associated with inflammation had a lower *p* value (<0.0001, Mann-Whitney U test) and higher AUC (ranging from 78% to 89%) than H-FABP and GSTP-1 (0.001<*p*<0.05, Mann-Whitney U test, AUCs of 69% and 64% respectively). Additionally, when only S2 patients were analysed,

CXCL10, IL-10 and TNF-α levels still demonstrated a significant difference between patients with or without trypanosomes in CSF (p<0.05, Dunn's post-hoc test, Table S1).

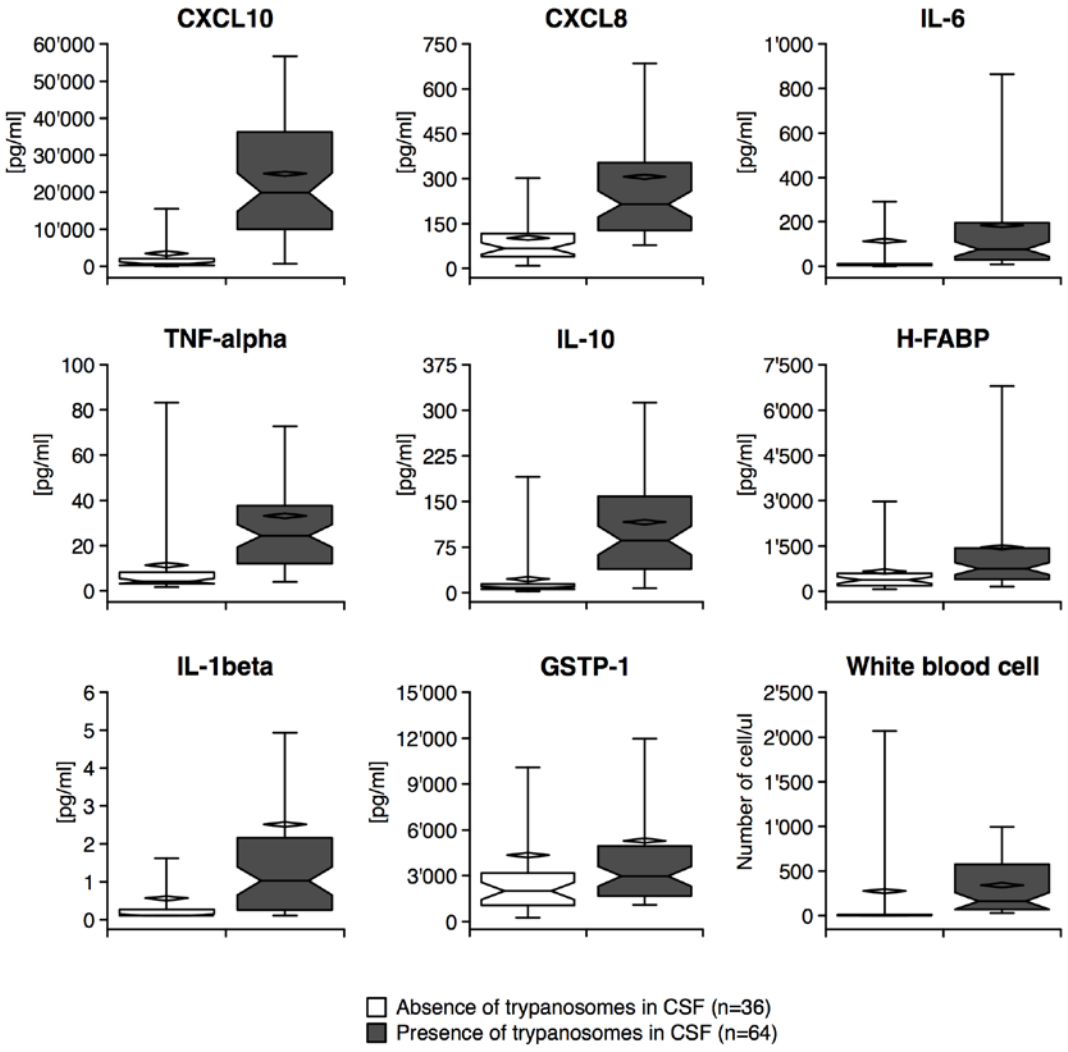

Figure 2 Box-plot of GR3 molecules and WBC classified according to the presence of the parasite in CSF. Median and mean are represented as a solid line in the box and a diamond respectively. Whisks are defined as 5th-95th percentile without outliers. Half-width of the notch was calculated automatically by the software.

Neurological signs and biomarker concentrations

The patients were classified with respect to the neurological signs reported (absence, moderate or severe) (Figure 3). All the GR3 molecules except GSTP-1 showed a significant increase in concentration associated with higher severity of neurological signs (p<0.05, Kruskal-Wallis test). Indeed, CXCL10, CXCL8, IL-6, IL-10, IL-1β, and TNF-α concentrations were significantly different between patients without neurological signs and severe neurological signs (p<0.05, Dunn's post-hoc test), as well as between patients with moderate and severe neurological signs (p<0.05, Dunn's post-hoc test). H-FABP level was significantly different between patients without neurological signs and severe neurological signs (p<0.05, Dunn's post-hoc test). Only the concentrations of CXCL10, IL-10 and TNF-α could distinguish between absence and moderate neurological signs (p<0.05, Dunn's post-hoc test).

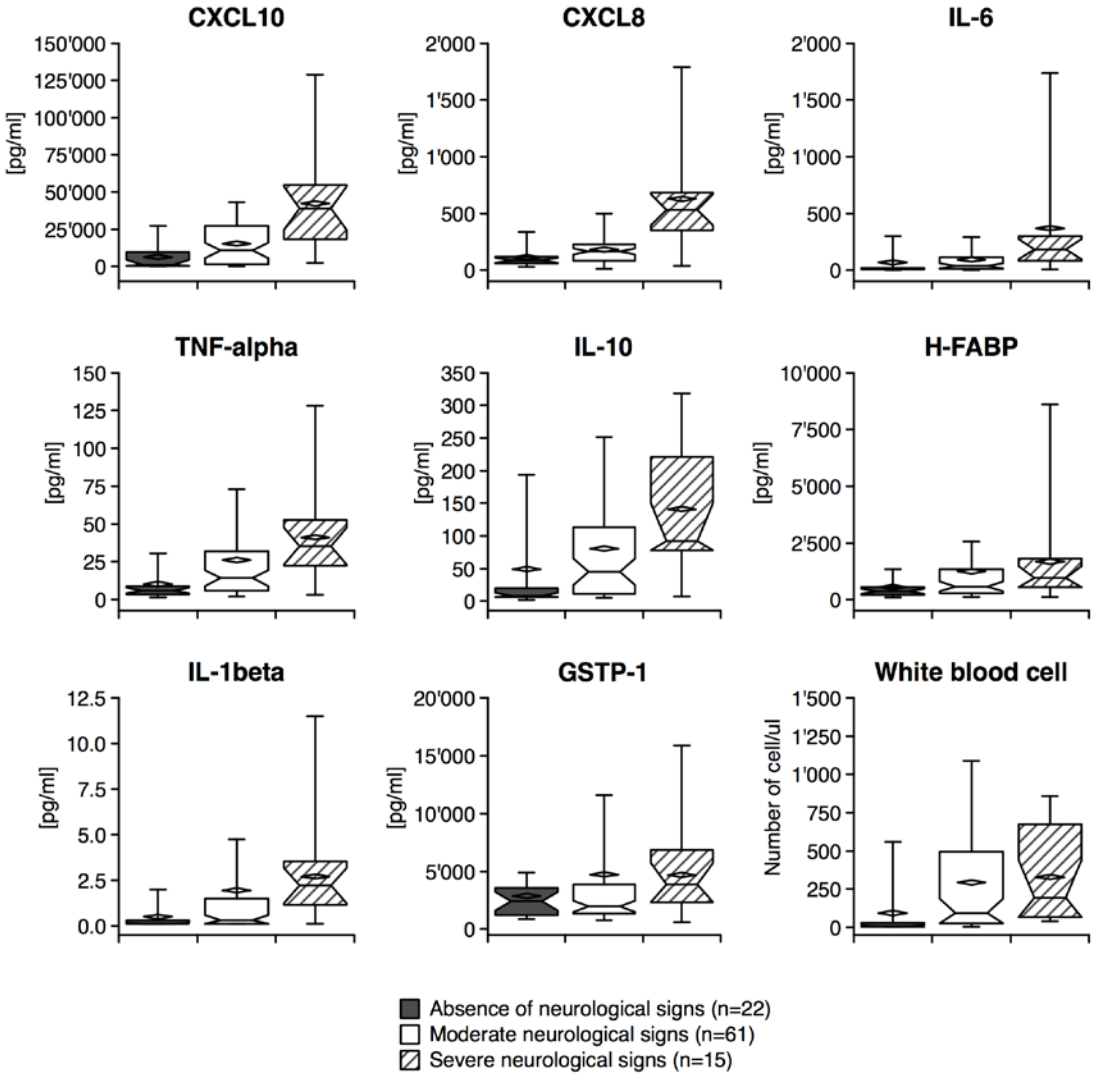

Figure 3 Box-plot of GR3 molecules and WBC classified according to the neurological signs. Median and mean are represented as a solid line in the box and a diamond respectively. Whisks are defined as 5th-95th percentile without outliers. Half-width of the notch was calculated automatically by the software. Neurological signs of two patients were not reported (n=98).

Table 3 Detailed results for the three molecule panel in respect with the stage of the disease

	Markers	Number of negative test	Number of positive test	Mann-Whitney U test , p value	% AUC (ROC curve)	Panel cutoff	Sensitivity, % (95% CI)[a]
Panel	CXCL10 CXCL8 H-FABP	23	77	<0.0001	99	≥1 molecule above its cutoff value[b]	97 (91-100)

[a]Sensitivity was set for a specificity of 100% (95% CI, 84-100)
[b]Cutoff values: CXCL10 > 2080.0 pg/mL, CXCL8 > 97.1 pg/mL and H-FABP > 571.8 pg/mL

Panel selection

In an effort to improve the global sensitivity of molecules in the prediction of second stage HAT, the GR3 molecules were combined using the rule induction like (RIL) approach. This resulted in the identification of a three-molecule panel characterized by CXCL10, CXCL8 and H-FABP (cutoff values were set at 2080.0, 97.1 and 571.8 pg/mL, respectively). A positive test (leading to identification of S2 patient) was obtained as soon as one of the three molecules included in the panel was above its cutoff value (Table

3). The panel had a sensitivity of 97% (95% CI, 91-100%) and, by definition, a specificity of 100% (95% CI, 84-100%). This means that the panel could identify 77 out of 79 stage 2 patients, and ruled-out all the 21 stage 1 patients. Out of the 77 ruled-in S2 patients, 5 were CXCL10 positive only (> 2080.0 pg/mL), 6 CXCL8 positive only (> 97.1 pg/mL) and 3 H-FABP positive only (> 571.8 pg/mL). The rest of ruled-in S2 patients were identified with either 2 positive molecules (n=23) or 3 positive molecules (n=40). When this panel was applied on the intermediate stage patients (eight patients having >5 and ≤20 WBC/µL and no trypanosomes in CSF) only one patient gave a negative test response and thus 7 out of 8 patients were classified as S2.

DISCUSSION

In this study, including early and late stage HAT patients (n=100), we evaluated sixteen molecules as potential staging markers of HAT, to replace or complement trypanosome detection and WBC count. Eight of these molecules, CXCL10, CXCL8, IL-6, IL-10, IL-1β, TNF-α, H-FABP and GSTP-1, presented concentrations significantly elevated in the CSF of late-stage HAT patients. We demonstrated that the CSF concentration of CXCL10 is highly elevated in stage 2 patients when compared to stage 1, highlighting this molecule as a potential new staging marker for sleeping sickness. A combinatorial approach has been applied in staging of HAT, in order to improve the sensitivity. This method has led to the identification of a panel consisting of CXCL10, CXCL8 and H-FABP, that identified late-stage patients with a sensitivity of 97% at 100% specificity.

H-FABP is a small protein belonging to the fatty acid-binding proteins (FABPs) and known to be expressed in the brain.[31] In myocardial infarction, HFABP is quickly released after the tissue damage.[32,33] It has been suggested that the release of H-FABP from damaged cells could be used for diagnosis of acute and chronic brain injuries.[31] GSTP-1 is a member of the Glutathione S-transferase superfamily, playing a role in oxidative stress. Its expression in brain has not been well studied, but GSTP-1 seems to be the main isoform in brain[34] and may function as a brain damage biomarker.[24] Our results showed a higher level of both H-FABP and GSTP-1 in CSF of late stage patients compared to early stage patients. These two molecules are known to be associated with early brain cell death,[24,31] which could be correlated with the observed increase of their concentration in late-stage HAT patients. From now, it is not know if these two molecules were also associated with the inflammatory process.

Cytokines and chemokines play an important role in inflammatory processes and blood-brain barrier (BBB) dysfunction,[35] and could therefore be potentially used as markers for staging HAT.[11,16,36] In the present study, the measured levels of inflammation-related proteins in CSF showed significant differences according to the disease progression. Indeed, concentrations of IL-1β, IL-6, IL-10, TNF-α, CXCL8 and CXCL10 were increased in the CSF of patients in late stage HAT compared to those in early stage of the disease. In addition, the levels of IL-1β, IL-6, CXCL8 and IL-10 were similar to those already reported for *T. b. gambiense* HAT.[11,16] IL-1β is a pro-inflammatory cytokine that induces leukocytes infiltration[37] and is rapidly expressed in response to brain damage.[35] The high level of IL-1β found in CSF of stage 2 patients confirmed its probable association with the inflammatory process. Furthermore, its level was clearly correlated to the presence of severe neurological signs, supporting a potential release in relation to neurodegeneration. IL-6 and IL-10 are both anti-inflammatory cytokines. Their increased level in the CSF according to the stage as well as the severity of the neurological signs confirmed their activation associated with disease progression. The concentration of the two molecules was significantly increased in patients with more than 20 WBC/µL, which may suggest a probable expression after an already activated inflammatory process. Indeed, it has been demonstrated in vervet monkey models of HAT that IL-10 is associated with down-regulation of pro-inflammatory cytokines (IFN-γ and TNF-α) in the late stage of *T. b. rhodesiense* disease.[38] The level of the pro-inflammatory chemokine CXCL8 was also significantly elevated in CSF of S2 patients and correlated well with both presence of trypanosomes in CSF and severity of neurological signs. CXCL8 is a strong neutrophil attractant,[16] which could thus not explain the good correlation of CXCL8 and the number of WBC (mainly B-lymphocytes) in CSF. However, its elevation in patients with a relatively low number of WBC (between 5 and 20/µL) suggests an early activation, which may play a role in BBB dysregulation.[11]

The pro-inflammatory cytokine TNF-α has been reported as being involved in blood-brain barrier dysfunction.[39] These authors also demonstrated that trypanosomes may induce synthesis of TNF-α. In the present study, the increasing level of TNF-α was associated with disease progression as well as the presence of the parasite in CSF. These results

suggested that parasites invasion into the CNS may lead to TNF-α production, which generated then CNS inflammation.[14] Additionally, an elevation according to the severity of the neurological symptoms was observed, which may support the neurotoxic effect of this cytokine in HAT.[35]

CXCL10, also known as IP-10, is a pro-inflammatory chemokine with a central role in inflammatory responses.[40] The main effect of CXCL10 as a chemotactic molecule is activation of T cell migration to the site of inflammation, after binding to its receptor, CXCR3.[41] The involvement of this chemokine in different CNS disorders has been demonstrated, such as viral meningitis[42] and multiple sclerosis,[43] where increased CXCL10 levels in the CSF correlated with tissue infiltration of T lymphocytes.[44] In our study, the concentration of CXCL10 increased with progression of the disease, and was highly correlated with the number of WBC in CSF. Many studies have pointed out astrocytes as the primary source of CXCL10 at the level of the CNS and showed that this molecule is responsible, as chemoattractant, for the influx of activated T lymphocytes in brain.[43,45-47] Indeed, there is a predominance of plasma cell infiltration in the brain of trypanosomiasis infected individuals. In addition, it has very recently been shown in a mouse model of HAT that CXCL10 may play an important role in T-cell recruitment into the brain parenchyma and is probably associated with brain invasion by trypanosomes.[48] Furthermore, the early activation of cytokine production (TNF-α, IL-6, and IFN-γ) by astrocytes and microglia in mice models infected with *T. brucei* before observation of an inflammatory response[49] has confirmed an important role of astrocyte activation in CNS inflammatory response. In consequence, early astrocyte activation, which induces CXCL10 production, is probably linked with BBB dysfunction and may occur before the inflammatory process. These hypotheses were supported by the increase CXCL10 concentration observed in patients having >5 and ≤20 WBC/µL but without trypanosomes detected in the CSF. The CXCL10 level was also demonstrated to be elevated in patients with cerebral malaria, and pointed out as potentially inducing apoptosis of endothelial cells leading to BBB breakdown.[17] Recent work has suggested that neuronal apoptosis associated with calcium dysregulation may be induced by CXCL10.[50] Even if mechanisms of CXCL10 mediated neurotoxicity remain unclear, we showed that the concentration of CXCL10 was correlated to the severity of neurological signs, supporting a possible involvement of this protein in neuronal injury pathways. Thus, CXCL10 expression in late stage HAT patients may be associated with both cell death and inflammatory process. Finally, active tuberculosis and pregnancy, two exclusion criteria in this study, have also been reported as modulating the level of CXCL10.[51,52] Although they have only been evaluated on serum and whole blood samples so far, it is not excluded that these criteria could potentially induce CXCL10 modulation in CSF. Nevertheless, our data demonstrated that CXCL10 is an efficient tool for staging patients, and suggested a potential role of CXCL10 as an early marker of parasite invasion into the CNS.

As the investigated proteins may be involved in different biological mechanisms, we evaluated in this study a strategy to combine results of each molecule, in order to find a panel able to discriminate more accurately early and late stage patients. This highlighted a panel of three molecules, including CXCL10 (the most promising single molecule), CXCL8 (another chemokine) and H-FABP (a marker of brain damage). With a specificity of 100%, this panel increased the sensitivity for staging of HAT patients up to 97% (compared to the 84% obtained with CXCL10 taken individually). Although the number of "intermediate" patients was small, the panel appeared to classify them rather as S2 patients (7/8 patients). This supports the current recommendation by WHO to consider such patients as S2 patients and treat them with drugs used for late stage disease. However, there is a need for more studies on *T. b. gambiense* and *T. b. rhodesiense* patients, before and after treatment, as well as on other parasitic diseases such as cerebral malaria, to verify these results and assess the feasibility of using the three-molecule panel as a complement to WBC count. There are obviously some drawbacks concerning this approach. Firstly the obtained panel is not 100% sensitive and thus some stage 2 patients will not be detected. The influence of other possible co-infections should also be evaluated in order to determine if they significantly modulate the evaluated molecules. Indeed, the three molecules included in the panel could potentially all be markers of other CNS disorders. It is also evident that the methods described in this study could not be implemented in such a way directly in the field and should be first transformed into a more simplified technique as for example a lateral flow immunoassay. Another limitation is the continued requirement of the invasive lumbar puncture since the molecules

highlighted in this study have been evaluated on CSF samples.

In conclusion, the present study demonstrated the utility of inflammation-related proteins and brain damage markers as indicators of the second stage of HAT but potentially in other CNS disorders as well. We highlighted the value of CXCL10 as an efficient staging biomarker for *T. b. gambiense* infected HAT patients. Additionally, a combination of CXCL10 with CXCL8 and H-FABP resulted in a highly sensitive tool for identification of late stage HAT patients.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

SUPPORTING INFORMATION

Figure S1 ROC curves of GR3 molecules and the panel. Cut-off value for each molecule [pg/mL] and for the panel is displayed by a point and a numeric value. In parenthesis, sensitivity (%) of each molecule was set for 100% specificity. Area under the ROC curve (AUC) is also given.

Figure S2 Box-plot of GR3 molecules classified according to the stage of the disease. Median and mean are represented as a solid line in the box and a diamond respectively. Whisks are defined as 5^{th}-95^{th} percentile without outliers. Half-width of the notch was calculated automatically by the software.

Table S1 Detailed results for GR3 molecules in function of the presence of trypanosomes in CSF (according or not to the stage) and the neurological signs.

REFERENCES

1. WHO. Human African trypanosomiasis (sleeping sickness): epidemiological update. Releve epidemiologique hebdomadaire / Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record / Health Section of the Secretariat of the League of Nations 2006;81:71-80.

2. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.

3. Simarro PP, Jannin J, Cattand P. Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS medicine 2008;5:e55.

4. Lejon V, Buscher P. Review Article: cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. Tropical medicine & international health : TM & IH 2005;10:395-403.

5. Kennedy PG. Diagnostic and neuropathogenesis issues in human African trypanosomiasis. International journal for parasitology 2006;36:505-12.

6. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.

7. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.

8. Lejon V, Buscher P. Stage determination and follow-up in sleeping sickness. Medecine tropicale : revue du Corps de sante colonial 2001;61:355-60.

9. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.

10. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.

11. Lejon V, Lardon J, Kenis G, et al. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of Trypanosoma brucei gambiense sleeping sickness patients before and after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 2002;96:329-33.

12. Sternberg JM. Human African trypanosomiasis: clinical presentation and immune response. Parasite immunology 2004;26:469-76.

13. Masocha W, Rottenberg ME, Kristensson K. Migration of African trypanosomes across the blood-brain barrier. Physiology & behavior 2007;92:110-4.

14. Kennedy PG. Cytokines in central nervous system trypanosomiasis: cause, effect or both? Transactions of the Royal Society of Tropical Medicine and Hygiene 2009;103:213-4.

15. Maclean L, Odiit M, Macleod A, et al. Spatially and genetically distinct African Trypanosome virulence variants

defined by host interferon-gamma response. The Journal of infectious diseases 2007;196:1620-8.
16. Courtioux B, Boda C, Vatunga G, et al. A link between chemokine levels and disease severity in human African trypanosomiasis. International journal for parasitology 2006;36:1057-65.
17. Armah HB, Wilson NO, Sarfo BY, et al. Cerebrospinal fluid and serum biomarkers of cerebral malaria mortality in Ghanaian children. Malaria journal 2007;6:147.
18. John CC, Panoskaltsis-Mortari A, Opoka RO, et al. Cerebrospinal fluid cytokine levels and cognitive impairment in cerebral malaria. The American journal of tropical medicine and hygiene 2008;78:198-205.
19. Jain V, Armah HB, Tongren JE, et al. Plasma IP-10, apoptotic and angiogenic factors associated with fatal cerebral malaria in India. Malaria journal 2008;7:83.
20. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics 2006;6:6326-53.
21. Lescuyer P, Allard L, Zimmermann-Ivol CG, et al. Identification of post-mortem cerebrospinal fluid proteins as potential biomarkers of ischemia and neurodegeneration. Proteomics 2004;4:2234-41.
22. Zimmermann-Ivol CG, Burkhard PR, Le Floch-Rohr J, Allard L, Hochstrasser DF, Sanchez JC. Fatty acid binding protein as a serum marker for the early diagnosis of stroke: a pilot study. Molecular & cellular proteomics : MCP 2004;3:66-72.
23. Guillaume E, Zimmermann C, Burkhard PR, Hochstrasser DF, Sanchez JC. A potential cerebrospinal fluid and plasmatic marker for the diagnosis of Creutzfeldt-Jakob disease. Proteomics 2003;3:1495-9.
24. Burgess JA, Lescuyer P, Hainard A, et al. Identification of brain cell death associated proteins in human post-mortem cerebrospinal fluid. Journal of proteome research 2006;5:1674-81.
25. Marchi N, Cavaglia M, Fazio V, Bhudia S, Hallene K, Janigro D. Peripheral markers of blood-brain barrier damage. Clinica chimica acta; international journal of clinical chemistry 2004;342:1-12.
26. Bloomfield SM, McKinney J, Smith L, Brisman J. Reliability of S100B in predicting severity of central nervous system injury. Neurocritical care 2007;6:121-38.
27. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:293.
28. Allard L, Turck N, Burkhard PR, et al. Ubiquitin fusion degradation protein 1 as a blood marker for the early diagnosis of ischemic stroke. Biomarker insights 2007;2:155-64.
29. Team RDC. R: A Language and Environment for Statistical Computing. Vienna, Austria 2008;Available at: http//www.R-project.org.
30. Reynolds MA, Kirchick HJ, Dahlen JR, et al. Early biomarkers of stroke. Clinical chemistry 2003;49:1733-9.
31. Lescuyer P, Allard L, Hochstrasser DF, Sanchez JC. Heart-fatty acid-binding protein as a marker for early detection of acute myocardial infarction and stroke. Molecular diagnosis : a journal devoted to the understanding of human disease through the clinical application of molecular biology 2005;9:1-7.
32. Glatz JF, van Bilsen M, Paulussen RJ, Veerkamp JH, van der Vusse GJ, Reneman RS. Release of fatty acid-binding protein from isolated rat heart subjected to ischemia and reperfusion or to the calcium paradox. Biochimica et biophysica acta 1988;961:148-52.
33. Knowlton AA, Apstein CS, Saouf R, Brecher P. Leakage of heart fatty acid binding protein with ischemia and reperfusion in the rat. Journal of molecular and cellular cardiology 1989;21:577-83.
34. Theodore C, Singh SV, Hong TD, Awasthi YC. Glutathione S-transferases of human brain. Evidence for two immunologically distinct types of 26500-Mr subunits. The Biochemical journal 1985;225:375-82.
35. Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nature reviews Neuroscience 2001;2:734-44.
36. Sternberg JM, Rodgers J, Bradley B, Maclean L, Murray M, Kennedy PG. Meningoencephalitic African trypanosomiasis: Brain IL-10 and IL-6 are associated with protection from neuro-inflammatory pathology. Journal of neuroimmunology 2005;167:81-9.
37. Ching S, He L, Lai W, Quan N. IL-1 type I receptor plays a key role in mediating the recruitment of leukocytes into the central nervous system. Brain, behavior, and immunity 2005;19:127-37.
38. Ngotho M, Maina N, Kagira J, Royo F, Farah IO, Hau J. IL-10 is up regulated in early and transitional stages in vervet monkeys experimentally infected with Trypanosoma brucei rhodesiense. Parasitology international 2006;55:243-8.
39. Girard M, Giraud S, Courtioux B, Jauberteau-Marchan MO, Bouteille B. Endothelial cell activation in the presence of African trypanosomes. Molecular and biochemical parasitology 2005;139:41-9.
40. Huang D, Han Y, Rani MR, et al. Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. Immunological reviews 2000;177:52-67.
41. Weng Y, Siciliano SJ, Waldburger KE, et al. Binding and functional properties of recombinant and endogenous CXCR3 chemokine receptors. The Journal of biological chemistry 1998;273:18288-91.
42. Lahrtz F, Piali L, Nadal D, et al. Chemotactic activity on mononuclear cells in the cerebrospinal fluid of patients with viral meningitis is mediated by interferon-gamma inducible protein-10 and monocyte chemotactic protein-1. European journal of immunology 1997;27:2484-9.
43. Sorensen TL, Tani M, Jensen J, et al. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. The Journal of clinical investigation 1999;103:807-15.
44. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol 2002;168:3195-204.

45. Farina C, Krumbholz M, Giese T, Hartmann G, Aloisi F, Meinl E. Preferential expression and function of Toll-like receptor 3 in human astrocytes. Journal of neuroimmunology 2005;159:12-9.
46. Hanum PS, Hayano M, Kojima S. Cytokine and chemokine responses in a cerebral malaria-susceptible or -resistant strain of mice to Plasmodium berghei ANKA infection: early chemokine expression in the brain. International immunology 2003;15:633-40.
47. van Heteren JT, Rozenberg F, Aronica E, Troost D, Lebon P, Kuijpers TW. Astrocytes produce interferon-alpha and CXCL10, but not IL-6 or CXCL8, in Aicardi-Goutieres syndrome. Glia 2008;56:568-78.
48. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.
49. Hunter CA, Jennings FW, Kennedy PG, Murray M. Astrocyte activation correlates with cytokine production in central nervous system of Trypanosoma brucei brucei-infected mice. Laboratory investigation; a journal of technical methods and pathology 1992;67:635-42.
50. Sui Y, Stehno-Bittel L, Li S, et al. CXCL10-induced cell death in neurons: role of calcium dysregulation. The European journal of neuroscience 2006;23:957-64.
51. Whittaker E, Gordon A, Kampmann B. Is IP-10 a better biomarker for active and latent tuberculosis in children than IFNgamma? PloS one 2008;3:e3901.
52. Gotsch F, Romero R, Friel L, et al. CXCL10/IP-10: a missing link between inflammation and anti-angiogenesis in preeclampsia? The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2007;20:777-92.

CHAPTER 4

Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis

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A central aspect, which characterizes HAT late stage, is the increased number of leukocytes in patients' CSF. The entrance of WBC into the brain compartment is the result of a complex, but well described, process of cell recruitment to the site of inflammation. This involves a number of interactions between leukocytes and blood-brain barrier endothelial cells, under the influence of chemotactic and activating cytokines of different origins.

We therefore tested the staging properties of 5 molecules, matrix metallopreoteinases (MMP-2 and MMP-9) and cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin), by measuring their concentrations in the CSF of 63 *T. b. gambiense* HAT patients. Since in our previous study we highlighted new powerful staging markers, we compared the molecules here investigated to CXCL10, CXCL8 and H-FABP. An increased accuracy in stage determination was achieved when measuring MMP-9 or ICAM-1 compared to CXCL10, and the combination of these two markers with H-FABP allowed to perfectly discriminate (100% SP and SE) between the two categories of patients.

These results have been published in 2011 in Tropical Medicine and International Health. I partially contributed to the experimental part, data analysis and writing of the manuscript.

Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis

Alexandre Hainard[1], **Natalia Tiberti[1],** Xavier Robin[1], Dieudonné Mumba Ngoyi[2], Enock Matovu[3], John C. K. Enyaru[4], Markus Müller[5], Natacha Turck[1], Joseph M. Ndung'u[6], Veerle Lejon[7], Jean-Charles Sanchez[1]

1 *Biomedical Proteomics Research Group, Medical University Centre, Geneva, Switzerland;* **2** *Institut National de Recherche Biomédicale, Kinshasa, D.R. Congo;* **3** *Department of Veterinary Parasitology and Microbiology, Faculty of Veterinary Medicine, Makerere University, Kampala, Uganda;* **4** *Department of Biochemistry, Faculty of Science, Makerere University, Kampala, Uganda;* **5** *Swiss Institute of Bioinformatics, Medical University Centre, Geneva, Switzerland;* **6** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland;* **7** *Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium*

SUMMARY

Objectives A critical step before treatment of human African trypanosomiasis (HAT) is the correct staging of the disease. As late stage is established when trypanosomes cross the blood brain barrier and invade the central nervous system, we hypothesized that matrix metalloproteinases and cell adhesion molecules could indicate, alone or in combination, the disease progression from the first to the second stage of HAT.

Methods We measured the levels of MMP-2, MMP-9, ICAM-1, VCAM-1 and E-selectin in the cerebrospinal fluid (CSF) of 63 *Trypanosoma brucei gambiense* infected patients (15 stage 1 and 48 stage 2). Staging was based on counting of white blood cells (WBC) and/or parasite detection in CSF. Concentrations were obtained either by ELISA or multiplex bead suspension assays and results were compared with three known HAT staging markers (CXCL10, CXCL8 and H-FABP).

Results ICAM-1 and MMP-9 accurately discriminated between stage 1 and stage 2 HAT patients with 95% sensitivity for 100% specificity, which was better than CXCL10 (93% SE for 100% SP), one of the most promising known markers. Combination of ICAM-1 and MMP-9 with H-FABP provided a panel that resulted in 100% of sensitivity and specificity for staging HAT.

Conclusions ICAM-1 and MMP-9, alone or in combination, appeared as powerful CSF staging markers of HAT. Final validation of all newly discovered staging markers on a large multi-centric cohort including both forms of the disease as well as patients with other infections should be performed.

INTRODUCTION

Human African trypanosomiasis (HAT), or sleeping sickness, is a tropical disease occurring in sub-Saharan Africa, caused by the protozoan parasite *Trypanosoma brucei* (*T. b.*). Two forms of the disease affect humans, a chronic form caused by the *Trypanosoma brucei gambiense* subspecies, and an acute form due to *Trypanosoma brucei rhodesiense*.[1] The disease evolves from a first haemolymphatic stage (stage 1, S1) to a second meningo-encephalitic stage (stage 2, S2), after parasites cross the blood-brain barrier (BBB) and invade the central nervous system (CNS).[2] If untreated, disease outcome is always fatal.[2] The treatment differs between the two stages and is more toxic and complicated in the case of second (late) stage disease. After diagnosis, patients therefore undergo a lumbar puncture for staging of sleeping sickness, which relies on cerebrospinal fluid (CSF) examination.[3] Correct staging of patients with HAT remains a critical issue.[4] According to the WHO guidelines, the late stage is defined by the detection in the CSF of more than five white blood cells (WBC)/µl and/or the presence of

trypanosomes.[5] However, higher thresholds for the WBC count as well as existence of a possible intermediate stage have been suggested.[6] Trypanosome detection in CSF defines the second stage, but direct finding suffers from sensitivity (SE).[3] In this context, discovery of new highly sensitive and specific staging markers is essential to replace or complement current staging methods.

Relations between chemokine levels in CSF and HAT progression have been demonstrated[7,8] and recently, CXC motif chemokine 10 (CXCL10), alone or in combination with interleukin-8 (CXCL8) and heart-type fatty acid-binding protein (H-FABP), has been highlighted as a powerful CSF staging biomarker.[9,10] Moreover, it has been suggested that trypanosomes may cross the BBB using a similar mechanisms as leukocyte infiltration into the CNS.[2] Cell adhesion molecules (CAMs) and matrix metalloproteinases (MMPs) are known to be involved in BBB permeability [11] and leukocyte extravasation.[12] Studies have demonstrated the up-regulation of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (endothelial leukocyte adhesion molecule 1) associated with parasite infiltration into the CNS.[13,14] The crucial role of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) for WBC infiltration into the brain parenchyma has also been shown.[15] In this context, we hypothesized that MMPs and CAMs could be used, alone or in combination, as HAT staging biomarkers. We measured the level of MMP-2, MMP-9, ICAM-1, VCAM-1 and E-selectin in the CSF of *T. b. gambiense* infected patients and evaluated their performances in the context of CNS invasion by the parasite. Levels of three other biomarkers (CXCL10, CXCL8 and H-FABP) previously shown to be efficient for staging were also measured and used for comparison.

MATERIALS AND METHODS

Samples

Samples originated from a prospective study on 360 *T. b. gambiense* infected patients (THARSAT), conducted between 2005 and 2008 at Dipumba hospital in Mbuji-Mayi (Kasai Oriental province, Democratic Republic of the Congo). Details of the THARSAT study design and results are reported elsewhere.[16] The study was approved by the Ministry of Health, Kinshasa, DRC, and by the Ethical Committee of the University of Antwerp, Belgium. Written informed consent was obtained from the patients or their responsible before enrolment. In this study, a total of 63 CSF samples comprising 15 stage 1 (S1) and 48 stage 2 (S2) patients, taken before treatment, were tested (Table 1). The patients were chosen sequentially and were also classified according to their neurological condition (Table 1). The stage 1 and stage 2 groups did not differ significantly in terms of age and sex (chi-square or Mann-Whitney *U* test). Three categories of neurological signs were defined: absence (no neurological signs), moderate (at least one major neurological sign but no generalised tremors) and severe (at least two major neurological signs including generalised tremors). Major neurological signs were defined as: daytime somnolence, sensory and gait disturbances, presence of primitive reflexes (Babinski's sign, palmo-mental reflex, perioral reflex), modified tendon reflexes (exaggeration or abolition), abnormal movements such as tremor (fine, diffuse and generalized). Disease staging was determined after CSF examination using the following criteria: patients with WBC ≤5/µl and no trypanosomes in CSF were classified as S1; patients with WBC >5/µl and/or trypanosomes in CSF corresponded to S2.

Table 1 Characteristics of the selected population of patients with human African trypanosomiasis

	Stage 1	Stage 2
Population (n)	15	48
Gender*		
Male	6	32
Female	9	16
Age, years (median, range)†	29 (14-51)	33 (13-65)
WBC/µl (median, range)‡	3 (1-5)	94 (6-2064)
Detected trypanosomes in CSF (n)‡	0	38
Neurological signs§		
Absence	9	7
Moderate	6	32
Severe	0	9

* Chi-square test: ns; † Mann-Whitney: ns; ‡ Mann-Whitney: $p < 0.001$; § Chi-square test: $p = 0.001$.

Matrix metalloproteinases (MMPs), cell adhesion molecules (CAMs) and chemokines measurements

The concentrations of MMP-2, MMP-9, ICAM-1, VCAM-1, E-selectin, CXCL10 and CXCL8 were measured using commercially available multiplex bead suspension assay (mBSA) kits (R&D Systems, UK; Biorad, Hercules, CA) according to manufacturer's instruction. Samples were diluted 1:4 with the corresponding provided buffer and assayed in duplicate. The concentration of each target was automatically calculated by the Bio-Plex Manager v4.1 software using corresponding standard curves (5-PL regression) obtained with recombinant protein standards.

H-FABP measurement

The concentration of H-FABP was measured using a commercially available ELISA (Hycult Biotechnology, Uden, Netherlands) following the protocol described elsewhere.[10]

Panel selection

Combinations of all evaluated proteins were performed to determine whether a panel could be more efficient for staging than the molecules alone. Optimized cut-off values were obtained by modified iterative permutation-response calculations (rule-induction-like, RIL) as described in a previous study.[10]

Data and statistical analysis

Statistical analyses were performed using PASW v18 and GraphPad Prism v4.03 software. Correlations and differences between groups were tested with non-parametric tests (i.e. Mann-Whitney *U* test, Kruskal-Wallis test, Dunn's *post hoc* test and Spearman test). Marker's SE was evaluated at 100% of specificity (SP). Overall accuracy was further assessed using the Youden index (*Y* = maximum [SE + SP − 1]) or partial area under ROC curve (pAUC) between 90 and 100% of SP.[17]

Table 2 Detailed results for the cell adhesion molecules and metalloproteinases (Part A) as well as three other known human African trypanosomiasis markers (Part B) tested in respect with the stage of the disease

	Stage 1 median (range)	Stage 2 median (range)	Ratio S2/S1	*P* value*	Correlation with WBC†	pAUC‡ (%)	Cut-off (pg/ml)	% SE (95% CI)§
Part A								
MMP-9	67.0 (14.8-414.0)	3085.3 (175.7-22643.0)	46.0	<0.0001	0.742**	95	424.7	90 (77-97)
ICAM-1	537.2 (116.1-1330.9)	8493.1 (604.6-39166.5)	15.8	<0.0001	0.794**	95	1450.6	90 (77-97)
VCAM-1	9100.5 (1466.4-23567.9)	50625.6 (8894.3-195040.3)	5.6	<0.0001	0.750**	93	25729.1	85 (72-94)
MMP-2	15354.8 (4010.0-38832.9)	39798.7 (10565.9-108146.1)	2.6	<0.0001	0.712**	78	39220.2	52 (37-67)
E-selectin	24.4 (1.1-59.1)	53.2 (1.1-1234.9)	2.2	0.0152	0.527**	73	61.0	48 (33-63)
Part B								
CXCL10	376.5 (24.3-2048.8)	11699.1 (24.3-65596.9)	31.1	<0.0001	0.667**	93	2079.7	83 (70-93)
CXCL8	48.8 (1.3-96.5)	172.5 (1.6-1755.0)	3.5	<0.0001	0.548**	89	97.1	79 (65-90)
H-FABP	264.8 (72.9-548.7)	759.8 (234.1-16680.7)	2.9	<0.0001	0.504**	84	552.6	67 (52-80)

SE, sensitivity; SP, specificity. Median values (and ranges) are expressed in pg/ml. *p value according to the Mann-Whitney *U* test. †Spearman rho value [**significant correlation at the 0.01 level (two-tailed)]. ‡Partial AUC between 90% and 100% of SP. §SE was set for a SP of 100%.

RESULTS

HAT CSF staging

As presented in Table 2 Part A, all the CAMs and the MMPs were able to discriminate the two stages of the disease with a high significance (p <0.0001, Mann-Whitney *U* test) except E-selectin (p value =0.0152). E-selectin and MMP-2 showed pAUC below 80% (respectively at 73% and 78%) whereas VCAM-1, ICAM-1 and MMP-9 presented higher pAUC ranging from 93% to 95% (Figure 1). The five molecules were able to identify S2 patients with SE ranging from 48% (E-selectin) up to 90% for ICAM-1 and MMP-9 (Figure 1) for 100% SP. As the number of WBC is the current staging reference, we evaluated the correlation between this parameter and the analyte levels. All these molecules significantly correlated with the number of WBC, with ICAM-1 showing the highest correlation (Spearman rho coefficient of 0.794). The three already known markers, CXCL10, CXCL8 and H-FABP were also able to significantly discriminate between the two stages (Table 2 part B) with pAUC, respectively, at 84%, 89% and 93%. These three molecules were also significantly correlated with the number of WBC.

Presence of trypanosomes in the CSF

To evaluate the relationship between the presence of parasites in the CSF and the concentration of the molecules, patients were classified into two groups: without (T-) and with (T+) detected trypanosomes in CSF (Figure 2 and Table S1). Concentrations of all MMPs and CAMs were significantly increased in T+ patients (p <0.01, Mann-Whitney *U* test). MMP-2 presented the lowest pAUC at 65%, whereas the other molecules (ICAM-1, E-selectin, VCAM-1, and MMP-9) had pAUC either at 72% or 73%. MMP-2, E-selectin, MMP-9 and VCAM-1 produced Youden indices ranging from 0.16 to 0.64. ICAM-1 showed the highest one with a value at 0.84. WBC count was also considered as an independent variable to compare its performance relatively to the evaluated analytes. The number of WBC was significantly increased in association with the presence of parasites in CSF (p <0.0001, Mann-Whitney *U* test) yielding a pAUC of

59% and a Youden index of 0.53. CXCL10, CXCL8 and H-FABP presented pAUC at, respectively, 79%, 65% and 55% and Youden indices ranging from 0.16 to 0.74 (Table S1).

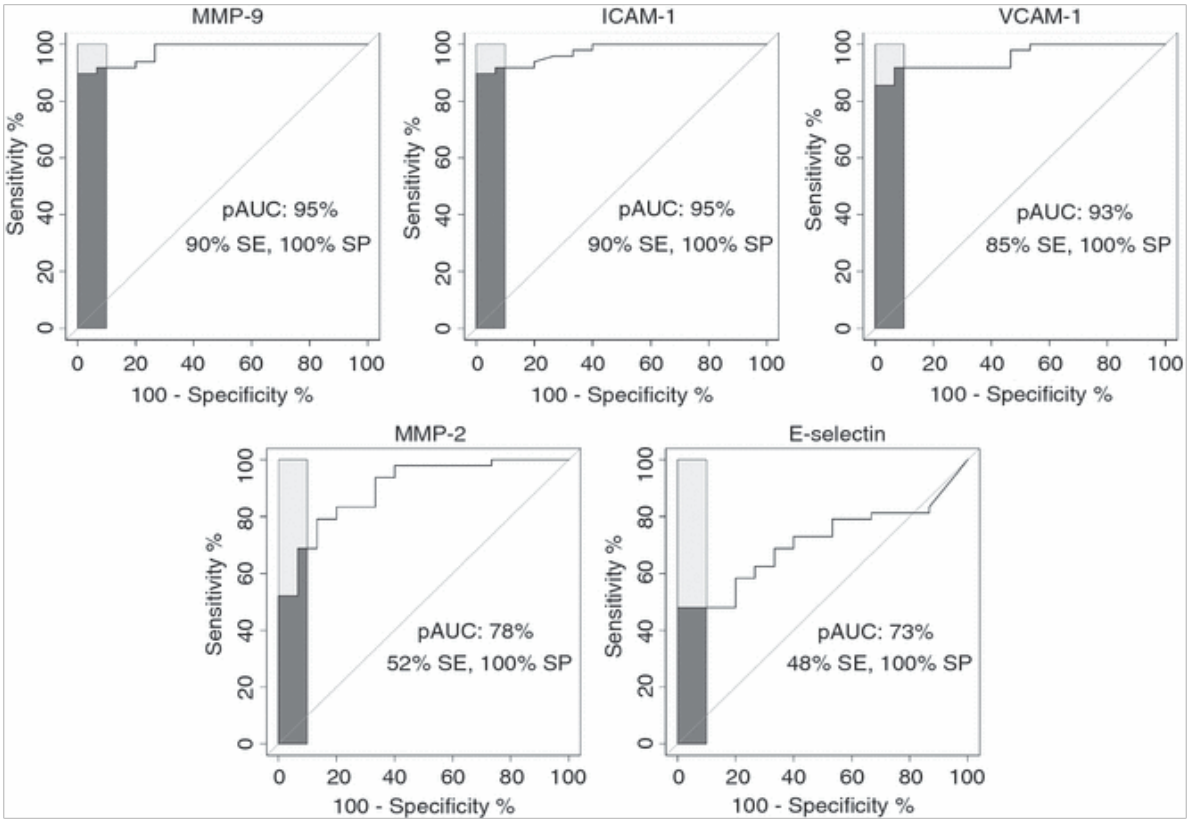

Figure 1 Roc curves of MMP-9, ICAM-1, VCAM-1, MMP-2 and E-selectin in cerebrospinal fluid for staging of human African trypanosomiasis. Dark grey boxes represent the partial area under the ROC (pAUC) displayed by the molecules between 90% and 100% of specificity (SP). Best sensitivity for 100% SP and pAUC% are indicated.

Neurological signs

As presented in Figure 3, patients were grouped according to their reported neurological signs (absent, moderate or severe). All the CAMs and MMPs, except E-selectin, presented a significant increase in concentration associated with the severity of the neurological signs (Table S1). However, MMP-9, ICAM-1 and VCAM-1 showed lower p values (<0.0005, Kruskal-Wallis test) than MMP-2 and E-selectin. VCAM-1, ICAM-1 and MMP-9 were able to significantly discriminate between absence and moderate as well as between absence and severe neurological signs (p value, respectively, <0.05 and <0.01, Dunn's *post hoc* test). E-selectin presented non-significant p values and MMP-2 only discriminated between absence and moderate neurological signs (p <0.05, Dunn's *post hoc* test). CXCL10, CXCL8 and H-FABP significantly distinguished between the different neurological conditions (p <0.005, Kruskal-Wallis test). These three molecules discriminated between absence and moderate as well as between absence and severe neurological signs, but CXCL8 was the only one able to discriminate between moderate and severe neurological signs (p <0.05; Dunn's *post hoc* test). WBC count presented significant differences between the three groups (p <0.005, Kruskal-Wallis test), discriminating between absence and moderate and between absence and severe neurological signs with p values, respectively, <0.05 and <0.01 (Dunn's *post hoc* tests).

Panel selection

All the evaluated markers were combined using the RIL method explained earlier. This approach resulted in the identification of a three molecules panel able to detect S2 patients with 100% of both SE and SP. This panel was characterized by MMP-9, ICAM-1 and H-FABP with cut-off values, respectively, at 424.7, 600.9 and 391.4 pg/ml. A positive test response (i.e. identification of a S2 patient) was obtained as soon as

two molecules were above their cut-off values (Table 3). This panel more accurately discriminated between S1 and S2 than the one made of CXCL10, CXCL8 and H-FABP (Table 3), previously described.[10]

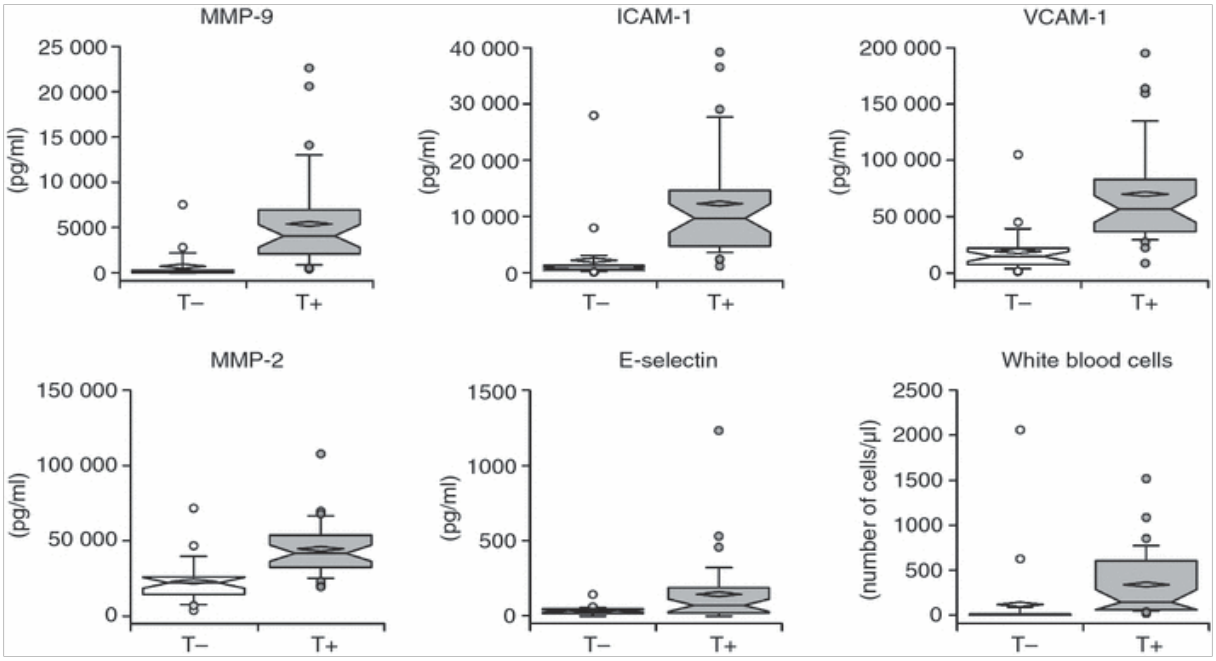

Figure 2 Box plots of cerebrospinal fluid (CSF) levels of MMP-9, ICAM-1, VCAM-1, MMP-2, E-selectin and the WBC according to the presence or absence of parasites in CSF. T-, no detected parasites in CSF; T+, detected parasites in CSF. Median and mean are represented as a solid line in the box and a diamond, respectively. Whisks are defined as 5^{th}-95^{th} percentile without outliers. All the p values are <0.0001.

DISCUSSION

This study has demonstrated that ICAM-1 and MMP-9 have high staging performance, with a SE of 90% for 100% SP. Furthermore, analysis of a combination of molecules allowed the selection of a panel comprising of MMP-9, ICAM-1 and H-FABP that detected S2 patients with 100% SE and SP.

The brain is protected from the free entry of molecules and pathogens by the BBB, mainly composed of endothelial cells. Second stage of HAT occurs when parasites cross the BBB, inducing a neuroinflammatory process associated with leukocyte infiltration into the CNS.[18,19] CAMs play an important role in leukocyte migration through the BBB during inflammatory processes[20,21] and are modulated in many CNS infections.[22,23] In this study, the measured concentration of ICAM-1, VCAM-1 and E-selectin in CSF showed a significant increase according to disease progression. E-selectin is synthesized and expressed by endothelial cells during acute or chronic inflammatory processes, and it is a mediator of leukocyte-endothelial interactions.[21] The increased level of E-selectin in the CSF of S2 patients is a further evidence of its probable association with the inflammatory process; additionally, the significant correlation between its concentration and WBC was coherent with a potential involvement of E-selectin in leukocyte trafficking.[12] ICAM-1 and VCAM-1 belong to the immunoglobulin superfamily of adhesion molecules and are principally expressed by endothelial cells.[24] They are also implicated in leukocyte trafficking[25] and *in vitro* studies demonstrated that the presence of *T. b. gambiense* parasites activates endothelial cells (the major cell population of the BBB) and enhances the expression of these two proteins.[14] The increased concentration of ICAM-1 and VCAM-1 observed in the CSF of late stage patients, and the strong correlation with the number of WBC is a clear indication of their involvement in disease progression. Higher

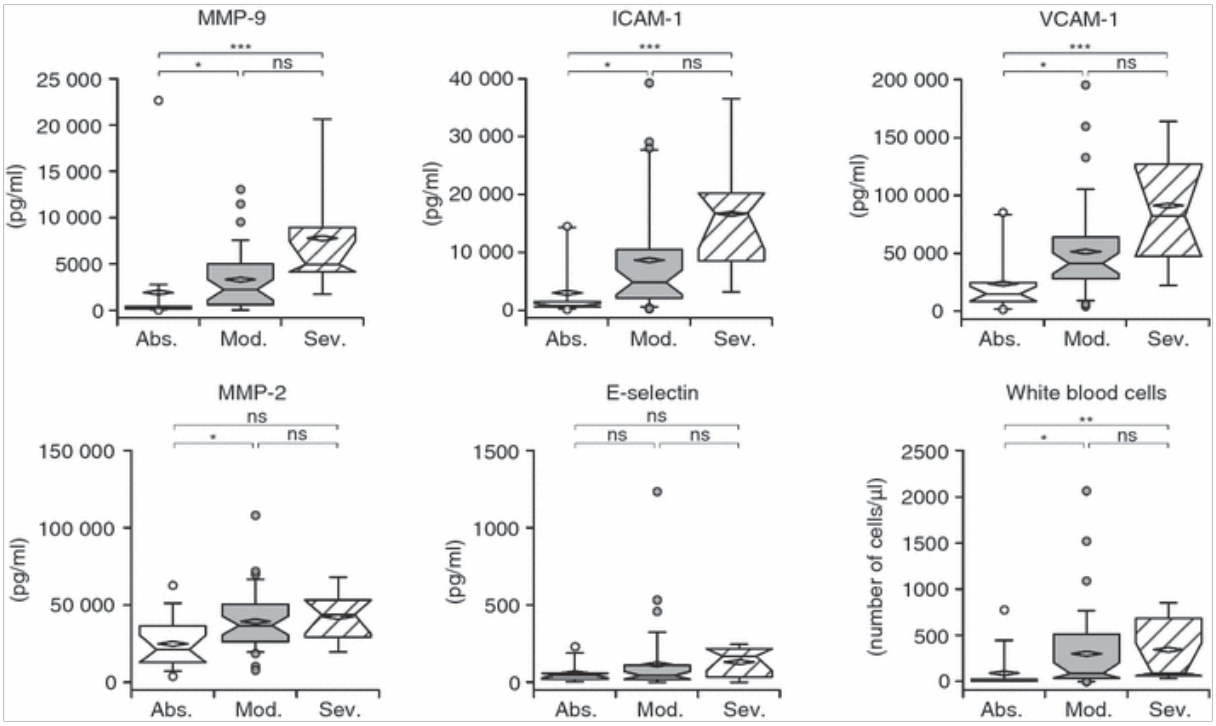

Figure 3 Box plots of levels of MMP-9, ICAM-1, VCAM-1, MMP-2, E-selectin and the WBC in cerebrospinal fluid of patients with human African trypanosomiasis according to the neurological signs. Abs., absence of neurological signs; Mod., moderate neurological signs; Sev., severe neurological signs. Median and mean are represented as a solid line in the box and a diamond, respectively. Whisks are defined as 5th-95th percentile without outliers. ns, non-significant p value; *: p <0.05; **: p <0.01; ***: p <0.001 (Dunn's *post hoc* test).

Table 3 Detailed results for the three molecules panels

Part C	Markers	No. of negative test	No. of positive test	Mann-Whitney *U* test , *P* value	% pAUC* (ROC curve)	Cut-off [no. of positive mol.]	% SE (95% CI)†
Panel 01	MMP-9 ICAM-1 H-FABP	15	48	<0.0001	100	≥2 molecules	100 (93-100)
Panel 02	CXCL10 CXCL8 H-FABP	16	47	<0.0001	99	≥1 molecule	98 (89-100)

SE, sensitivity; SP, specificity; pAUC, partial area under the ROC curve. *Partial AUC between 90% and 100% of SP. †SE was set for a SP of 100%.

concentrations of ICAM-1 and VCAM-1 were also observed for patients with detected trypanosomes in CSF (T+), suggesting a possible up-regulation associated with the passage of trypanosomes across the BBB.[13]

Matrix metalloproteinases are a family of proteases produced by a variety of cells, such as neurons, astrocytes and microglia.[26] A subgroup of MMPs, the gelatinases, made of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), play an important role in neuroinflammation. They are involved in BBB permeability by attacking the extracellular matrix,[11] as demonstrated in other CNS disorders, such as cerebral malaria,[26] meningitis[27] or multiple sclerosis.[28] In our study, MMP-2 and MMP-9 were significantly elevated in the CSF of patients with second-stage HAT and were highly correlated with the number of WBC in CSF, confirming their role in BBB dysfunction associated with leukocyte penetration into the CNS.[11] Indeed, it has been demonstrated that MMP-2 and MMP-9 create a localized temporary opening of the *glia limitans* (part of the BBB) by selective cleavage of the *β*-dystroglycan subunit anchoring the astrocyte end-feets to the parenchymal membrane.[15] A clear association between these two MMPs and the presence of parasites in CSF has also been observed, supporting a probable association with trypanosome infiltration into the CNS. The observed modulation of

MMP-9 according to the severity of the neurological signs suggested a potential role in neuronal injury as described by Leib *et al.*[29] in a rat model of meningitis. MMP-9 performed better than MMP-2 as a marker for staging HAT. Because MMP-9 is secreted during inflammatory processes, whereas MMP-2 is constitutively expressed in CSF,[28] greater differences in concentrations could be observed for MMP-9, resulting in better staging performances. The roles of MMPs and CAMs in the pathogenesis of HAT remain to be clarified.

CXCL10 was recently reported as a powerful CSF staging biomarker,[9,10] which was confirmed by the present study, although the accuracy of ICAM-1 and MMP-9 for staging HAT was better. These two molecules showed also better accuracies than the WBC count regarding to the presence of parasites in the CSF and the progression of the neurological signs.

In an effort to further improve SE and SP for the detection of S2 patients, we applied a marker combination strategy. The two best performing molecules, ICAM-1 and MMP-9, associated with H-FABP, resulted in a panel able to detect all the S2 patients and to rule out all the S1 (i.e. 100% of both SE and SP). Interestingly, the brain damage marker (H-FABP), which was part of the panel reported in our previous study, was not a good marker on its own, but clearly improved the accuracy of staging when used in combination with ICAM-1 and MMP-9.

This work therefore highlighted MMP-9 and ICAM-1 as powerful staging markers for *T. b. gambiense* HAT and demonstrated the utility, alone or in combination, of MMPs as well as CAMs to indicate the second stage of HAT. Nevertheless, deeper investigations on more patients are needed to validate the performance of these biomarkers. The influence of other concomitant diseases, such as malaria and HIV, should also be evaluated, as well as staging ability for patients infected with the *T. b. rhodesiense* parasite. All these molecules were also evaluated on plasma samples from a small cohort of patients with HAT (n=30; 15 S1 and 15 S2), without showing any significant differences between the two stages of the disease. Thus, a major drawback remains the continuous need of lumbar puncture for staging.

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SUPPORTING INFORMATION

Table S1 Detailed results for the evaluated molecules and the WBC according to the presence of parasites in CSF (T-/T+) and the neurological signs (absence, moderate or severe).

REFERENCES

1. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet 2010;375:148-59.

2. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Progress in neurobiology 2010;91:152-71.

3. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.

4. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.

5. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.

6. Rodgers J. Human African trypanosomiasis, chemotherapy and CNS disease. Journal of neuroimmunology 2009;211:16-22.

7. Lejon V, Lardon J, Kenis G, et al. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of Trypanosoma brucei gambiense sleeping sickness patients before and after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 2002;96:329-33.

8. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Tropical medicine & international health : TM & IH 2009;14:529-34.

9. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.

10. Hainard A, Tiberti N, Robin X, et al. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS neglected tropical diseases 2009;3:e459.

11. Rosenberg GA. Matrix metalloproteinases and their multiple roles in neurodegenerative diseases. Lancet neurology 2009;8:205-16.

12. Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature reviews Immunology 2003;3:569-81.
13. Mulenga C, Mhlanga JD, Kristensson K, Robertson B. Trypanosoma brucei brucei crosses the blood-brain barrier while tight junction proteins are preserved in a rat chronic disease model. Neuropathology and applied neurobiology 2001;27:77-85.
14. Girard M, Giraud S, Courtioux B, Jauberteau-Marchan MO, Bouteille B. Endothelial cell activation in the presence of African trypanosomes. Molecular and biochemical parasitology 2005;139:41-9.
15. Agrawal S, Anderson P, Durbeej M, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. The Journal of experimental medicine 2006;203:1007-19.
16. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. The Journal of infectious diseases 2010;201:453-63.
17. McClish DK. Analyzing a portion of the ROC curve. Medical decision making : an international journal of the Society for Medical Decision Making 1989;9:190-5.
18. Enanga B, Burchmore RJ, Stewart ML, Barrett MP. Sleeping sickness and the brain. Cellular and molecular life sciences : CMLS 2002;59:845-58.
19. Grab DJ, Kennedy PG. Traversal of human and animal trypanosomes across the blood-brain barrier. Journal of neurovirology 2008;14:344-51.
20. Radi ZA, Kehrli ME, Jr., Ackermann MR. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2001;15:516-29.
21. Man S, Ubogu EE, Ransohoff RM. Inflammatory cell migration into the central nervous system: a few new twists on an old tale. Brain pathology 2007;17:243-50.
22. Brown HC, Chau TT, Mai NT, et al. Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. Neurology 2000;55:104-11.
23. Jaber SM, Hamed EA, Hamed SA. Adhesion molecule levels in serum and cerebrospinal fluid in children with bacterial meningitis and sepsis. Journal of pediatric neurosciences 2009;4:76-85.
24. Petruzzelli L, Takami M, Humes HD. Structure and function of cell adhesion molecules. The American journal of medicine 1999;106:467-76.
25. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. Nature immunology 2005;6:1182-90.
26. Van den Steen PE, Van Aelst I, Starckx S, Maskos K, Opdenakker G, Pagenstecher A. Matrix metalloproteinases, tissue inhibitors of MMPs and TACE in experimental cerebral malaria. Laboratory investigation; a journal of technical methods and pathology 2006;86:873-88.
27. Tsai HC, Chung LY, Chen ER, et al. Association of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinase-4 in cerebrospinal fluid with blood-brain barrier dysfunction in patients with eosinophilic meningitis caused by Angiostrongylus cantonensis. The American journal of tropical medicine and hygiene 2008;78:20-7.
28. Liuzzi GM, Trojano M, Fanelli M, et al. Intrathecal synthesis of matrix metalloproteinase-9 in patients with multiple sclerosis: implication for pathogenesis. Mult Scler 2002;8:222-8.
29. Leib SL, Leppert D, Clements J, Tauber MG. Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. Infection and immunity 2000;68:615-20.

CHAPTER 5

Cerebrospinal fluid neopterin as marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness

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In the study presented in this chapter we validated, on a large multicenter cohort, the staging ability of the 8 most promising markers for *T. b. gambiense* HAT: MMP-9, ICAM-1, VCAM-1, CXCL10, CXCL13, IgM, B2MG and neopterin. These eight molecules were chosen among a list comprising those discovered and verified by our group (Chapter 2, 3, and 4) and some markers already proposed in the literature for the staging of HAT.

Through a two-step validation process we confirmed, first on a training cohort and then on a validation one, the high power of neopterin for the staging of *T. b. gambiense* patients, and we showed the high potential of this marker in replacing the counting of WBC.

This study was published in 2012 in PLoS ONE. My contribution consisted in performing part of the experiments and the whole data analysis and interpretation. I also wrote the manuscript.

Cerebrospinal fluid neopterin as marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness

Natalia Tiberti[1*], Alexandre Hainard[1*], Veerle Lejon[2], Bertrand Courtioux[3], Enock Matovu[4], John Charles Enyaru[5], Xavier Robin[1], Natacha Turck[1], Krister Kristensson[6], Dieudonné Mumba Ngoyi[7], Gedeão M. L. Vatunga[8], Sanjeev Krishna[9], Philippe Büscher[2], Sylvie Bisser[3], Joseph Mathu Ndung'u[10], Jean-Charles Sanchez[1]

1 *Biomedical Proteomics Research Group, Department of Human Protein Sciences, University of Geneva, Geneva, Switzerland;* **2** *Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium;* **3** *INSERM UMR1094, Tropical Neuroepidemiology, Limoges, France & Institute of Neuroepidemiology and Tropical Neurology, School of Medicine, CNRS FR 3503 GEIST, University of Limoges, Limoges, France;* **4** *Department of Veterinary Parasitology and Microbiology, School of Veterinary Medicine, Makerere University, Kampala, Uganda;* **5** *Department of Biochemistry, College of Natural Sciences, Makerere University, Kampala, Uganda;* **6** *Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden;* **7** *Department of Parasitology, Institut National de Recherche Biomédicale, Kinshasa, D. R. Congo;* **8** *Instituto de Combate e Controlo das Tripanossomíases, Luanda, Angola;* **9** *Centre for Infection, Division of Cellular and Molecular Medicine, St. George's, University of London, London, Great Britain;* **10** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland.*

** These authors contributed equally*

SUMMARY

Background Sleeping sickness, or human African trypanosomiasis (HAT), is a protozoan disease that affects rural communities in sub-Saharan Africa. Determination of the disease stage, essential for correct treatment, represents a key issue in the management of patients. In the present study we evaluated the potential of CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, B2MG, neopterin and IgM to complement current methods for staging *Trypanosoma brucei gambiense* patients.

Methods and Findings Five hundred and twelve *T. b. gambiense* HAT patients originated from Angola, Chad and the Democratic Republic of the Congo (D.R.C.). Their classification as stage 2 (S2) was based on the number of white blood cells (WBC) (> 5/μL) or presence of parasites in the cerebrospinal fluid (CSF). The CSF concentration of the eight markers was first measured on a training cohort encompassing 100 patients (44 S1 and 56 S2). IgM and neopterin were the best in discriminating between the two stages of disease with 86.4% and 84.1% specificity respectively, at 100% sensitivity. When a validation cohort (412 patients) was tested, neopterin (14.3 nmol/L) correctly classified 88% of S1 and S2 patients, confirming its high staging power. On this second cohort, neopterin also predicted both the presence of parasites, and of neurological signs, with the same ability as IgM and WBC, the current reference for staging.

Conclusions This study has demonstrated that neopterin is an excellent biomarker for staging *T. b. gambiense* HAT patients. A rapid diagnostic test for detecting this metabolite in CSF could help in more accurate stage determination.

INTRODUCTION

Sleeping sickness, or human African trypanosomiasis (HAT), is a parasitic disease that affects rural communities in sub-Saharan Africa.[1] More than 90% of HAT cases are caused by *Trypanosoma brucei gambiense*, responsible for the chronic form of the infection in Western and Central Africa.[2] Sleeping sickness typically progresses from a haemolymphatic first stage (S1) to a meningo-encephalitic second stage (S2), when parasites cross the blood-brain barrier (BBB) and enter the central nervous system (CNS).[3] As S1 and S2 patients are treated with different drugs, a correct determination of the stage of disease is crucial. *T. b. gambiense* S1 patients are treated with

pentamidine, a drug that cannot be used to treat S2 patients due to its low diffusion into the CNS.[4] Most S2 patients were until recently treated with melarsoprol, a toxic arsenic derivate that can cause fatal encephalopathy and for which increased treatment failure rates have been observed in different foci endemic for *T. b. gambiense* HAT.[4,5] Since 2006 the use of eflornithine has increased and, with the subsequent introduction of nifurtimox-eflornithine combination therapy (NECT), there has been a drastic reduction of the use of melarsoprol in all countries endemic for *T. b. gambiense* HAT.[6-9] Despite these new advances, difficulties in administration of eflornithine and NECT and associated high costs keep stage determination as a central issue in the management of HAT patients.

Determination of the stage of disease is currently based on examination of the cerebrospinal fluid (CSF) for presence of parasites and counting of white blood cells (WBC). Detection of parasites by microscopy is a specific method and relatively easy to use in the field,[10] but limited in sensitivity. To reduce the number of false negatives, i.e. stage 2 patients that would be wrongly treated with ineffective S1 drugs, parasite detection is complemented with counting of white blood cells in the CSF. According to WHO guidelines, S1 is defined as ≤ 5 WBC/µL and absence of trypanosomes in CSF, while S2 is defined as > 5 WBC/µL and/or parasites in CSF.[11] Despite WHO recommendations, a number of countries apply different thresholds to select the appropriate treatment,[12] highlighting the lack of a generally accepted gold standard for staging.[13] Furthermore, it has been shown that some patients having between 5 and 20 WBC per microliter of CSF without detected parasites, or patients having ≤ 5 WBC and presence of parasites (i.e., early-late stage patients) can be cured with S1 drugs.[14,15]

During the last decade, many studies have been conducted to rationalise stage determination in sleeping sickness. The use of PCR as surrogate for parasite detection in CSF showed promising results,[16,17] but there is not enough evidence in the literature to define the diagnostic accuracy of PCR in terms of sensitivity and specificity for stage determination.[10] The most promising results were obtained with the detection of intrathecal IgM as indicator of CNS involvement in HAT patients.[15,18,19] Detection of IgM was also translated into a rapid field test, LATEX/IgM.[18,20]

Many other studies that investigated alternative staging markers highlighted a strong correlation between the levels of cytokines, chemokines and other immune-mediators in CSF, and disease progression.[21] In the present study, we assess on a multi-centre cohort the CSF levels of six promising staging markers: B2MG, CXCL10, CXCL13, ICAM-1, VCAM-1 and MMP-9[22-27] plus neopterin, an indicator of activation of Th1 immune response,[28] already investigated in the staging of *T. b. gambiense* (Krishna S., unpublished) and *T. b. rhodesiense*[29] HAT. The concentration of total IgM was also measured on the same samples, as it currently represents the best alternative to counting WBC. The ability of all these markers to correctly stratify patients classified according to WHO staging criteria was first evaluated on a training cohort comprising 100 *T. b. gambiense* patients. The staging power of the best performing marker, was then validated on a larger multi-centre cohort (n=412) and compared to IgM and WBC.

METHODS

Ethics statement

The National Ethical Committees of the countries where samples were collected (Angola, Chad and the Democratic Republic of the Congo – D.R.C.) approved the studies. HAT patients gave written informed consent before enrolment. Children (< 18 years) or patients with altered mental status, a common condition in late stage HAT, were included only after written informed consent from a parent or a guardian. All patients had the option of withdrawing from the studies at any time.

Patients

Cerebrospinal fluid samples of 1028 patients with evidence of trypanosomes in blood, lymph or CSF were received at the University of Geneva. The samples originated from patients identified through active and passive case finding between 2005 and 2009 in Angola, Chad and the Democratic Republic of the Congo. Sample collection was done in the context of prospective diagnostic studies (THARSAT,[30] NEUROTRYP[22,23] and FIND/CD19), or was directed by the WHO for a HAT specimen bank (Supporting Table S1 and Supporting Figure S1).

Stage determination and treatment were performed in the designated treatment centres or by medical teams at the site of active screening, according to the policies of the national sleeping sickness control programs. The number of WBCs in CSF was determined by microscopy, and presence of parasites was determined during WBC counting or by using the modified single centrifugation method.[31] Samples were aliquoted and stored at -80°C or in liquid nitrogen. The levels of biomarkers were evaluated on

Table 1 Description of the training and the validation cohorts

	Training cohort (n=100)		Validation cohort (n=412)	
	Stage 1 (n=44)	**Stage 2 (n=56)**	**Stage 1 (n=184)**	**Stage 2 (n=228)**
Demography				
Female, n *	19	17	102	98
Age (mean [SD]) †	34.4 [±13.4]	31.7 [±12.2]	32.4 [±14.9]	32.8 [±12.7]
Geographical origin				
Angola, n	8	13	38	68
Chad, n	8	3	17	13
D.R.C., n	28	40	129	147
CSF examinations				
Trypanosome positive, n	0	50	0	166 ‡
WBC/µL (median [range])	2 [1-5]	278.5 [11-1350]	2 [0-5]	161 [1-2000]
Neurological signs				
Absent, n	33	3	142	39
Present, n	10	51	36	186
NA, n ‖	1	2	6	3

* Fisher's exact test: training cohort, non significant differences; validation cohort, p value = 0.0133

† Mann-Whitney *U* test: training and validation cohort, non significant differences

‡ Information not available for one patient

‖ NA: not available information

Stage was defined according to WHO guidelines.

the CSF samples between December 2009 and August 2010.

For the present study, all patients were classified as S1 or S2 according to WHO guidelines, i.e. S1 when ≤5 WBC/µL and no parasites in CSF, S2 when >5 WBC/µL and/or trypanosomes in CSF, regardless of the cut-offs applied at the country level. Exclusion criteria were: age <12 years, spontaneous withdrawal from the study, insufficient volume of CSF, haemorrhagic CSF (>100 red cells/µL), or missing information to classify patients as S1 or S2 according to WHO criteria. Information on the neurological status of patients, including daytime somnolence, sensory and gait disturbances, presence of primitive reflexes (palmomental and perioral reflexes), modified tendon reflexes (exaggeration or abolition), Babinski's sign, abnormal movements such as tremors (fine, diffused and generalized), was recorded when available. Due to the absence of systematic screening for malaria or HIV co-infections, we considered their prevalence not different between S1 and S2 patients.

Five hundred and twelve patients were considered eligible for the present study and were used to create two study cohorts. A first training cohort of 100 patients was established with 44 S1 and 56 S2 randomly chosen from among all eligible patients. For the selection of S2 patients, those considered to be early-late stage (i.e., patients having 5 <WBC/µL ≤20 and absence of parasites, or patients having ≤5 WBC/µL and presence of parasites)[14,15] were not included. The remaining 412 patients were included in the second cohort (i.e. the validation cohort). Estimating a staging AUC ≥90% for each marker based on the results obtained on the training cohort, the calculated power of the test on the validation cohort was equal to 1.

Immunoassay procedures

Commercially available human ELISA kits were used to determine CSF levels of CXCL13 (R&D Systems, UK), IgM (ICL, OR, USA), B2MG (Calbiotech, CA, USA) and neopterin (BRAHMS, Germany). Multiplex bead suspension assays (mBSA) were used to measure CXCL10 (Bio-Rad, CA, USA), MMP-9, VCAM-1 and ICAM-1 (R&D Systems). The assays were performed following manufacturers' instructions. For each assay the limit of detection (LOD) was calculated as the mean concentration of the lowest standard less 2 standard deviations. To all outliers (≤ LOD), a value corresponding to the mean of LODs divided by 2 was assigned. Variability (CV) between assays, evaluated using internal quality controls, was below 25%.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics version 20.0.0 (IBM, NY, USA), GraphPad PRISM version 4.03 (GraphPad Software Inc.) and S+ version 8.1 (TIBCO, Software Inc.). All statistical tests were two-tailed. Differences between two groups were assessed with the Mann-Whitney *U* test. For each biomarker, receiver-operating characteristic (ROC) curves, area under the curve (AUC), specificity (SP) and sensitivity (SE) were calculated from the pROC package.[32] To minimize the number of false negatives, the staging cut-offs on the training cohort were calculated as the threshold corresponding to 100% sensitivity.

On the validation cohort, the specificity and sensitivity for discrimination between early and late stage patients were obtained by applying the cut-offs calculated on the training cohort. The same analysis was done on patients classified according to the absence (T-) or presence (T+) of parasites in the CSF; or according to the absence (NS-) or presence (NS+) of neurological signs. Confidence intervals at the 95% level on AUC, sensitivity, specificity and cut-off were computed with pROC.

This work conforms to the STARD guidelines for reporting of studies on diagnostic accuracy.

RESULTS

Training cohort

When assessed on a the training cohort, comprising 44 early stage and 56 late stage *T. b. gambiense* patients (Table 1), the seven markers (CXCL10, CXCL13, neopterin, B2MG, ICAM-1, VCAM-1 and MMP-9) showed high ability to discriminate between the two groups of patients (AUC > 90% and p value < 0.0001, Mann-Whitney *U* test) (Table 2). The same results were obtained with IgM, confirming its value as a staging marker.[18] To reduce the number of false negatives, i.e. S2 patients wrongly classified as S1, the threshold corresponding to 100% sensitivity was calculated for each marker. Only IgM and neopterin were >80% specific at 100% sensitivity. In particular, with a threshold concentration of 14.3 nmol/L (13.4-30.3 95% CI), neopterin was 84% specific. Similar results were obtained with IgM (86.4% specificity) at a threshold concentration of 3.4 µg/mL (3.3-21 95% CI) (Table 2).

Table 2 Results obtained for the eight markers assessed on the training cohort

Marker	[S2]/[S1]	AUC% (95%CI)	Cut-off (95% CI)	SP% (95% CI) *	p value†
IgM [µg/mL]	71.9	99.6 (98.9-100)	3.4 (3.3-21)	86.4 (75-95.5)	< 0.0001
Neopterin [nmol/L]	17.6	99.6 (99-100)	14.3 (13.4-30.3)	84.1 (72.7-93.2)	< 0.0001
MMP-9 [pg/mL]	38.4	99.4 (98.4-100)	141.2 (126.9-1040)	72.7 (59.1-86.4)	< 0.0001
VCAM-1 [ng/mL]	3.8	95.5 (91.7-99.4)	15.2 (14.9-21.1)	68.2 (54.6-81.8)	< 0.0001
B2MG [ng/mL]	5.1	98.4 (96.7-100)	965 (927.5-1577)	63.6 (50-77.3)	< 0.0001
ICAM-1 [ng/mL]	5.4	97.7 (95.3-100)	1.3 (1.2-2.3)	61.4 (47.7-75)	< 0.0001
CXCL10 [pg/mL]	32.8	93.3 (88.9-97.8)	757.3 (706.4-1531.7)	43.2 (29.6-59.1)	< 0.0001
CXCL13 [pg/mL]	641.4	97.6 (94.9-100)	<8.2 (<8.2-64.6)	0 (0-0)	< 0.0001

Training cohort (n=100): Stage 1 n=44; Stage 2 n=56. Early-late stage patients were not included.

* Sensitivity was set to 100%

† Mann-Whitney *U* test

95%CI = 95% confidence interval; SP% = specificity %; SE% = sensitivity %.

The reported cut-off and SP% correspond to 100% SE.

Validation cohort

Neopterin and IgM were then measured on a validation cohort comprising 184 S1 and 228 S2 *T. b. gambiense* patients (Table 1). CSF neopterin at a threshold concentration of 14.3 nmol/L discriminated between early and late stage patients with 87.5% specificity (82.6-91.9 95% CI) and 88.2% sensitivity (83.8-92.1 95% CI) (Table 3). This means that 23 out of 184 S1 patients were classified as stage 2 by neopterin (false positives) and 27 out of 228 S2 were classified as S1 (false negatives). Among these 27 false negatives, twenty three belonged to the early-late stage group, i.e. patients having between 5 and 20 WBC/µL with no trypanosomes in CSF or ≤5 WBC/µL and presence of trypanosomes in CSF. When all early-late stage patients (n=41) were excluded from the S2 group, the number of false negatives was only 4 patients, thus increasing the sensitivity of neopterin in determining the disease stage to 97.9% (95.7-99.5 95% CI), (Table 4). Two of these 4 patients had ≤32 WBC/µL and neither showed evidence of parasites in CSF nor presence of neurological signs (Table 5).

With a staging cut-off of 3.4 µg/mL, IgM correctly discriminated S1 and S2 patients, classified according to WHO guidelines, with 86.4% (81-91.3 95% CI) specificity (25/184 false positives) and 91.7% (87.7-95.2 95% CI) sensitivity (19/228 false negatives) (Table 3). When early-late stage patients were excluded, the number of false negatives decreased to 2 patients thus increasing the sensitivity of IgM in disease staging to 98.9% (97.3-100 95%CI) (Table 4, Table 5).

To compare neopterin and IgM with WBC, patients were classified according to absence or presence of trypanosomes in CSF or according to absence or presence of neurological signs, as these two conditions can be indicative of an advanced stage of disease. The ability of the two markers and WBC to discriminate between the two groups was then assessed through ROC curves. Neopterin (14.3 nmol/L) was the best discriminator between patients without parasites in CSF (n=245) and those with detected

Table 3 Results obtained for IgM and neopterin on the validation cohort after application of the cut-off calculated on the training cohort

Marker	[S2/S1]	AUC% (95% CI)	Applied cut-off	SP% (95% CI)	SE% (95% CI)	p value *
IgM [µg/mL]	76.5	96.2 (94.4-98.0)	3.4	86.4 (81-91.3)	91.7 (87.7-95.2)	< 0.0001
Neopterin [nmol/L]	14.8	95.2 (93.2-97.1)	14.3	87.5 (82.6-91.9)	88.2 (83.8-92.1)	< 0.0001

Validation cohort (n=412): Stage 1 n=184; Stage 2 n=228. Early-late stage patients are included in S2 group.
*Mann-Whitney *U* test. 95%CI = 95% confidence interval; SP% = specificity %; SE% = sensitivity %.

Table 4 Results obtained for IgM and neopterin on the validation cohort after application of the cut-off calculated on the training cohort and removal of early-late stage patients (n=41)

Marker	AUC% (95% CI)	Applied cut-off	SP% (95% CI)	SE% (95% CI)
IgM [µg/mL]	99.2 (98.3-100)	3.4	86.4 (81.5-91.3)	98.9 (97.3-100)
Neopterin [nmol/L]	99 (98-100)	14.3	87.5 (82.6-91.9)	97.9 (95.7-99.5)

95%CI = 95% confidence interval; SP% = specificity %; SE% = sensitivity %.
Early-late stage patients (n=41), i.e. patients having CSF WBC/µL ≤ 5 and presence of parasites in CSF (n=4) or patients having 5<WBC/µL≤20 and absence of parasites in CSF (n=37), were excluded from the group of S2 patients.

parasites (n=166), showing higher specificity (75.1%, 69.8-80.4 95% CI) than WBC (68.6%, 62.9-74.3 95% CI) for similar levels of sensitivity (Table 6).

When patients were grouped based on the absence (n=181) or presence (n=222) of neurological signs, the specificity and sensitivity of both neopterin and IgM were comparable to WBC (Table 7).

Finally we assessed how neopterin and IgM classified early-late stage patients (n=41), defined as S2 according to WHO guidelines. Based on the two markers, respectively 23 (56%) and 17 (41%) of these patients were classified as S1 instead of S2. Most of the early-late stage patients (71%) were classified as stage 1 by both neopterin and IgM. However, no relationship between the presence of neurological signs and the levels of the two markers could be demonstrated in the analyzed patients (Figure 1).

DISCUSSION

The lack of a generally accepted gold standard highlights the need of new tools that could replace WBC counting and complement trypanosome detection in the staging of *T. b. gambiense* sleeping sickness patients.[13,33] The aim of the present study was to compare on a large multi-centre cohort of *T. b. gambiense* patients the ability of the best staging markers in CSF proposed in the literature[13,21,33] to discriminate between S1 and S2 HAT patients. This is the first time that neopterin, IgM, CXCL13, ICAM-1, VCAM-1, MMP-9, CXCL10 and B2MG have been assessed on the same training population encompassing 100 *T. b. gambiense* HAT patients. This evaluation allowed a better comparison of their individual performances, leading to the selection of neopterin as a promising new staging marker for *T. b. gambiense* sleeping sickness.

Due to the risk of relapses and even death when S2 patients are misdiagnosed and treated as S1 patients, a highly sensitive marker is needed in order to reduce the number of false negative patients. A cut-off corresponding to 100% sensitivity was thus established on the training cohort, and neopterin emerged as the most powerful staging marker with specificity close to that of IgM, the best alternative to WBC proposed so far.

Neopterin and IgM were further tested on a validation cohort encompassing 412 *T. b. gambiense* patients and the cut-offs calculated on the training cohort applied. The results obtained confirmed previously published data on the high staging power of IgM, for which a rapid, field applicable agglutination test was developed,[18] as well as preliminary data on the high correlation between neopterin and WBC counts (S. Krishna, unpublished). The decreased sensitivity observed on the validation cohort was due to the presence of early-late stage patients in the S2 group. These patients are the most critical because in some cases they have been reported to be cured with pentamidine.[14,34] When these patients were omitted from the validation cohort, the sensitivity of neopterin increased to 97.9% meaning that only 4 S2 patients, including 2 with trypanosomes in the CSF, were classified as false negatives. The value of neopterin as a staging tool was also supported by the results obtained for IgM, which showed very similar performance. Furthermore, both IgM and neopterin were shown to correlate with presence of parasites in CSF and neurological signs with the same accuracy as WBC count.

A rapid assay for detection of neopterin in CSF might have potential to replace WBC counting for diagnosis of the meningo-encephalitic stage of HAT, in

Table 5 Detailed description of false negative patients obtained according to neopterin and IgM after removal of early –late stage patients from S2 group

FN Patient	Neopterin<14.3 nmol/L	IgM<3.4 µg/mL	WBC/µL	Parasites	Neurological signs
# 1	Yes	Yes	25	Absent	Absent
# 2	Yes	No	32	Absent	Absent
# 3	Yes	No	261	Present	Present
# 4	Yes	Yes	282	Present	Present

FN: false negatives

Table 6 Ability of WBC, IgM and neopterin in discriminating between patients without or with parasites in CSF

T- vs. T+

Marker	AUC% (95% CI)	Applied cut-off	SP% (95% CI)	SE% (95% CI)
WBC (Cells/µL)	95.1 (92.8-97.4)	5.0	68.6 (62.9-74.3)	98.2 (95.8-100)
IgM [µg/mL]	95.2 (93-97.3)	3.4	71.4 (65.7-77.1)	98.2 (95.8-100)
Neopterin [nmol/L]	96.0 (93.9-98.0)	14.3	75.1 (69.8-80.4)	97.6 (95.2-99.4)

T-: patients without evidence of parasite in CSF (n=245); T+: patients with parasite detected in CSF (n=166). Missing information for 1 S2 patient.
95%CI = 95% confidence interval; SP% = specificity %; SE% = sensitivity %.

Table 7 Ability of WBC, IgM and neopterin in discriminating between patients without or with neurological signs

NS- vs. NS+

Marker	AUC% (95% CI)	Applied cut-off	SP% (95% CI)	SE% (95% CI)
WBC (Cells/µL)	86.2 (82.5-90.0)	5.0	72.4 (65.8-79.0)	83.8 (78.8-88.7)
IgM [µg/mL]	84.5 (80.6-88.5)	3.4	71.8 (65.2-78.5)	80.6 (75.2-85.6)
Neopterin [nmol/L]	88.2 (84.9-91.4)	14.3	78.5 (72.4-84)	81.5 (76.6-86.5)

NS-: patients without neurological signs (n=181); NS+: patients with neurological signs (n=222). Missing information for 9 patients.
95%CI = 95% confidence interval; SP% = specificity %; SE% = sensitivity %.

combination with parasite detection in CSF. These important perspectives in the field of staging do not find application in the diagnosis of the disease, which is based on detection of parasite-specific antibodies using the CATT test as a screening tool, followed by parasitological confirmation of disease. It might also show potential for assessment of cure after treatment, within a shorter period compared to IgM and WBC count.[30] Neopterin is a stable catabolic product of guanosine triphosphate (GTP) that is produced by interferon (IFN)-γ activated macrophages and dendritic cells.[35] It has been proposed as a marker of disease activity in tuberculosis[36,37] and of CNS involvement in HIV and *T. b. rhodesiense* HAT.[29] A simple dipstick assay for semi-quantitative detection of neopterin in serum has already been found useful and practicable for other infectious diseases,[38] indicating the potential to assess neopterin in CSF with a rapid diagnostic test.

The present study had a number of limitations that need to be considered. An important weakness was the absence of highly detailed and standardised information on patients' neurological status or presence of concomitant infections such as HIV and malaria, which could help in interpretation of data. The markers reported here for staging are not specific for HAT. Sleeping sickness patients are exposed to other infections, and neopterin has already been proposed as marker for other diseases that are endemic in Africa.[36] Thus, the effect of concomitant diseases on the levels of the markers should be investigated further.

Another drawback is the absence of a gold standard to which the performance of neopterin, as well as the other molecules analysed, could be compared. In an effort to overcome this deficiency, the staging performance was correlated to the presence of parasites in CSF, neurological abnormalities and WBC counts, parameters used for disease staging in practice. Furthermore, neopterin was also compared to IgM, already validated as a staging marker.[15,18] However, within the study cohorts, the methods used for parasite detection, WBC count and assessing neurological signs were not standardised, thus reducing the accuracy of original staging. Finally, the utility of the tested marker, including neopterin, in correct staging of early-late stage patients, i.e. patients having between 5 and 20 WBC/µL and no parasites in CSF, or patients having ≤5 WBC/µL and trypanosomes in CSF, cannot be assessed on the

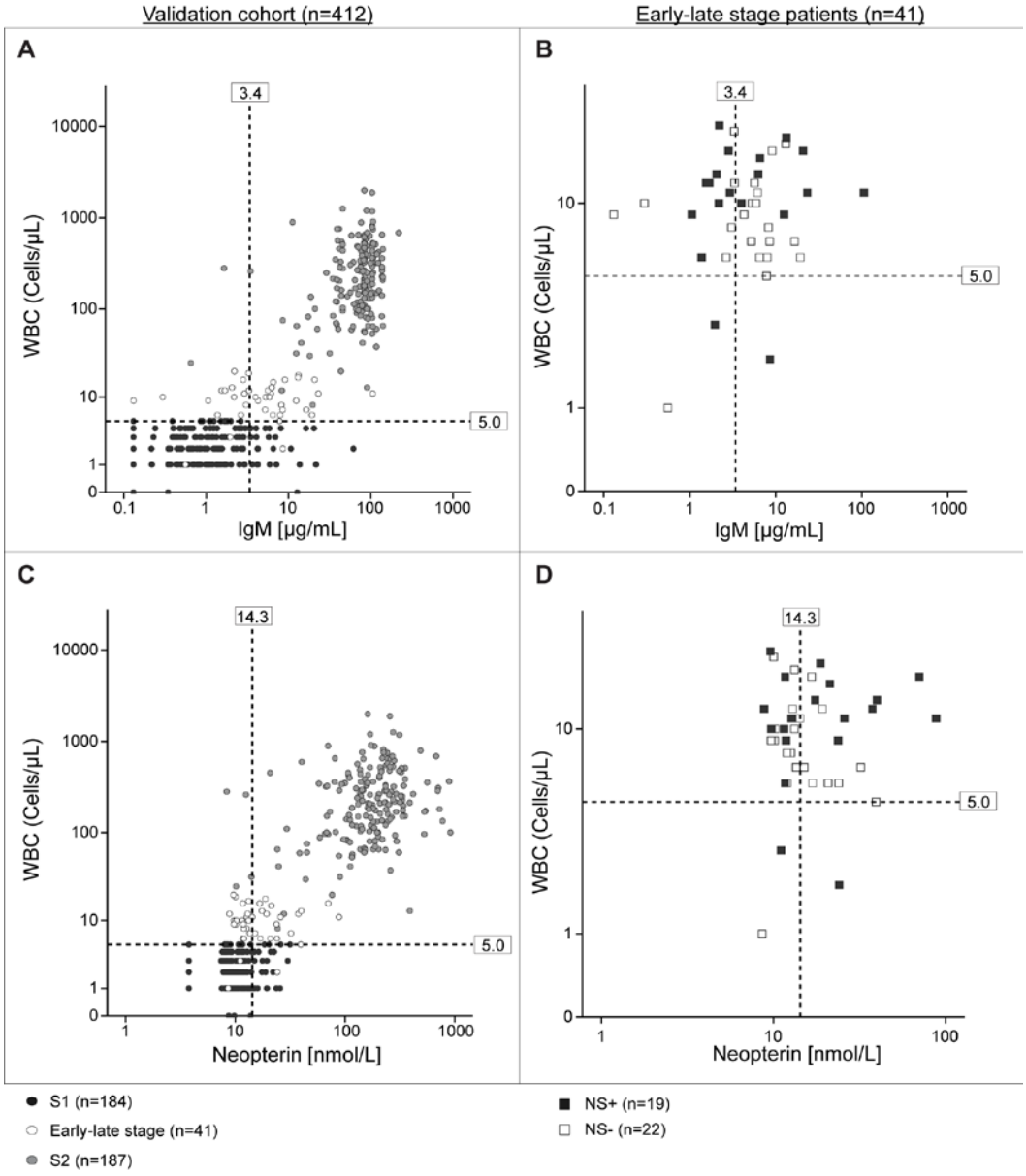

Figure 1 Classification of HAT patients according to WBC and IgM, or according to WBC and neopterin. **A)** Comparison of the classification of HAT patients (validation cohort) according to WBC and IgM. The staging cut-off of 5 WBC/µL recommended by WHO is reported on the graph as well as the staging cut-off of 3.4 µg/mL for IgM calculated on the training cohort. Black dots represent stage 1 patients, white dots represent early-late stage patients and gray dots represent stage 2 patients. **B)** Detailed classification of early-late stage patients (n=41) according to IgM. Colours indicate the absence (white) or presence of neurological (black) signs. The staging cut-off of 5 WBC/µL recommended by WHO is reported on the graph as well as the staging cut-off of 3.4 µg/mL for IgM calculated on the training cohort. **C)** Comparison of the classification of HAT patients (validation cohort) according to WBC and neopterin. The staging cut-off of 5 WBC/µL recommended by WHO is reported on the graph as well as the staging cut-off of 14.3 nmol/L for neopterin calculated on the training cohort. Black dots represent stage 1 patients, white dots represent early-late stage patients and gray dots represent stage 2 patients. **D)** Detailed classification of early-late stage patients (n=41) according to neopterin. Colours indicate the absence (white) or presence (black) of neurological signs. The staging cut-off of 5 WBC/µL recommended by WHO is reported on the graph as well as the staging cut-off of 14.3 nmol/L for neopterin calculated on the training cohort. NS+: presence of neurological signs; NS-: absence of neurological signs.

current cohort. Some early-late stage patients have previously been reported to be cured with pentamidine.[34] In the studied cohort, some of the early-late stage patients could have benefited from treatment with S1 drugs, as suggested by levels of neopterin and IgM that were below the proposed threshold values.

The markers evaluated in the current study still rely on examination of CSF, which is the basis of actual staging. Ideally, alternative markers for staging should be detectable in plasma, thus avoiding lumbar punctures. Unfortunately, none of the markers investigated here was able to distinguish between S1 and S2 patients when measured in plasma (data not shown).

In conclusion this study highlights the value of neopterin in CSF as a marker for the identification the meningo-encephalitic stage of *gambiense* HAT. A rapid diagnostic test for detection of this metabolite in patients' CSF could become a valuable alternative to counting of WBC, still to be combined with parasite detection in CSF. The value of neopterin for follow-up after treatment should be investigated as well as its ability to stage *Trypanosoma brucei rhodesiense* HAT.

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COMPETING INTERESTS

SK, VL and PB were consultant for the Foundation for Innovative New Diagnostics (FIND) at the moment of the analyses. JMN is employee of the Foundation for Innovative New Diagnostics (FIND). All other authors have declared that no competing interests exist.

SUPPORTING INFORMATION

Figure S1 Flow chart representing the collected CSF samples.

Table S1 Description of the prospective diagnostic studies from which patients were obtained.

REFERENCES

1. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet 2009;375:148-59.
2. Malvy D, Chappuis F. Sleeping sickness. Clin Microbiol Infect 2011;17:986-95.
3. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clin Microbiol Rev 2005;18:133-46.
4. Burri C. Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitology 2010;137:1987-94.
5. Moore AC. Prospects for improving African trypanosomiasis chemotherapy. J Infect Dis 2005;191:1793-5.
6. WHO. Model list of essential medicines (EML). 2009.
7. Priotto G, Kasparian S, Mutombo W, et al. Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet 2009;374:56-64.
8. Simarro PP, Franco J, Diarra A, Postigo JA, Jannin J. Update on field use of the available drugs for the chemotherapy of human African trypanosomiasis. Parasitology 2012:1-5.
9. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS Negl Trop Dis 2011;5:e1007.
10. Mugasa CM, Adams ER, Boer KR, et al. Diagnostic accuracy of molecular amplification tests for human African trypanosomiasis--systematic review. PLoS Negl Trop Dis 2012;6:e1438.
11. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser 1998;881:I-VI, 1-114.
12. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Trans R Soc Trop Med Hyg 2008;102:306-7.
13. Kennedy PG. Difficulties in diagnostic staging of human African trypanosomiasis. J Neuroparasitol 2011;2.
14. Doua F, Miezan TW, Sanon Singaro JR, Boa Yapo F, Baltz T. The efficacy of pentamidine in the treatment of early-late stage Trypanosoma brucei gambiense trypanosomiasis. Am J Trop Med Hyg 1996;55:586-8.
15. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. J Infect Dis 2003;187:1475-83.
16. Deborggraeve S, Lejon V, Ekangu RA, et al. Diagnostic accuracy of PCR in gambiense sleeping sickness diagnosis,

staging and post-treatment follow-up: a 2-year longitudinal study. PLoS Negl Trop Dis 2011;5:e972.
17. Truc P, Jamonneau V, Cuny G, Frezil JL. Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. Bull World Health Organ 1999;77:745-8.
18. Lejon V, Legros D, Richer M, et al. IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Trop Med Int Health 2002;7:685-92.
19. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. J Neurol Sci 2002;193:127-35.
20. Lejon V, Buscher P, Sema NH, Magnus E, Van Meirvenne N. Human African trypanosomiasis: a latex agglutination field test for quantifying IgM in cerebrospinal fluid. Bull World Health Organ 1998;76:553-8.
21. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Prog Neurobiol 2010;91:152-71.
22. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. J Infect Dis 2009;200:1556-65.
23. Amin DN, Ngoyi DM, Nhkwachi GM, et al. Identification of stage biomarkers for human African trypanosomiasis. Am J Trop Med Hyg 2010;82:983-90.
24. Hainard A, Tiberti N, Robin X, et al. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS Negl Trop Dis 2009;3:e459.
25. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Trop Med Int Health 2009;14:529-34.
26. Hainard A, Tiberti N, Robin X, et al. Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis. Trop Med Int Health 2011;16:119-26.
27. Tiberti N, Hainard A, Lejon V, et al. Discovery and verification of osteopontin and Beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Mol Cell Proteomics 2010;9:2783-95.
28. Hoffmann G, Wirleitner B, Fuchs D. Potential role of immune system activation-associated production of neopterin derivatives in humans. Inflamm Res 2003;52:313-21.
29. Maclean L, Odiit M, Sternberg JM. Intrathecal cytokine responses in Trypanosoma brucei rhodesiense sleeping sickness patients. Trans R Soc Trop Med Hyg 2006;100:270-5.
30. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. J Infect Dis 2010;201:453-63.
31. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Trans R Soc Trop Med Hyg 2000;94:293.
32. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.
33. Kennedy PG. Novel biomarkers for late-stage human African trypanosomiasis--the search goes on. Am J Trop Med Hyg 2010;82:981-2.
34. Lejon V, Legros D, Savignoni A, Etchegorry MG, Mbulamberi D, Buscher P. Neuro-inflammatory risk factors for treatment failure in "early second stage" sleeping sickness patients treated with pentamidine. J Neuroimmunol 2003;144:132-8.
35. Sucher R, Schroecksnadel K, Weiss G, Margreiter R, Fuchs D, Brandacher G. Neopterin, a prognostic marker in human malignancies. Cancer Lett 2010;287:13-22.
36. Hagberg L, Cinque P, Gisslen M, et al. Cerebrospinal fluid neopterin: an informative biomarker of central nervous system immune activation in HIV-1 infection. AIDS Res Ther 2010;7:15.
37. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet 2006;368:1012-21.
38. Buhrer-Sekula S, Hamerlinck FF, Out TA, Bordewijk LG, Klatser PR. Simple dipstick assay for semi-quantitative detection of neopterin in sera. J Immunol Methods 2000;238:55-8.

CHAPTER 6

Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness

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Staging sleeping sickness is not the only priority in the management of this neglected tropical disease. Due to the limited efficacy of some of the treatment currently used, the early detection of cases of relapse is essential to properly re-treat patients.

In the work presented in this chapter, we hypothesized that the CSF markers previously shown to be able to indicate the advanced stage of the disease before the treatment, could potentially be used to indicate the re-appearance of the S2 disease during the follow-up. The 8 markers tested for the staging of *T. b. gambiense* HAT were, therefore, assessed on a small population of *T. b. gambiense* patients followed after treatment to assess their potential in detecting relapses. The best performing markers, i.e. neopterin and CXCL13, were then assessed on a larger population of patients followed after treatment. Neopterin showed the highest accuracy of discrimination between S2 cured and S2 relapsed patients. It was able to shorten the follow-up for patients as soon as 6 or 12 months after treatment with high accuracy.

This work has been published in PLoS Neglected Tropical Diseases in 2013. I performed part of the experiments, I analyzed and interpreted the data and I wrote the manuscript.

Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness

Natalia Tiberti[1], Veerle Lejon[2], Alexandre Hainard[1], Bertrand Courtioux[3,4], Xavier Robin[1], Natacha Turck[1], Krister Kristensson[5], Enock Matovu[6], John C. Enyaru[7], Dieudonné Mumba Ngoyi[8], Sanjeev Krishna[9], Sylvie Bisser[3,4], Joseph Mathu Ndung'u[10], Philippe Büscher[2], Jean-Charles Sanchez[1*]

1 *Biomedical Proteomics Research Group, Department of Human Protein Sciences, University of Geneva, Geneva, Switzerland;* **2** *Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium;* **3** *INSERM UMR1094, Tropical Neuroepidemiology, Limoges, France;* ***4*** *Institute of Neuroepidemiology and Tropical Neurology, School of Medicine, CNRS FR 3503 GEIST, University of Limoges, Limoges, France;* **5** *Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden;* **6** *Department of Veterinary Parasitology and Microbiology, School of Veterinary Medicine, Makerere University, Kampala, Uganda;* **7** *Department of Biochemistry, College of Natural Sciences, Makerere University, Kampala, Uganda;* **8** *Department of Parasitology, Institut National de Recherche Biomédicale, Kinshasa, D. R. Congo;* **9** *Centre for Infection, Division of Cellular and Molecular Medicine, St. George's, University of London, London, Great Britain;* **10** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland.*

SUMMARY

Background Post-therapeutic follow-up is essential to confirm cure and to detect early treatment failures in patients affected by sleeping sickness (HAT). Current methods, based on finding of parasites in blood and cerebrospinal fluid (CSF) and counting of white blood cells (WBC) in CSF, are imperfect. New markers for treatment outcome evaluation are needed. We hypothesized that alternative CSF markers, able to diagnose the meningo-encephalitic stage of the disease, could also be useful for the evaluation of treatment outcome.

Methodology/Principal findings Cerebrospinal fluid from patients affected by *Trypanosoma brucei gambiense* HAT and followed for two years after treatment was investigated. The population comprised stage 2 (S2) patients either cured or experiencing treatment failure during the follow-up. IgM, neopterin, B2MG, MMP-9, ICAM-1, VCAM-1, CXCL10 and CXCL13 were first screened on a small number of HAT patients (n=97). Neopterin and CXCL13 showed the highest accuracy in discriminating between S2 cured and S2 relapsed patients (AUC 99% and 94%, respectively). When verified on a larger cohort (n=242), neopterin resulted to be the most efficient predictor of outcome. High levels of this molecule before treatment were already associated with an increased risk of treatment failure. At six months after treatment, neopterin discriminated between cured and relapsed S2 patients with 87% specificity and 92% sensitivity, showing a higher accuracy than white blood cell numbers.

Conclusions/Significance In the present study neopterin was highlighted as a useful marker for the evaluation of the post-therapeutic outcome in patients suffering from sleeping sickness. Detectable levels of this marker in the CSF have the potential to shorten the follow-up for HAT patients to six months after the end of the treatment.

INTRODUCTION

Sleeping sickness, also known as human African trypanosomiasis (HAT), is a neglected parasitic disease widespread in sub-Saharan Africa where it mainly afflicts rural communities.[1] According to the most recent published data, 10'000 new cases were reported in 2009.[2] More than ninety percent of HAT

AUTHOR SUMMARY

The reduction of the number of lumbar punctures performed during the follow-up of patients affected by sleeping sickness (HAT) is considered a research priority. Follow-up, consisting of the examination of cerebrospinal fluid (CSF) for presence of parasites and for the number of leukocytes, is necessary to assess treatment outcome. However, diagnosis of treatment failure is still imperfect and WHO encourages improvements in defining criteria.

Many studies have attempted to standardize actual methods and to define a cut-off for the number of white blood cells in CSF to define relapses, while only few have proposed alternatives to current practice. Here we showed that neopterin, already proven to be a powerful marker for staging *T. b. gambiense* HAT, is also useful in evaluating post-therapeutic outcome. The measurement of neopterin concentrations in CSF during the follow-up may allow reduction in the number of lumbar punctures from five to three for the majority of cured patients.

cases are caused by *Trypanosoma brucei gambiense* parasite, which is responsible for a chronic disease in Western and Central Africa.[2] Without treatment the disease progresses through two stages. Immediately after infection the proliferation of the parasites in blood and lymph, gives rise to the haemolymphatic first stage (stage 1, S1). If stage 1 patients are not treated, the disease progresses to the second meningo-encephalitic stage (stage 2, S2) as a consequence of the penetration of the parasites into the central nervous system (CNS).[2] HAT patients need to be treated according to their stage. Thus, S1 patients should receive pentamidine treatment, while S2 patients can be treated using melarsoprol, eflornithine or NECT (nifurtimox-eflornithine combination therapy).[3,4] However, patients cannot be considered cured immediately after treatment as parasites may persist in the host and the disease may reappear later on as a consequence of treatment failure.[5] To assess the efficacy of the treatment, patients need to be followed for two years to detect relapses, or to confirm recovery.[6] WHO still recommends 5 follow-up visits performed at the end of the treatment (EoT) and at 6, 12, 18 and 24 months after treatment.[6] Visits consist of clinical assessments, examination of blood and cerebrospinal fluid (CSF) for the presence of trypanosomes and evaluation of the number of white blood cells (WBC) in the CSF. Optimal criteria to detect relapses accurately and early, when trypanosomes are not yet detectable, are being investigated. Several studies have tried to determine a cut-off for the number of WBCs, to predict relapses and to shorten the follow-up to less than 24 months after treatment.[5,7,8] Recently, an algorithm based on the CSF WBC count at 6 and 12 months has been proposed and showed a high potential in shortening patient follow-up as soon as 6 months after treatment.[5,8] However, the counting of WBC still has weaknesses, such as limited specificity and reproducibility, as already highlighted for the staging of HAT patients.[9] New surrogate markers to assess the post-therapeutic outcome therefore represent an unmet need, as highlighted by WHO.[10,11]

Very few alternative markers in CSF have been evaluated so far. These included DNA detection by PCR,[12,13] IgM and trypanosome specific antibodies, total proteins and the level of the anti-inflammatory cytokine IL-10.[14] However, when assessed at 6 and 12 months after treatment, they showed lower accuracy as outcome predictors compared to WBC.

We hypothesize that newly described CSF staging markers,[15-23] able to indicate the presence of the second stage of sleeping sickness, could also indicate a reappearance of the infection in S2 patients after treatment. IgM, B2MG, CXCL13, CXCL10, MMP-9, VCAM-1, ICAM-1 and neopterin were first tested on a small cohort of HAT patients followed after treatment to carry out a preliminary selection of molecules with highest accuracy as outcome predictors. Markers with the highest accuracy (neopterin and CXCL13) were further validated on a larger number of patients and compared to WBC to assess the treatment outcome.

METHODS

Ethics statement

The THARSAT study, from which patients originated, was approved by the Ministry of Health of the Democratic Republic of the Congo and by the Commission for Medical Ethics of the Institute of Tropical Medicine Antwerp, Belgium (reference 04441472). All patients, or their legal representatives, gave written informed consent before enrolment. All patients had the possibility to withdraw from the study at any moment.

Patients

The present study was designed into two parts: a first screening of 8 markers on a small population, followed by the verification of the two most promising markers on a larger cohort.

All patients were enrolled by either active or passive case finding in the Democratic Republic of the Congo

as part of the THARSAT study.[8] Inclusion and exclusion criteria are reported elsewhere.[8]

All patients had parasitologically confirmed HAT, either as primary cases (no previous HAT treatment) or as secondary cases (previously treated for HAT). Stage determination was performed through CSF examination for number of leukocytes and presence of parasites following modified single centrifugation.[24] Stage was defined according to WHO guidelines, i.e. stage 1 when WBC ≤ 5 cells/µL and absence of parasites, stage 2 when WBC > 5 cells/µL and/or parasites detected in CSF.[6] Patients were treated according to their stage as reported by Mumba Ngoyi *et al*.[8] After treatment, patients were followed up with visits planned at the end of the treatment (EoT) and at 3, 6, 12, 18 and 24 months after treatment. Blood and CSF examinations were performed at each follow-up visit and outcome was determined as recommended by WHO.[8,10] Briefly, cured patients were defined based on absence of trypanosomes during the follow-up and CSF WBC ≤ 20/µL at 24 months for stage 2, or CSF WBC ≤ 5/µL for stage 1. Confirmed relapses were diagnosed following the finding of parasites in CSF at any follow-up visit. Probable relapses were diagnosed following an increased count of WBC (more than 30 WBC/µL compared to the lowest number of WBC obtained during the previous FU examinations) and/or aggravation of neurological signs, or WBC > 20/µL at 24 months.[8] Patients classified either as confirmed or probable relapses were considered as treatment failures.

The patients investigated in the present study were selected among the 360 participants of the THARSAT study,[8] after exclusion of those who died prior or during the follow-up, relapses of early stage patients, patients lost during the follow-up or for whom 2 or more interim follow-up visits were missing. Furthermore, patients whose diagnosis of relapse was based on the finding of parasites in blood (n=8) were also excluded, as they could potentially represent re-infection cases.

The screening cohort comprised S1 (n=19) and S2 (n=78) primary cases. All S2 patients included in the screening cohort received melarsoprol treatment and only cases of treatment failure (i.e. S2 relapse) defined as confirmed relapse were chosen. Selected cured and relapsed S2 patients were matched for age and sex. Characteristics at baseline, i.e. observed at the moment of the diagnosis and before the treatment, of patients included in the screening cohort are reported in Supporting Table S1.

Table 1 Characteristics at baseline of the verification cohort

	S1 cured (n=21)	S2 cured (n=114)	S2 relapsed* (n=107)
Demography			
Sex, F (n)†	14 (66.7%)	28 (24.6%)	33 (35.3%)
Age, years [mean ± SD]‡	37.3 [± 13]	34 [± 12.4]	34.3 [± 12.4]
Pre-treatment CSF examination			
Trypanosome positive, n	0	104	100
WBC/µL (median, range)	3 [1-5]	213 [2-1940]	267 [8-2064]
Neurological signs			
Absent	13	15	13
Present	8	99	94
Treatment			
P	21	0	0
E	0	42	2
M	0	40	99
M-E	0	1	0
M-N	0	31	6
Secondary case, n‖	0	66	9

***** n=27, probable relapse

† No significant difference between S2 cured and S2 relapsed, Fisher's exact test

‡ No significant difference between S2 cured and S2 relapsed, Mann-Whitney *U* test

‖ Secondary case: patients already treated once for HAT

P: pentamidine treatment; E: eflornithine treatment; M: melarsoprol treatment; M-E: combination of melarsoprol and eflornithine; M-N: combination of melarsoprol and nifurtimox. More details on treatment regimens are reported in[8].

The verification cohort (n=242) comprised all patients of the THARSAT study considered eligible for the present study and for whom enough CSF sample volume was available to perform all the analyses. Eighty six patients were included in both screening and verification cohort. The characteristic at baseline of the verification cohort are reported in Table 1. More details on the verification cohort are reported in Supporting Figure S1.

Immunoassays

Cerebrospinal fluid levels of neopterin (Brahms, Thermo Fisher Scientific, Germany), IgM (ICL, OR, USA), B2MG (Calbiotech, CA, USA) and CXCL13 (R&D Systems, UK and RayBiotech, GA, USA), were measured using commercially available ELISA assays. The levels in CSF of CXCL10, MMP-9, ICAM-1 and VCAM-1 were measured using multiplex bead suspension assays (R&D Systems, UK).

All assays were performed according to manufacturer's instructions and the inter-assay variability was evaluated using quality controls (coefficient of variation - CV < 20%). A limit of detection (LOD, corresponding to the mean measured concentration for the lowest standard less 2 standard deviations) was calculated for each assay. To all outliers (≤LOD) a value corresponding to the mean of LODs divided by 2 was assigned.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 20.0.0 (IBM, NY, USA) and STATA version 11.0 (StataCorp LP, TX, USA). Receiver operating characteristic (ROC) curves, area under the ROC curve (AUC), corrected partial AUC (pAUC), sensitivity (SE) and specificity (SP) were computed using the pROC package for S+ version 8.1 (TIBCO, Software Inc.).

All statistical tests were two tailed and significance level was set at 0.05. Comparison between two groups was performed with the Mann-Whitney *U* test for independent variables or using the Wilcoxon signed rank test for dependent variables. Comparison between more than two independent variables was obtained through the Kruskal-Wallis test followed by post-test to assess differences between pairs. The accuracy of the markers in discriminating between cure and relapse was evaluated considering only stage 2 patients.

First analysis - screening

ROC analysis was performed to assess the accuracy of the markers in discriminating between cured and relapsing patients. The AUC and the cut-off corresponding to the highest combination of specificity and sensitivity were calculated. Comparison was done between the levels of the markers measured in S2 patients at the moment of the relapse taking cured S2 patients at matched time points. The two best markers were selected for further analyses.

Second analysis - verification

Baseline risk factors, i.e. pre-treatment, for relapse were assessed using logistic regression and calculating the relative risk, prior and after adjustment for treatment. The following baseline variables were investigated for their association with an increased risk of treatment failure: sex, age, treatment, secondary cases, incomplete treatment, presence of parasites in CSF, number of WBC in CSF, presence of neurological signs and CSF concentrations of neopterin and CXCL13. The threshold in concentration of the two markers was determined using ROC curves and comparing their baseline levels in the two categories of late stage patients (cured and relapsed).

The accuracy for the prediction of treatment outcome was evaluated for neopterin, CXCL13 and WBC at three, six and twelve months after treatment. Eighteen and 24 months were not tested due to the low number of patients experiencing a relapse later than 12 months after treatment in the THARSAT study. For each marker and at each time point of the follow-up a highly sensitive cut-off was selected within an area under the ROC curve comprised between 90 and 100% sensitivity (partial AUC - pAUC).[25] Positive and negative likelihood ratios (LR+ and LR-, respectively) were calculated to better evaluate the association of the levels of the markers and the final outcome at the 3 time points of follow-up considered.[26]

RESULTS

First analysis - screening

The first analysis consisted in the evaluation of IgM, B2MG, CXCL10, CXCL13, MMP-9, VCAM-1, ICAM-1 and neopterin on a cohort of 97 patients.

According to the AUC, neopterin and CXCL13 showed the highest accuracy in discriminating S2 cured and S2 relapsed patients (Table 2). Neopterin showed a higher AUC, and both neopterin and CXCL13 showed higher sensitivity than the counting of leukocytes.

The ability of neopterin and CXCL13 in following the disease progression in the different categories of patients was further confirmed through the kinetic profiles, where an increased concentration of the two markers in relapsing patients was highlighted (Supporting Figure S2).

Table 2 Performance of the markers on the screening cohort

Marker	AUC% (95%CI)	Cut-off	SP% (95%CI)	SE% (95%CI)
Neopterin [nmol/L]	99.1 (97.7-100)	64.4	100 (100-100)	92.3 (82.1-100)
CXCL13 [pg/mL]	94.0 (88.9-99.2)	125.5	84.6 (71.8-94.9)	92.3 (82.1-100)
ICAM-1 [ng/mL]	93.2 (87.0-99.4)	2.1	92.3 (82.1-100)	87.2 (76.9-97.4)
CXCL10 [pg/mL]	90.0 (83.6-98.2)	2308.4	84.6 (71.8-94.9)	89.7 (79.5-97.4)
B2MG [ng/mL]	89.2 (81.4-97.1)	2486.5	94.9 (87.2-100)	76.9 (64.1-89.7)
MMP-9 [pg/mL]	88.8 (80.8-96.9)	814.8	84.6 (71.8-94.9)	87.2 (76.9-97.4)
VCAM-1 [ng/mL]	86.2 (77.4-95)	38.5	94.9 (87.2-100)	69.2 (53.9-82.1)
IgM [µg/mL]	81.2 (71.6-90.8)	75.9	66.7 (51.3-79.5)	87.2 (76.9-97.4)
WBC (Cells/µL)	96.9 (93.9-100)	44.5	94.9 (87.2-100)	89.7 (79.5-97.4)

Number of patients: 39 S2 relapsed vs. 39 S2 cured
SP%: specificity %; SE%: sensitivity%; 95%CI: 95% confidence interval
The reported cut-off corresponds to the best combination of specificity and sensitivity obtained for each marker

Second analysis - verification

Pre-treatment evaluation of the markers

Neopterin and CXCL13 were investigated on a larger population comprising 242 patients (Table 1, Supporting Figure S1). Baseline characteristics of S2 patients, cured and relapsed, were first analysed to evaluate whether they could already represent a risk of treatment failure (Table 3). An increased risk of relapse was associated with the received treatment and with the condition of being a primary case, i.e. never treated before for HAT. As these two conditions are strictly associated (primary cases were preferentially treated with melarsoprol), new relative risks were calculated after adjustment for treatment. This analysis highlighted an increased risk of relapse for patients presenting parasites in the CSF and a high count of WBC (≥ 100/µl) at diagnosis.

At the time of the diagnosis, i.e. before treatment, the levels of neopterin and CXCL13 were higher in S2 patients, cured or relapsed, compared to S1 (p value<0.0001, Kruskal-Wallis test, followed by post-test paired comparison). Only neopterin was able to significantly discriminate (p<0.05) between cured S2 patients and relapsed S2 patients already at baseline with AUC of 60% (52.8-67.74 95% CI), while CXCL13 and WBC showed AUC of 55% (49.2-61, 95%CI) and 56.8% (49.3-64.4), respectively. However, no significant differences were observed between the ROC curves (DeLong's test for two correlated ROC curves) (data not shown).

When the best cut-offs for discrimination between S2 cured and relapsed patients at baseline (neopterin 261.6 nmol/L and CXCL13 1734.1 pg/mL) were used to dichotomize the population, patients presenting high baseline levels of the markers were associated to a significantly higher risk for treatment failure (Table 3).

Evolution of the markers after treatment

A significant decrease in CSF concentration of neopterin, CXCL13 and white blood cells was observed in all S2 patients after treatment (p value < 0.0001, Wilcoxon signed rank test for paired samples) (data not shown).

The evolution of the two markers and of WBC during further follow-up of HAT patients is represented in Figure 1. The levels of neopterin, CXCL13 and of WBC in S1 cured patients remained constantly lower than in S2 patients. In late stage patients confirmed to be cured at the end of the follow-up neopterin, CXCL13 and WBC reached levels closer to those observed in S1 patients cured already 3 months after treatment (Figure 1).

In those patients experiencing a relapse, the levels of the two markers and of WBC, after having decreased following treatment administration, started to increase at 3 months after treatment when 43 patients out of 105 already had a relapse or a probable relapse. Similarly, higher median concentration of neopterin and CXCL13 were observed in relapsing patients 6 and 12 months after treatment compared to cured S2 patients.

ROC curves were built to assess the performance of neopterin, CXCL13 and WBC count in discriminating between cured S2 patients and S2 patients having a relapse 3, 6 and 12 months after treatment. Partial AUC (pAUC) between 90 and 100% of sensitivity and the best cut-off within this area were calculated (Table 4). When measured in the CSF taken 3 months after treatment, neopterin and CXCL13 showed accuracy comparable to the one of WBC. However, six months after treatment, neopterin was a better predictor of the outcome than both CXCL13 and WBC with a negative likelihood ratio < 0.1 indicating a strong correlation between the levels of this marker and the ruling out of cured patients.

Table 3 Baseline risk factors for treatment failure in late stage patients

	Treatment failure	*Unadjusted*		*Adjusted for TT*	
Baseline variable	**n/N**	**RR**	**p value**	**RR**	**p value**
Sex					
F	33/61	1		1	
M	74/160	0.85	0.28	0.88	0.31
Age					
<25	27/55	1		1	
25-39	43/94	0.93	0.69	1	0.99
>40	37/72	1.05	0.80	0.91	0.54
Treatment					
E	2/44	1			
M	99/139	15.67	<0.0001		
M-E	0/1	0.00	<0.0001		
M-N	6/37	3.57	0.11		
Secondary case					
No	98/144	1		1	
Yes	9/77	0.17	<0.0001	0.94	0.90
Incomplete treatment					
No	92/197	1		1	
Yes	15/24	1.34	0.10	1	0.96
Parasites in CSF					
T-	7/17	1		1	
T+	100/204	1.19	0.56	1.83	0.04
CSF WBC/µL					
<100	17/46	1		1	
100-199	26/50	1.40	0.15	1.64	0.02
200-399	32/69	1.25	0.39	1.52	0.04
≥400	32/56	1.54	0.05	1.32	0.18
Neurological signs					
NS-	12/27	1		1	
NS+	95/194	1.1	0.67	1.28	0.24
Neopterin [nmol/L]					
≤ 261.6	35/96	1		1	
>261.6	72/125	1.58	0.003	1.75	<0.0001
CXCL13 [pg/mL]					
≤ 1734.1	17/50	1		1	
> 1734.1	90/171	1.55	0.04	1.55	0.02

TT: treatment; E: eflornithine treatment; M: melarsoprol treatment; M-E: combination of melarsoprol and eflornithine; M-N: combination of melarsoprol and nifurtimox
n/N: n=number of patients experiencing treatment failure, N=total number of patients
RR: risk ratio
T-: absence of parasite in CSF; T+: presence of parasites in CSF
NS-: absence of neurological sings, NS+: presence of neurological sings
Age and WBC/µL categories were defined according to Mumba Ngoyi D. *et al.*[8]

At this time point, neopterin, at a CSF concentration of 28 nmol/L was able to correctly classify as cured 97 out of 111 patients, with only 5 false negatives. Similarly, a WBC count of < 11 cells/µL could correctly predict 90 out of 111 cured patients with 6 false negatives, while CXCL13 could only predict 71 out of 111 cured patients.

Finally, twelve months after treatment neopterin showed strong power to rule in (LR+ 35) and to rule out (LR- 0.06) S2 patients. At a concentration of 41.4 nmol/L, it could correctly predict cure in 97% (93.6-100 95%CI) of cured patients with only 1 relapsing patient miss-classified, compared to the 93% predicted by WBC (87.3-97 95%CI). CXCL13 showed again a lower accuracy, being able to predict cure in 87% of cured patients (80.9-92.7% CI) (Table 4).

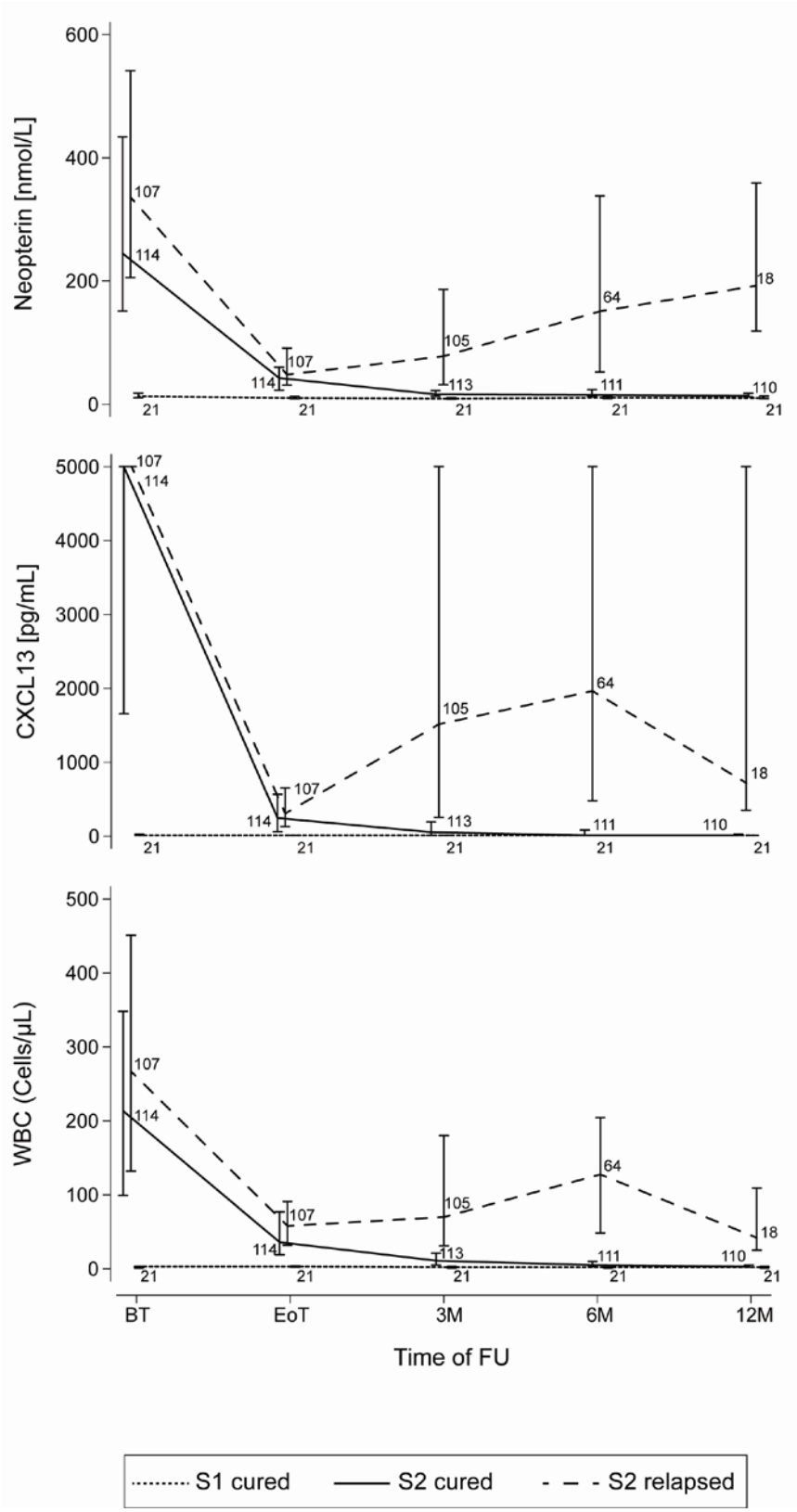

Figure 1 Kinetics of neopterin, CXCL13 and WBC during the follow-up. The variation in concentrations of the three markers in S1 cured patients, S2 cured patients and S2 relapsing patients are represented. Median concentrations at each time point are reported. Bars represent inter-quartile intervals. Numbers on the graphs represent the number of CSF samples assessed at each time point for each category of HAT patients. BT: before treatment; EoT: end of treatment; 3M, 6M, 12M: 3, 6, 12 months after treatment. FU: follow-up.

Table 4 Performances of neopterin, CXCL13 and WBC in discriminating between S2 cured and S2 relapsed patients

Marker, Tx	AUC%	pAUC%	Cut-Off	SP% (95%CI)	SE% (95%CI)	LR+	LR-	TN	FN
Neopterin [nmol/L]									
3M	90	69.3	17.3	57.5 (48.7-66.4)	93.3 (88.6-98.1)	2.2	0.12	65	7
6M	93.9	75.8	27.9	87.4 (81.1-92.8)	92.2 (84.4-98.4)	7.3	0.09	97	5
12M	98.4	93.3	41.4	97.3 (93.6-100)	94.4 (83.3-100)	35.0	0.06	107	1
CXCL13 [pg/mL]									
3M	86	60.4	58.9	52.2 (42.5-61.1)	90.5 (84.8-96.2)	1.9	0.18	59	10
6M	92.4	69.7	53.2	64 (55-73)	90.6 (82.8-96.9)	2.5	0.15	71	6
12M	94.7	78.3	76.7	87.3 (80.9-92.7)	94.4 (83.3-100)	7.4	0.06	96	1
WBC (Cells/µL)									
3M	87.1	64.5	14.5	62.8 (54-71.7)	90.5 (84.8-96.2)	2.4	0.15	71	10
6M	93.3	71.4	11.5	81.1 (73.9-88.3)	90.6 (82.8-96.9)	4.8	0.12	90	6
12M	94.4	70.9	9.5	92.7 (87.3-97.3)	94.4 (83.3-100)	12.9	0.06	102	1

3M, 6M, 12M: 3, 6, 12 months after treatment. Number of patients: 3M cured n=113, relapsed n=105; 6M cured n=111, relapsed n=64; 12M cured n=110, relapsed n=18. pAUC was calculated between 90 and 100% of sensitivity; SP% = specificity%; SE% = sensitivity%. Cut-off corresponds to the best combination if SP and SE within the pAUC. LR+: positive likelihood ratio, LR-: negative likelihood ratio; TN: true negatives, number of cured patients correctly classified; FN: false negatives, number of relapsing patients wrongly classified as cured.

DISCUSSION

The long post-therapeutic follow-up for patients affected with sleeping sickness is a major limitation in the management of HAT patients.[6] A gold standard to detect treatment failures is still missing.[10] New tools to achieve a better evaluation of treatment outcome, in terms of early detection of relapses and reduction of the time of follow-up are absolutely needed.[11] The reduction of the current follow-up of two-years would not only have the advantage of reducing the number of lumbar punctures, but would also potentially increase the compliance rate, as after 6 months a decrease of the attendance rate has been reported.[27]

In the present study, we investigated the ability of a number of molecules associated with an advanced stage of disease at diagnosis, as markers for treatment outcome.[15]

The evaluation of IgM, B2MG, CXCL10, CXCL13, MMP-9, ICAM-1, VCAM-1 and neopterin was performed on a first cohort of patients (n=97) followed after treatment. Neopterin, already highlighted as a powerful marker to stage *T. b. gambiense* HAT,[15] resulted here to be the most accurate discriminator between cured and relapsed patients, together with the chemoattractant chemokine CXCL13.

The association of these molecules with the advanced stage of HAT at diagnosis, already reported,[15,19] was confirmed. Furthermore, their high CSF concentration at baseline showed a strong association with an increased risk of treatment failure. When assessed during the complete follow-up, both neopterin and CXCL13 were able to indicate the recurrence of brain disease, as their concentration significantly increased in association with relapse.

From a functional point of view, both markers might be associated with the immune-pathogenesis of HAT. CXCL13 is a chemokine involved in the recruitment of B and T lymphocytes to the site of inflammation and its potential involvement in HAT progression has already been proposed.[19] Neopterin is a catabolic product of the GTP known as an indicator of the immune response activation, a central process in HAT late stage pathogenesis.[28,29] However, further investigations are needed to better understand the role of these molecules in both disease progression and reappearance.

Neopterin was here shown to be the most accurate marker for treatment outcome evaluation. When measured in the CSF of HAT patients 6 months after the end of the treatment, it was able to shorten the follow-up in 97 out of 111 S2 cured patients, thus potentially reducing the follow-up period and the number of lumbar punctures.

The present study has a number of limitations. In the verification population, treatment failures were diagnosed either based on the reappearance of the parasites in the CSF, or based on a high number of white blood cell count (probable relapses). This could represent a bias as white blood cells are not considered as a gold standard for the detection of relapses, but for some patients it was considered as a diagnostic criterion to which our markers were compared. Interestingly, the concentration of neopterin and the number of WBC, but not CXCL13, was significantly lower in suspected relapses

compared to confirmed relapses 6 months after treatment (data not shown), suggesting that some physiopathological differences may characterize the two groups.

Most relapse cases included in the present study had received melarsoprol treatment, which was, at the moment of the study, the first line treatment in the Democratic Republic of the Congo. However, due to the high relapsing rate observed after melarsoprol treatment, the first treatment of choice for *T. b. gambiense* HAT has now changed to eflornithine and NECT therapies.[3,4] Deeper investigations on a multi-centric cohort including recoveries and failures after treatments other than melarsoprol are needed, as already done for the counting of leukocytes,[5] even if low failure rates with NECT have been reported so far.[30]

Another drawback may rely in the lack of specificity of neopterin. This metabolite has been reported to be an indicator of the immune activation in other pathological conditions including HIV.[31,32] However, we observed significantly lower levels of neopterin in patients affected by cerebral malaria or meningitis when compared to HAT patients (data not shown). Better insights on the role of neopterin in the physiopathology of disease relapses could be achieved through the use of animal models, as already done for IL-10.[33]

Due to its high ability in stage determination,[15] developments for the translation of neopterin in an ASSURED test[34] for staging are ongoing. Here we extended the potential utility of this marker by showing its power as outcome predictor. In the present study, different cut-offs in neopterin concentration have been calculated, corresponding to the best performance of the marker at each time point of the follow-up. However, to be translated into clinical practice, a unique and highly accurate cut-off for the interim-follow up visits as well as a cut-off for the TOC visit should be determined, as it has been recently done for the WBC.[5,8]

The reduction of the follow-up from 24 months to a maximum of 12 months would have the major advantage of a decreased number of lumbar punctures for patients and, as a consequence, an increased attendance rate. However, a further improvement in patients' management would be the finding of test-of-cure markers in plasma. All molecules investigated here in CSF, were also assessed in the plasma of a small number of patients, but none of them was able to indicate the reappearance of the disease (data not shown).

In conclusion, the present study demonstrated the accuracy of neopterin in predicting and detecting treatment outcome for HAT patients. Due to its power as both staging[15] and follow-up marker for *T. b. gambiense* sleeping sickness, it is a promising candidate for further investigations in the field.

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COMPETING INTERESTS

SK, VL and PB were consultants for the Foundation for New Innovative Diagnostics (FIND) at the moment of the analyses. JMN is employee of the Foundation for New Innovative Diagnostics (FIND). All other authors declared that no competing interests exist.

SUPPORTING INFORMATION

Table S1. Characteristics at baseline of the screening cohort.

Supporting Figure S1 Flow-chart describing the patients investigated for the verification analysis.

Supporting Figure S2 Kinetics of the eight molecules and of WBC assessed on the screening cohort.

REFERENCES

1. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.

2. Malvy D, Chappuis F. Sleeping sickness. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2011;17:986-95.

3. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS neglected tropical diseases 2011;5:e1007.

4. Simarro PP, Franco J, Diarra A, Postigo JA, Jannin J. Update on field use of the available drugs for the chemotherapy of human African trypanosomiasis. Parasitology 2012;139:842-6.
5. Priotto G, Chappuis F, Bastard M, Flevaud L, Etard JF. Early prediction of treatment efficacy in second-stage gambiense human african trypanosomiasis. PLoS neglected tropical diseases 2012;6:e1662.
6. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.
7. Mumba Ngoyi D, Lejon V, N'Siesi FX, Boelaert M, Buscher P. Comparison of operational criteria for treatment outcome in gambiense human African trypanosomiasis. Tropical medicine & international health : TM & IH 2009;14:438-44.
8. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. The Journal of infectious diseases 2010;201:453-63.
9. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.
10. WHO. Recommendations of the informal consultation on issues for clinical product development for human African trypanosomiasis; WHO/CDS/NTD/IDM/2007.1. 2007.
11. Lejon V, Buscher P. Review Article: cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. Tropical medicine & international health : TM & IH 2005;10:395-403.
12. Truc P, Jamonneau V, Cuny G, Frezil JL. Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. Bulletin of the World Health Organization 1999;77:745-8.
13. Deborggraeve S, Lejon V, Ekangu RA, et al. Diagnostic accuracy of PCR in gambiense sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. PLoS neglected tropical diseases 2011;5:e972.
14. Lejon V, Roger I, Mumba Ngoyi D, et al. Novel markers for treatment outcome in late-stage Trypanosoma brucei gambiense trypanosomiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2008;47:15-22.
15. Tiberti N, Hainard A, Lejon V, et al. Cerebrospinal Fluid Neopterin as Marker of the Meningo-Encephalitic Stage of Trypanosoma brucei gambiense Sleeping Sickness. PloS one 2012;7:e40909.
16. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.
17. Amin DN, Ngoyi DM, Nhkwachi GM, et al. Identification of stage biomarkers for human African trypanosomiasis. The American journal of tropical medicine and hygiene 2010;82:983-90.
18. Hainard A, Tiberti N, Robin X, et al. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS neglected tropical diseases 2009;3:e459.
19. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Tropical medicine & international health : TM & IH 2009;14:529-34.
20. Hainard A, Tiberti N, Robin X, et al. Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis. Tropical medicine & international health : TM & IH 2011;16:119-26.
21. Tiberti N, Hainard A, Lejon V, et al. Discovery and verification of osteopontin and Beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Molecular & cellular proteomics : MCP 2010;9:2783-95.
22. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.
23. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. Journal of the neurological sciences 2002;193:127-35.
24. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:293.
25. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC bioinformatics 2011;12:77.
26. Deeks JJ, Altman DG. Diagnostic tests 4: likelihood ratios. BMJ 2004;329:168-9.
27. Robays J, Nyamowala G, Sese C, et al. High failure rates of melarsoprol for sleeping sickness, Democratic Republic of Congo. Emerging infectious diseases 2008;14:966-7.
28. Hoffmann G, Wirleitner B, Fuchs D. Potential role of immune system activation-associated production of neopterin derivatives in humans. Inflammation research : official journal of the European Histamine Research Society [et al] 2003;52:313-21.
29. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Progress in neurobiology 2010;91:152-71.
30. Priotto G, Kasparian S, Ngouama D, et al. Nifurtimox-eflornithine combination therapy for second-stage Trypanosoma brucei gambiense sleeping sickness: a randomized clinical trial in Congo. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2007;45:1435-42.
31. Berdowska A, Zwirska-Korczala K. Neopterin measurement in clinical diagnosis. Journal of clinical pharmacy and therapeutics 2001;26:319-29.
32. Hagberg L, Cinque P, Gisslen M, et al. Cerebrospinal fluid neopterin: an informative biomarker of central nervous

system immune activation in HIV-1 infection. AIDS research and therapy 2010;7:15.
33. Ngotho M, Kagira JM, Jensen HE, Karanja SM, Farah IO, Hau J. Immunospecific immunoglobulins and IL-10 as markers for Trypanosoma brucei rhodesiense late stage disease in experimentally infected vervet monkeys. Tropical medicine & international health : TM & IH 2009;14:736-47.
34. Peeling RW, Mabey D, Herring A, Hook EW, 3rd. Why do we need quality-assured diagnostic tests for sexually transmitted infections? Nature reviews Microbiology 2006;4:S7-19.

CHAPTER 7

New biomarkers for stage determination in *Trypanosoma brucei rhodesiense* sleeping sickness patients

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In this chapter we addressed the problem of the stage determination in patients suffering from *T. b. rhodesiense* sleeping sickness, the acute form of the disease.

The eight markers previously tested for the stage determination and treatment outcome evaluation in *T. b. gambiense* HAT, were assessed on a small population of *T. b. rhodesiense* patients. Interestingly, different staging abilities were observed on this population compared to the results obtained in our previous studies on *gambiense* HAT. IgM, MMP-9 and CXCL13 showed here the most promising results, individually or combined into panels of markers comprising CXCL13-CXCL10-MMP-9 or CXCL13-CXCL10-IgM.

Even if deeper investigations are needed, the results reported in this manuscript are particularly interesting since they indicate the possibility of ameliorating the management of *T. b. rhodesiense* patients and pave the way for deeper investigation on the pathophysiological differences between the chronic and acute forms of HAT.

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New biomarkers for stage determination in *Trypanosoma brucei rhodesiense* sleeping sickness patients

Natalia Tiberti[1], Enock Matovu[2], Alexandre Hainard[1], John C. Enyaru[3], Veerle Lejon[4], Xavier Robin[1], Natacha Turck[1], Dieudonné Mumba Ngoyi[5], Sanjeev Krishna[6], Sylvie Bisser[7,8], Bertrand Courtioux[7,8], Philippe Büscher[4], Krister Kristensson[9], Joseph M. Ndung'u[10], Jean-Charles Sanchez[1*]

1 *Translational Biomarker Group, Department of Human Protein Sciences, University of Geneva, Geneva, Switzerland;* ***2*** *Department of Biotechnical and Diagnostics Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda;* ***3*** *Department of Biochemistry, College of Natural Sciences, Makerere University, Kampala, Uganda;* ***4*** *Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium;* ***5*** *Department of Parasitology, Institut National de Recherche Biomédicale, Kinshasa, D. R. Congo;* ***6*** *Centre for Infection, Division of Cellular and Molecular Medicine, St. George's, University of London, London, Great Britain;* ***7*** *INSERM UMR1094, Tropical Neuroepidemiology, Limoges, France;* ***8*** *Institute of Neuroepidemiology and Tropical Neurology, University of Limoges, School of Medicine, CNRS FR 3503 GEIST, Limoges, France;* ***9*** *Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden;* ***10*** *Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland.*

SUMMARY

Background Accurate stage determination is crucial in the choice of treatment for patients suffering from sleeping sickness, also known as human African trypanosomiasis (HAT). Current staging methods, based on the counting of white blood cells (WBC) and the detection of parasites in the cerebrospinal fluid (CSF) have limited accuracy. We hypothesized that immune mediators reliable for staging *T. b. gambiense* HAT could also be used to stratify *T. b. rhodesiense* patients, the less common form of HAT.

Methods A population comprising 85 *T. b. rhodesiense* patients, 14 stage 1 (S1) and 71 stage 2 (S2) enrolled in Malawi and Uganda, was investigated. The CSF levels of IgM, MMP-9, CXCL13, CXCL10, ICAM-1, VCAM-1, neopterin and B2MG were measured and their staging performances evaluated using receiver operating characteristic (ROC) analyses.

Results IgM, MMP-9 and CXCL13 were the most accurate markers for stage determination (partial AUC 88%, 86% and 85%, respectively). The combination in panels of three molecules comprising CXCL13-CXCL10-MMP-9 or CXCL13-CXCL10-IgM significantly increased their staging ability to partial AUC 94% (p value < 0.01).

Conclusions The present study highlighted new potential markers for stage determination of *T. b. rhodesiense* patients. Further investigations are needed to better evaluate these molecules, alone or in panels, as alternatives to WBC to make reliable choice of treatment.

INTRODUCTION

Human African trypanosomiasis (HAT), commonly known as sleeping sickness, is a neglected tropical disease caused by the *Trypanosoma brucei* parasite and transmitted to humans through the bite of the tsetse fly.[1] Two morphologically identical subspecies of parasites are responsible for the disease: *Trypanosoma brucei gambiense* and *T. b. rhodesiense*.[2] In both cases, the disease progresses from a haemolymphatic first stage (S1), to a meningo-encephalitic second stage (S2). The latter reflects invasion of the central nervous system (CNS) by the parasites across the blood-brain barrier (BBB) with severe neurological complications, which can ultimately lead to coma and death, when untreated.[3] The two forms of HAT differ in their clinical presentations and geographic distribution. The

gambiense form is widespread in Central and Western Africa and is commonly considered to be a chronic infection, which slowly progresses from the first to the second stage. The *rhodesiense* form of sleeping sickness, that affects communities in Eastern Africa, is a more aggressive illness, which rapidly progresses to the meningo-encephalitic stage[3] and accounts for less than 5% of all HAT cases.[4] Contrary to *T. b. gambiense*, for which a relatively safe drug combination has recently been introduced for treatment of S2 patients,[4-6] treatment of S2 *T. b. rhodesiense* patients still relies on melarsoprol.[7-9] Melarsoprol has been reported to cause reactive encephalopathies in 8% of *T. b. rhodesiense* treated patients, which are fatal in 57% of them.[8] As a drug to safely treat both stage 1 and stage 2 patients is yet to be identified, and as S2 treatment is associated with severe side effects and toxicity,[8] stage determination remains a key step in the management of patients suffering from *T. b. rhodesiense* HAT.

Staging is based on the examination of the cerebrospinal fluid (CSF) by microscopy. According to WHO, patients having ≤ 5 white blood cells (WBC) per microliter of CSF and absence of parasites are considered to be in the first stage of the disease, while patients having more than 5 WBC/µL and/or presence of parasites in the CSF are considered as S2.[10] These methods suffer from limited specificity and reproducibility of the counting of WBC and lack of sensitivity in finding of parasites in CSF[11,12] (Dieudonné Mumba Ngoyi, personal communication).

The discovery of surrogate markers to complement or replace the counting of WBC in the staging of HAT is highly desired.[11,13,14] Many studies have focused on the staging in *T. b. gambiense* HAT,[13,15-20] while less attention has been paid to *T. b. rhodesiense*, with a paucity of data on staging markers.[13,21,22] Some pro- and counter-inflammatory factors have been shown to be associated with the late stage of *T. b. rhodesiense* sleeping sickness, including IL-10, IL-6, CXCL10 and neopterin.[13,21,22]

The aim of the present study was to investigate eight immune-related factors, shown to be powerful markers for stratification of *T. b. gambiense* HAT patients,[13,15-20,23] as staging markers for *T. b. rhodesiense* sleeping sickness.

METHODS

Patients

Eighty five patients (14 stage 1 and 71 stage 2) with evidence of parasites in blood, lymph or CSF were investigated in the present study (Table 1). Patients were enrolled by active or passive case finding in Malawi (NEUROTRYP study[13]) and Uganda (FINDTRYP study), in regions endemic for *T. b. rhodesiense* HAT. The studies were approved by the Ministry of Health and Population, Lilongwe, Malawi and by the Uganda National Council for Science and Technology (UNCST).

All patients signed a written informed consent before inclusion into the study. Children (< 18 years old) or patients with altered mental status were only included in the studies after written consent of a parent or a guardian. All enrolled patients had the possibility to withdraw at any moment. Details on sample collection, inclusion and exclusion criteria of the two cohorts are reported in Supporting Table S1.

CSF samples were collected by lumbar puncture and the number of WBC counted. The presence of parasites was determined using either the modified single centrifugation (Malawi)[24] or double centrifugation (Uganda)[25] methods. CSF samples were stored in liquid nitrogen at the site of collection, followed by storage at -80°C. Patients were diagnosed, staged and treated for HAT according to the guidelines of the national sleeping sickness control program of the country of sample collection.

In the present study, patients' stage was assigned according to WHO recommendations,[10] i.e., stage 1 when CSF WBC ≤ 5/µL and absence of parasite in CSF, stage 2 when CSF WBC > 5/µL and/or parasites detected in the CSF. Patients were excluded when information to classify them according to these criteria was not available.

Table 1 Pre-treatment characteristics of the investigated patients

	Stage 1 (n=14)	Stage 2 (n=71)
Demography		
Sex, F (n)*	7	32
Age, years [mean ± SD]†	37.1 [± 19.3]	36.9 [± 15.8]
Geographical origin		
Malawi, n	3	27
Uganda, n	11	44
CSF examinations		
Trypanosome positive, n	0	64
WBC/µL (median, range)	3 [2-5]	21 [4-1140]

* Fisher's exact test, no significant differences

† Mann-Whitney *U* test, no significant differences

Immunoassays

The levels of the markers were measured in pre-treatment CSF using commercially available immunoassays (ELISA or multiplex bead suspension assay) following manufacturers' instructions as reported elsewhere.[23] These included IgM (ICL, OR, USA), B2MG (Calbiotech, CA, USA), neopterin (BRAHMS, Germany), CXCL10 (Bio-Rad, CA, USA),

VCAM-1, ICAM-1, CXCL13 and MMP-9 (R&D Systems, UK).

Statistics

Statistical analyses were performed using IBM SPSS Statistics version 20.0.0 (IBM, NY, USA). Comparisons between groups were performed using the Mann-Whitney *U* test, setting the level of significance at 0.05. Correlations between molecules and the number of WBC were assessed through the Spearman correlation rho coefficient. ROC analyses were performed using pROC package for S+ version 8.1 (TIBCO, Software Inc.).[26] All tests were two-tailed.

To assess the staging ability of each marker and to compare their performances at high specificity, corrected partial areas under the ROC curves (pAUC) were calculated between 90 and 100% of specificity (SP).[26] A cut-off corresponding to 100% specificity was also computed.

To evaluate the power of the markers in predicting the presence of trypanosomes in CSF, patients were classified based on the absence (n=21) or presence (n=64) of parasites in their CSF. Complete AUC were then computed as well as the cut-off corresponding to the best combination of specificity (SP) and sensitivity (SE).

Panels of markers were obtained using an in-house software, PanelomiX, based on a method of optimization of cut-off values by iterative combination of biomarkers and thresholds (rule-induction-like method).[18] Highly specific combinations of three molecules were computed. Those showing the highest pAUC for discrimination between stage 1 and stage 2 patients were kept. Statistical comparison between the pAUC of the panels and those of the individual markers was obtained through the Bootstrap test for two correlated ROC curves.

RESULTS

Concentration of markers in patients' CSF

The CSF levels of IgM, B2MG, MMP-9, CXCL13, CXCL10, ICAM-1, VCAM-1 and neopterin were measured on a population of 85 patients, comprising 14 stage 1 and 71 stage 2 (Table 1).

All molecules showed a higher CSF concentration in S2 patients compared to S1. IgM, MMP-9 and CXCL13 showed the highest fold increase (S2/S1 concentration ratio of 68, 12 and 187, respectively). However, the comparison using the Mann-Whitney *U* test, highlighted significant differences between the two stages for all markers (Table 2). These differences were confirmed when patients from Uganda (S1 n=11; S2 n=44) were considered separately, while only MMP-9, IgM and B2MG could significantly discriminate between Malawian S1 (n=3) and S2 (n=27) patients (Supporting Figure S1). Furthermore, all markers significantly correlated (Spearman correlation) with the number of WBC counted in the CSF, the current staging method, with MMP-9, CXCL13 and CXCL10 having a rho coefficient > 0.5 (Table 2).

Table 2 Concentration of markers in early (S1) and late (S2) stage *T.b. rhodesiense* patients

Marker	[S1], median	[S2], median	[S2]/[S1]	p value*	Spearman rho†
IgM [µg/mL]	0.96	65.4	68.1	<0.0001	0.491
MMP-9 [pg/mL]	108.6	1309.9	12.1	<0.0001	0.554
CXCL13 [pg/mL]	8.2	1531.2	186.7	<0.0001	0.529
VCAM-1 [ng/mL]	22.6	67.3	3.0	<0.0001	0.372
B2MG [ng/mL]	964	3447	3.6	<0.0001	0.426
ICAM-1 [ng/mL]	1.99	9.6	4.8	<0.0001	0.457
Neopterin [nmol/L]	41.2	112.9	2.7	0.001	0.360
CXCL10 [ng/mL]	8.9	41.9	4.7	0.005	0.508

* Mann-Whitney *U* test

† Correlation between the number of CSF WBC and CSF levels for each marker. Correlation was significant at 0.01 level

Staging ability of the markers

ROC analyses were performed to further assess the ability of the markers to discriminate between S1 and S2 patients in terms of specificity and sensitivity.

To highlight markers able to correctly rule out the highest number of S1 patients, partial AUC between 90 and 100% of specificity were calculated together with a cut-off in marker concentration at 100% of SP (Figure 1, Supporting Table S2).

IgM, MMP-9 and CXCL13 had a pAUC higher than 80%, but for 100% of specificity IgM showed higher sensitivity (SE 77%, 76.6-87.3 95% CI), compared to MMP-9 (72% SE, 60.6-81-7 95% CI) and CXCL13 (69% SE, 57.8-80.3 95% CI).

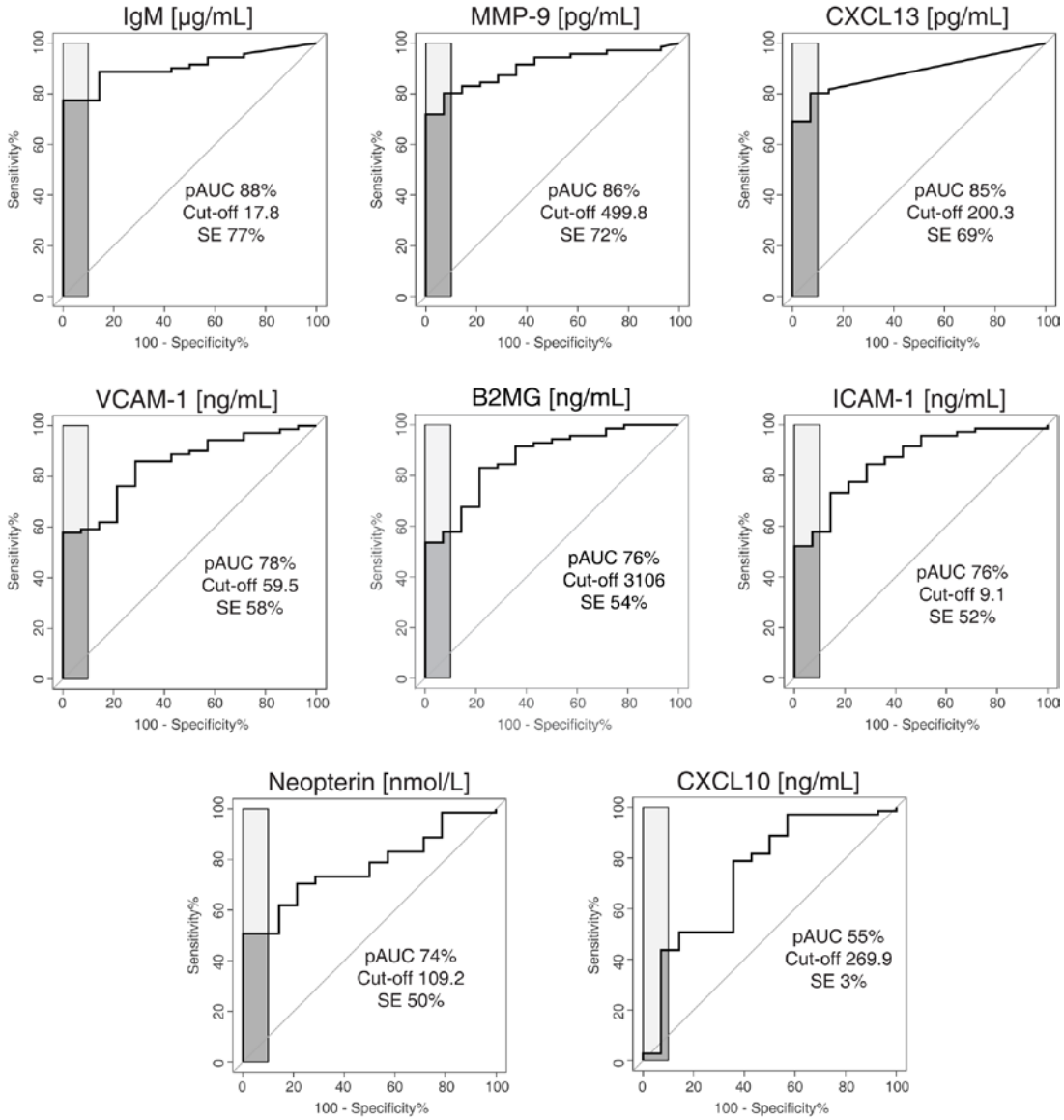

Figure 1 ROC curves representing the staging abilities of the eight markers. Dark gray areas represent the corrected pAUC between 90 and 100% of specificity obtained for each marker. Light gray zones represent a pAUC of 100%. The value of the cut-off corresponding to 100% specificity comprised within the pAUC is reported on each graph together with the corresponding sensitivity% (SE%). Additional results are reported in Additional Table 2.

A second group of markers with a pAUC between 70-80% comprised VCAM-1, B2MG, ICAM-1 and neopterin. CXCL10 was highlighted as the less accurate marker, with a pAUC of only 55% and 3% sensitivity for 100% specificity.

To further assess the ability of the eight molecules to identify patients with advanced S2 HAT, patients were classified based on the absence (T-, n=21) or the presence (T+, n=64) of parasites in the CSF. The total area under the ROC curve was considered. All markers, except neopterin and CXCL10, discriminated between T- and T+ patients with AUC > 80% and with performances comparable to those of WBC (95% CI around AUC overlapping) (Table 3).

Interestingly, when the specificity and sensitivity corresponding to the best cut-off were taken into account, the three best markers, i.e. IgM, MMP-9 and CXCL13, turned out to be more specific (SP > 85%) than WBC, which in turn was more sensitive (SE > 95%) (Table 3).

Table 3 Ability of markers to classify *T.b. rhodesiense* patients according to the presence of parasites in CSF

Marker	p value*	AUC% (95% CI)	Cut-off	SP% (95% CI)	SE% (95% CI)
IgM [µg/mL]	< 0.0001	84.5 (73.9-95.2)	17.8	85.7 (66.7-100)	81.3 (71.9-90.6)
MMP-9 [pg/mL]	< 0.0001	85.2 (74.3-96)	499.8	90.5 (76.2-100)	76.6 (65.6-85.9)
CXCL13 [pg/mL]	< 0.0001	80.4 (69.1-91.8)	200.3	90.5 (76.2-100)	73.4 (62.5-84.4)
VCAM-1 [ng/mL]	< 0.0001	83.2 (73.7-92.7)	43.9	76.2 (57.1-90.5)	78.1 (67.2-87.5)
B2MG [ng/mL]	< 0.0001	82.0 (71.3-92.7)	1462	66.7 (47.6-85.7)	85.9 (76.6-93.8)
ICAM-1 [ng/mL]	< 0.0001	83.3 (73-93.6)	4.7	81.0 (61.9-95.2)	73.4 (62.5-84.4)
Neopterin [nmol/L]	< 0.0001	75.5 (63.9-87.1)	69.4	81.0 (61.9-95.2)	65.6 (54.7-76.6)
CXCL10 [ng/mL]	0.002	73.1 (59.3-86.8)	7.5	47.6 (28.6-66.7)	92.2 (84.4-98.4)
WBC (Cells/µL)	< 0.0001	87.4 (77.4-97.4)	6.5	71.4 (52.4-90.5)	95.3 (89.1-100)

Patients without parasites detected in CSF, n=21; patients with parasite detected in CSF, n=64

* Mann-Whitney *U* test

SP% = specificity%; SE% = sensitivity%; 95% CI = 95% confidence interval

Combination of markers into panels

To evaluate whether a combination of markers could increase the staging ability, panels of three molecules corresponding to 100% specificity were calculated. Two different combinations showing the same staging performances (pAUC 94%, 89.9-97.3 95% CI; SE 87.3%, 78.9-94.4 95% CI) were obtained. Both panels comprised CXCL10 (cut-off 2.24 ng/mL) and CXCL13 (cut-off 23.3 pg/mL) in combination with either MMP-9 (cut-off 499.8 pg/mL) or IgM (cut-off 17.8 µg/mL) (Table 4). Both panels were considered positive when at least two out of three molecules were above their cut-offs.

When compared to the individual molecules, the two panels were significantly more accurate for stage determination (Bootstrap test for two correlated ROC curves, $p<0.05$). These combinations enabled the correct classification of all S1 patients (100% SP) and 62 out of 71 S2 patients (87% SE).

Table 4 Panels of markers for staging *T.b. rhodesiense* patients obtained through a combination of 3 molecules

Panel	Markers	Cut-off	pAUC% (95% CI)*	SE% (95%CI)*	p value†
1	CXCL10 [ng/mL]	2.2	94 (89.9-97.3)	87.3 (78.9-94.4)	0.0001
	CXCL13 [pg/mL]	23.3			0.01
	MMP-9 [pg/mL]	499.8			0.01
2	CXCL10 [ng/mL]	2.2	94 (89.9-97.3)	87.3 (78.9-94.4)	0.0001
	CXCL13 [pg/mL]	23.3			0.01
	IgM [µg/mL]	17.8			0.02

* pAUC and SE% were calculated for 100% SP

† Comparison between the pAUC (90-100% SP) of the panel and those of the markers individually considered obtained through the Bootstrap test for two correlated ROC curves

Both panels are positive when ≥2 molecules are above their cut-off

DISCUSSION

Stage determination in *T. b. rhodesiense* sleeping sickness patients is a critical step in ensuring that the appropriate treatment is used.[14] An imperfect gold standard for staging and the lack of a safe S2 drug highlight the need for new tools for staging this form of disease.[27,28] In the present study we investigated, on a small population of patients suffering from *T. b. rhodesiense* HAT, a number of molecules (MMP-9, CXCL10, CXCL13, IgM, neopterin, ICAM-1, VCAM-1 and B2MG) known to be over-expressed in the CSF of late stage *T. b. gambiense* patients.[23] Since melarsoprol is still the only treatment for S2 *rhodesiense* patients, we evaluated their staging ability as highly specific markers, to try to limit unnecessary exposure of patients to this toxic drug. IgM, MMP-9 and CXCL13 were shown to be the most accurate discriminators between early and late stage disease (pAUC ≥ 85%) and showed the same accuracy as WBC in distinguishing between patients having parasites in their CSF from those without. Furthermore, combination of the molecules into panels of three markers (IgM-CXCL13-CXCL10 or MMP-9-CXCL13-CXCL10) significantly increased the staging accuracy, leading to the correct classification of all S1 patients and 62 out of 71 S2 patients.

All the markers investigated here are known to be involved in the immune response elicited by the presence of the parasite in the host. Interestingly, a different behavior of the 8 molecules was observed in

T. b. gambiense patients, which may reflect the differences in immunopathogenesis[29] and clinical presentation[3,30] of the two forms of HAT. It has already been proposed, for example, that different activation pathways of macrophages and astrocytes may take place in the two forms of HAT.[22] Such differences may be responsible of the less accurate staging ability of neopterin on *T. b. rhodesiense* patients, compared to its very high staging power on *T. b. gambiense* patients.

The role of IgM, the best individual marker in the present study, in disease progression has been extensively studied. An increased CSF concentration of IgM of intrathecal origin was shown to be a good indicator of brain involvement in HAT,[15] leading to the development of a rapid latex agglutination test (Latex/IgM) for stage determination in the field.[31] However, when assessed under field conditions, this assay did not represent an advantage compared to counting of WBC.[31] Furthermore, when used for evaluation of the outcome after treatment, IgM levels were not an optimal indicator of recovery due to their slow normalization.[32] Studies in animal models have shown that HAT meningo-encephalitis is characterized by an increased number of leukocytes in the CNS.[33] CXCL13, also known as BCA-1, is a chemokine mainly produced by dendritic cells,[34] which specifically attracts B and T lymphocytes to the site of inflammation.[35] Its over-expression in CSF has been associated with increased WBC and intrathecal production of immunoglobulins in many pathological conditions,[36,37] including late stage *T. b. gambiense* HAT.[19] On the other hand, MMP-9 (matrix-metalloproteinase 9), an enzyme involved in tissue homeostasis and remodeling,[38,39] has been extensively studied in a number of pathologies affecting the CNS,[39-42] in addition to *T. b. gambiense* HAT.[17] Due to its ability to degrade β-dystroglycan, this protein has been proposed to be involved in the passage of leukocytes through the *glia limitans* to reach the brain parenchyma.[43] However, the temporal relationship between the events leading to CNS invasion and the appearance of various signs and symptoms of nervous system dysfunction needs to be investigated further.

The markers investigated in the present study were combined into panels in order to increase their accuracy in stage determination. The utility of this approach to achieve a better diagnostic accuracy has already been shown.[18,44] Using this method, we highlighted highly specific combinations comprising CXCL13, CXCL10 and MMP-9 or IgM. Interestingly, CXCL10 was present in both panels. This molecule was not efficient in staging *T. b. rhodesiense* patients when considered individually. However, when combined to CXCL13 and MMP-9 or IgM, it helped in reaching a significantly increased staging accuracy. This chemokine, which specifically attracts T lymphocytes to the site of inflammation,[45] was reported to be produced by activated astrocytes in trypanosome-infected mice.[46] The activation of astrocytes and macrophages are early events in stage 2 infection,[47-49] suggesting that CXCL10 may represent an early indicator of CNS involvement in HAT.

Interestingly, the markers did not show the same staging performances when assessed on patients classified according to their geographic origin (i.e. Malawi or Uganda). Although the low number of Malawian S1 patients (n=3) certainly represents a bias and may be responsible for the differences observed, this result could reflect the variable clinical presentation of *rhodesiense* disease observed in different foci.[27] This may suggest that potentially different markers will be needed to stage *T. b. rhodesiense* patients according to their geographical origin and the parasite strain.

The present study has a number of limitations that should be considered. First, the data presented resulted from analyses on a small number of patients. This is a common problem associated to the investigation of this form of HAT. Collecting samples from *T. b. rhodesiense* patients is considerably difficult, not only due to the lower incidence of this disease compared to the *gambiense* form, but also as a consequence of a less effective active screening, since the CATT test can only detect *T. b. gambiense* cases.[50] To further evaluate the staging properties of the markers, larger cohorts of patients should be investigated. Moreover, due to the reported differences between *T. b. rhodesiense* HAT among foci, the results presented here should be validated in a more controlled set of patients (i.e. in which the same parasitological examinations were performed).

Another drawback could be represented by the choice of selecting highly specific markers, with the consequence of compromising the sensitivity. Management of *T. b. rhodesiense* patients is far from being optimal, thus both choices of high specificity or sensitivity would be associated either to a risk of missing the diagnosis of late stage patients, or to the exposure of S1 patients to a highly toxic stage 2 drug, respectively. However, it should be emphasized that a new staging biomarker for *rhodesiense* HAT would be combined with the detection of parasites in CSF, which would increase the sensitivity, and with clinical evaluation of the neurological status of the patients.

The absence of information on neurological signs exhibited by patients in the present study prevented

an efficient assessment of the association between the levels of the markers and the signs of CNS involvement. This aspect is particularly important in the light of a recent publication on *T. b. rhodesiense* HAT reporting the poor association between disease progression, the levels of a number of cytokines and patients' neurological manifestations.[51]

The 8 markers investigated here behaved differently when assessed on *T. b. gambiense* or *T. b. rhodesiense* samples, underlining the differences between the two forms of disease, and suggesting that potentially new *rhodesiense*-specific markers could be discovered.

Despite the high staging accuracy shown by the combinations of markers described in the present study (i.e. CXCL10-CXCL13-MMP-9 and CXCL10-CXCL13-IgM), their translation into a rapid field diagnostic test could be difficult, due to a potential increase in the costs of production, suggesting that deeper investigations should be performed. The individual staging power of the molecules should be assessed on a larger cohort of *T. b. rhodesiense* HAT patients, including CSF samples collected during the post-therapeutic follow-up, and the possibility of their translation into a point-of-care test for stage determination in the field should be evaluated. Furthermore, their study in animal models, as already done for IL-10,[52] could help in the further characterization of the role of these markers in disease progression.

CONCLUSIONS

The results presented in this work on *T. b. rhodesiense* sleeping sickness highlight the potential utility of IgM, MMP-9 and CXCL13, alone or combined with CXCL10 in staging patients. We believe that this work has paved the way for further investigations on the role of these markers in detecting the meningo-encephalitic stage of *T. b. rhodesiense* HAT, and therefore making a more accurate choice of treatment.

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COMPETING INTERESTS

Joseph Ndung'u is an employee of the Foundation for Innovative New Diagnostics (FIND). Veerle Lejon, Philippe Büscher and Sanjeev Krishna were consultants for FIND at the time of the study. All other authors declare that they have no competing interests.

SUPPORTING INFORMATION

Table S1 Details of the studies from which samples were obtained.

Table S2 Detailed calculation for the evaluation of the staging ability of the eight markers.

Figure S1 Comparison of the levels of the markers between stage 1 (S1) and stage 2 (S2) *T.b. rhodesiense* patients classified according to the country of sample collection.
For each country, differences between S1 and S2 were assessed using the Mann-Whiney *U* test.
* corresponds to a p value < 0.05; ** corresponds to a p value < 0.001; *** corresponds to a p value < 0.0001.

REFERENCES

1. Kennedy PG. Cytokines in central nervous system trypanosomiasis: cause, effect or both? Transactions of the Royal Society of Tropical Medicine and Hygiene 2009;103:213-4.
2. Sternberg JM, Maclean L. A spectrum of disease in human African trypanosomiasis: the host and parasite genetics of virulence. Parasitology 2010;137:2007-15.
3. Malvy D, Chappuis F. Sleeping sickness. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2011;17:986-95.
4. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS neglected tropical diseases 2011;5:e1007.
5. Priotto G, Kasparian S, Mutombo W, et al. Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet 2009;374:56-64.
6. Simarro PP, Franco J, Diarra A, Postigo JA, Jannin J. Update on field use of the available drugs for the chemotherapy of

human African trypanosomiasis. Parasitology 2012;139:842-6.
7. Kennedy PG. An alternative form of melarsoprol in sleeping sickness. Trends in parasitology 2012;28:307-10.
8. Burri C. Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitology 2010;137:1987-94.
9. Kuepfer I, Schmid C, Allan M, et al. Safety and efficacy of the 10-day melarsoprol schedule for the treatment of second stage rhodesiense sleeping sickness. PLoS neglected tropical diseases 2012;6:e1695.
10. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organization technical report series 1998;881:I-VI, 1-114.
11. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.
12. Simarro PP, Jannin J, Cattand P. Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS medicine 2008;5:e55.
13. Amin DN, Ngoyi DM, Nhkwachi GM, et al. Identification of stage biomarkers for human African trypanosomiasis. The American journal of tropical medicine and hygiene 2010;82:983-90.
14. Kennedy PG. The continuing problem of human African trypanosomiasis (sleeping sickness). Annals of neurology 2008;64:116-26.
15. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.
16. Tiberti N, Hainard A, Lejon V, et al. Discovery and verification of osteopontin and Beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Molecular & cellular proteomics : MCP 2010;9:2783-95.
17. Hainard A, Tiberti N, Robin X, et al. Matrix metalloproteinase-9 and intercellular adhesion molecule 1 are powerful staging markers for human African trypanosomiasis. Tropical medicine & international health : TM & IH 2011;16:119-26.
18. Hainard A, Tiberti N, Robin X, et al. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PLoS neglected tropical diseases 2009;3:e459.
19. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Tropical medicine & international health : TM & IH 2009;14:529-34.
20. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. Journal of the neurological sciences 2002;193:127-35.
21. MacLean L, Odiit M, Sternberg JM. Nitric oxide and cytokine synthesis in human African trypanosomiasis. The Journal of infectious diseases 2001;184:1086-90.
22. Maclean L, Odiit M, Sternberg JM. Intrathecal cytokine responses in Trypanosoma brucei rhodesiense sleeping sickness patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006;100:270-5.
23. Tiberti N, Hainard A, Lejon V, et al. Cerebrospinal Fluid Neopterin as Marker of the Meningo-Encephalitic Stage of Trypanosoma brucei gambiense Sleeping Sickness. PloS one 2012;7:e40909.
24. Miezan TW, Meda HA, Doua F, Dje NN, Lejon V, Buscher P. Single centrifugation of cerebrospinal fluid in a sealed pasteur pipette for simple, rapid and sensitive detection of trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene 2000;94:293.
25. Cattand P, Miezan BT, de Raadt P. Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes. Bulletin of the World Health Organization 1988;66:83-6.
26. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC bioinformatics 2011;12:77.
27. MacLean LM, Odiit M, Chisi JE, Kennedy PG, Sternberg JM. Focus-specific clinical profiles in human African Trypanosomiasis caused by Trypanosoma brucei rhodesiense. PLoS neglected tropical diseases 2010;4:e906.
28. Kennedy PG. Difficulties in diagnostic staging of human African trypanosomiasis. J Neuroparasitol 2011;2.
29. Kennedy PG. Diagnostic and neuropathogenesis issues in human African trypanosomiasis. International journal for parasitology 2006;36:505-12.
30. Gibson W. Will the real Trypanosoma brucei rhodesiense please step forward? Trends in parasitology 2002;18:486-90.
31. Lejon V, Legros D, Richer M, et al. IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Tropical medicine & international health : TM & IH 2002;7:685-92.
32. Lejon V, Roger I, Mumba Ngoyi D, et al. Novel markers for treatment outcome in late-stage Trypanosoma brucei gambiense trypanosomiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2008;47:15-22.
33. Rodgers J. Trypanosomiasis and the brain. Parasitology 2010;137:1995-2006.
34. Lalor SJ, Segal BM. Lymphoid chemokines in the CNS. Journal of neuroimmunology 2010;224:56-61.
35. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature 1998;391:799-803.
36. Krumbholz M, Theil D, Cepok S, et al. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain : a journal of neurology 2006;129:200-11.
37. Rainey-Barger EK, Rumble JM, Lalor SJ, Esen N, Segal BM, Irani DN. The lymphoid chemokine, CXCL13, is dispensable for the initial recruitment of B cells to the acutely inflamed

central nervous system. Brain, behavior, and immunity 2011;25:922-31.
38. Dziembowska M, Wlodarczyk J. MMP9: a novel function in synaptic plasticity. The international journal of biochemistry & cell biology 2012;44:709-13.
39. Ramos-Fernandez M, Bellolio MF, Stead LG. Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association 2011;20:47-54.
40. Unsal Y, Kivilcim G, Aysegul A, et al. Matrix metalloproteinase-7 and matrix metalloproteinase-9 in pediatric multiple sclerosis. Pediatric neurology 2012;47:171-6.
41. Prato M, Giribaldi G. Matrix Metalloproteinase-9 and Haemozoin: Wedding Rings for Human Host and Plasmodium falciparum Parasite in Complicated Malaria. Journal of tropical medicine 2011;2011:628435.
42. Szklarczyk A, Stins M, Milward EA, et al. Glial activation and matrix metalloproteinase release in cerebral malaria. Journal of neurovirology 2007;13:2-10.
43. Agrawal S, Anderson P, Durbeej M, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. The Journal of experimental medicine 2006;203:1007-19.
44. Robin X, Turck N, Hainard A, Lisacek F, Sanchez JC, Muller M. Bioinformatics for protein biomarker panel classification: what is needed to bring biomarker panels into in vitro diagnostics? Expert review of proteomics 2009;6:675-89.
45. Weng Y, Siciliano SJ, Waldburger KE, et al. Binding and functional properties of recombinant and endogenous CXCR3 chemokine receptors. The Journal of biological chemistry 1998;273:18288-91.
46. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.
47. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Progress in neurobiology 2010;91:152-71.
48. Kennedy PG. Human African trypanosomiasis of the CNS: current issues and challenges. The Journal of clinical investigation 2004;113:496-504.
49. Hunter CA, Jennings FW, Kennedy PG, Murray M. Astrocyte activation correlates with cytokine production in central nervous system of Trypanosoma brucei brucei-infected mice. Laboratory investigation; a journal of technical methods and pathology 1992;67:635-42.
50. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.
51. Maclean L, Reiber H, Kennedy PG, Sternberg JM. Stage Progression and Neurological Symptoms in Trypanosoma brucei rhodesiense Sleeping Sickness: Role of the CNS Inflammatory Response. PLoS neglected tropical diseases 2012;6:e1857.
52. Ngotho M, Kagira JM, Jensen HE, Karanja SM, Farah IO, Hau J. Immunospecific immunoglobulins and IL-10 as markers for Trypanosoma brucei rhodesiense late stage disease in experimentally infected vervet monkeys. Tropical medicine & international health : TM & IH 2009;14:736-47.

CHAPTER 8

Discussion & Conclusions

1. Finding new HAT biomarkers

The improvement of sleeping sickness patients' stratification and management through the finding of new cerebrospinal fluid biomarkers has been a challenge since a long time.[1,2] The current clinical practice for stage determination, based on the counting of the white blood cells and the finding of trypanosomes in patients' CSF, is unsatisfactory and not considered as a gold standard.[1,3] As a consequence of an incorrect stage determination, patients may be unnecessary exposed to toxic drugs or to the risk of not getting cured. Additionally, the controversial stage determination, together with the decreased efficacy of some S2 drugs,[4,5] imposes a post-therapeutic follow-up of two years to detect disease relapses. Follow-up visits, performed each 6 months for two years,[6] consist as it happens for the staging, in the detection of trypanosomes in blood and CSF and the counting of WBC in CSF. In addition to the technical limitations already highlighted, precise WHO indications for the diagnosis of treatment failures are missing.

The first objective of the project presented in this manuscript was to identify new markers for the stratification of patients suffering from *T. b. gambiense* HAT and to assess their value as alternatives to the counting of the WBC. A classical three phase workflow consisting in biomarker discovery, verification and validation was applied (Figure 1). The discovery was performed using two different approaches:

I. The CSF proteome of S1 and S2 patients was investigated using either 2-DE (n=9) or six-plex TMT quantitative mass spectrometry (n=9), to highlight proteins differentially expressed between the two groups of patients (Chapter 2);

II. Based on the hypothesis that inflammation plays an important role in disease progression, the CSF concentration of 23 immune-related factors or brain damage markers was assessed using immunoassays (n=16).

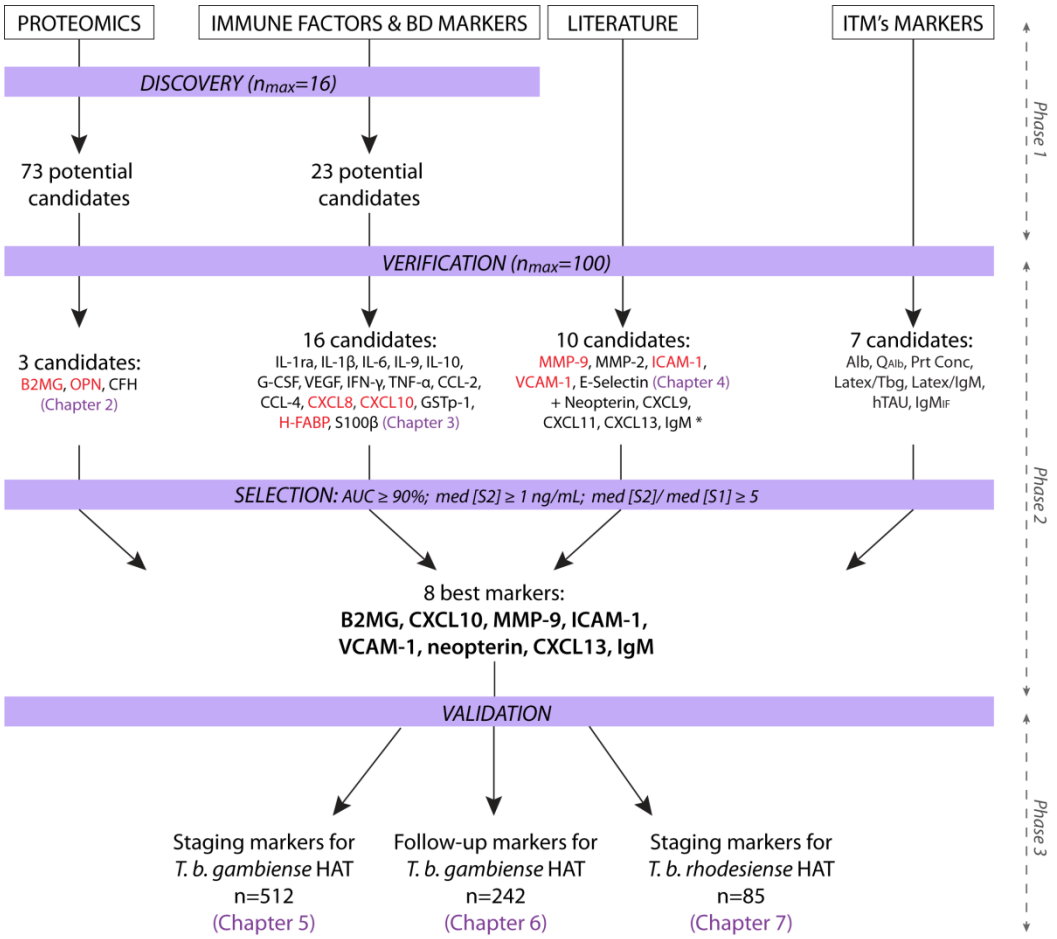


Figure 1 Graphic representation of the workflow applied for the identification of new markers for HAT. n max = highest number of patients tested during the discovery and verification phases. * = unpublished data. In red are indicated those markers highlighted as the most promising in each published study. BD markers = brain damage markers.

A verification cohort comprising 100 patients collected in the Democratic Republic of the Congo (DRC), as part of the THARSAT study,[7] was obtained through the collaboration with the Institute of Tropical Medicine (ITM) in Antwerp (Belgium). The most interesting molecules were verified on this cohort. B2MG and osteopontin (Chapter 2), previously discovered by proteomics, and a panel composed of CXCL10-CXCL8-H-FABP (Chapter 3) were shown as the most promising markers among those discovered.

To expand the set of potential biomarkers for *T. b. gambiense* HAT, we did not limit our evaluations to those newly discovered, but we took advantage from molecules already proposed in the literature. A number of adhesion molecules (ICAM-1, VCAM-1, E-selectin) and matrix

metalloproteinases (MMP-2 and MMP-9) were tested on the verification cohort and the high staging power of MMP-9 and ICAM-1 was highlighted (Chapter 4). Altogether considered, we obtained a list of 24 verified proteins to which we added 12 more candidates tested either by our group or by ITM (unpublished data).

The subsequent step towards the identification of new staging tools was the validation. A large multicenter cohort was collected comprising 512 *T. b. gambiense* patients enrolled in DRC, Chad and Angola (Chapter 5, Supporting Figure S1). Among the 36 candidates, we selected those having the highest potential as staging markers, by applying selection criteria based on the final objective of the study, i.e. to find a new tool for stage determination under field conditions. We selected only markers having i) AUC ≥ 90%, indicating a high discrimination ability between S1 and S2 patients; ii) median concentration in S2 patients ≥ 1 ng/mL and iii) 5-fold increased concentration in S2 patients compared to S1, in order to highlight those molecules, which could be more easily translated into a semi-quantitative point-of-care test (POCT). The results of this selection are reported in Table 1.

Marker	n	[S2]	[S2]/[S1]	AUC%	Score
Neopterin	27	74.90	19.7	100	3
CXCL13	96	9.44	142.3	100	3
ICAM-1	65	8.62	16.0	98	3
MMP-9	64	3.10	42.5	98	3
VCAM-1	65	53.3	5.9	96	3
IgM	100	232000	69.5	95	3
CXCL10	100	14.31	40.7	95	3
B2MG	58	3688	5.5	91	3
IL-6	100	0.06	12.8	94	2
TNF-α	100	0.02	6.8	93	2
Total protein	100	1176000	1.9	92	2
Albumin	100	275000	3.3	90	2
MMP-2	65	39.80	2.6	90	2
CXCL9	38	0.10	8.9	89	1
IL-10	100	0.07	11.1	89	1
Osteopontin	57	686.10	3.8	85	1
CXCL8	100	0.18	3.1	94	1
hTAU	94	0.17	2.8	87	1
H-FABP	100	0.75	3.3	86	1
IL-1β	100	0.0001	6.0	80	1
GSTπ-1	100	3.01	2.4	79	1
CFH	52	2505	1.5	72	1
E-Selectin	56	0.08	2.7	82	0
CXCL11	37	0.06	1.4	73	0
CCL2	100	0.59	1.4	70	0
IFN-γ	100	0.10	1.5	70	0
S100β	100	0.08	1.8	70	0
IL-9	100	0.03	1.3	70	0
G-CSF	100	0.06	1.5	62	0
IL-1ra	100	0.78	1.0	55	0
CCL4	100	0.09	1.0	54	0
VEGF	100	0.05	1.0	54	0
Latex/IgM	100	128	na	91	
IgM_{IF} (%)	100	89	na	93	
Q_{Alb}	100	8.3	na	85	
Latex/Tbg	94	2	na	81	

Table 1 Classification of the 36 markers according to the established selection criteria, i.e. AUC ≥ 90%, [S2] ≥ 1ng/mL, [S2]/[S1] ≥ 5. The concentration of all markers is reported in ng/mL except for: Latex/IgM and Latex/Tbg, for which the test end titres are reported; IgM_{IF} (IgM intrathecal fraction), which is expressed as a percentage of IgM in CSF produced intratechally; Q_{Alb} since it is a ratio of concentrations of albumin in CSF/serum. na = not applicable. n = number of patients of the validation cohort tested; [S1] = median S1 concentration; [S2] = median S2 concentration. Score: a point was assigned for each selection criteria accomplished.

B2MG, CXCL10, CXCL13, IgM, MMP-9, neopterin, ICAM-1 and VCAM-1 were selected for validation. Neopterin was highlighted as the most accurate staging marker for *T. b. gambiense* HAT, able to correctly classify 161 out of 184 stage 1 patients and 201 out of 228 stage 2 patients (Chapter 5). The high correlation of neopterin with the presence of trypanosomes and neurological signs further supported its association with the disease progression.

To extend the potential clinical utility of the markers, we assessed whether they could detect the reappearance of the CNS disease after treatment and shorten the follow-up for patients. A population of 242 *T. b. gambiense* patients (DRC) followed for 2 years after treatment was

investigated. Neopterin showed again the highest accuracy in detecting relapses and it resulted to be able to shorten the follow-up as soon as 6 months after treatment with 87% specificity, and at 12 months with 97% specificity (Chapter 6).

As a final step, we tested whether the 8 markers could also be useful in staging patients suffering from the acute form of disease, i.e. caused by *T. b. rhodesiense* parasites. When assessed on a population comprising 85 *T. b. rhodesiense* patients collected either in Eastern Uganda or in Malawi, IgM, MMP-9 and CXCL13 resulted to be the most accurate staging markers, individually or combined into panels comprising CXCL13-CXCL10-MMP-9 or CXCL13-CXCL10-IgM (Chapter 7).

2. HAT markers and neuro-pathogenesis

The diagnostic accuracy, generally considered in terms of specificity and sensitivity, is not the only aspect that should be taken into account during the investigation of new disease biomarkers. An added value can be achieved through the evaluation of the potential involvement of the biomarkers in those mechanisms leading to the onset, progression or manifestations of a specific pathological state.

As already stated in the introduction (Paragraph 1.6), the mechanisms characterizing the invasion of the CNS by the parasites and the development of extensive neurological disorders in HAT are still not completely understood. However, many findings in animal models suggest that the host inflammatory response may play a central role in disease pathogenesis.[8-10]

Interestingly, but not surprisingly, the 8 markers here validated are all involved either in leukocyte extravasation, a mechanism likely associated to the increased number of WBC observed in late stage patients, or in the humoral and cellular host immune response (Table 2). This aspect is even more interesting for those molecules, such as B2MG and MMP-9, never proposed before for staging HAT. The potential implication of the 8 markers in the scenario of HAT neuro-pathogenesis is represented in Figure 2.

Marker	Origin	Main functions	Evidences in HAT	Evidences in other diseases
ICAM-1	Expressed by a variety of cell types including endothelial cells [11]	Mediator of leukocytes transmigration process and of antigen presentation [11]	Girard M, *et al.* 2005 [12] Masocha W, *et al.* 2007 [13]	Bacterial meningitis [14], CNS infections[15]
VCAM-1	Expressed by a variety of cell types including endothelial cells [11]	Mediator of rolling and adhesion steps during leukocyte extravasation processes [11]	Girard M, *et al.* 2005 [12] Masocha W, *et al.* 2007 [13]	Bacterial meningitis [14], CNS infections[15]
MMP-9	Produced in the CNS by a variety of cells including T cells activated monocytes and macrophages [16]	Involved in the degradation of extracellular matrix and in leukocyte recruitment to the site of inflammation [16]	None	Acute ischemic stroke [17], multiple sclerosis [18], cerebral malaria [19,20]
Neopterin	Produced by IFN-γ activated macrophages and dendritic cells [21,22]	Indicator of the activation of the inflammation; involved in oxidative stress but most of its functions are still unknown [21]	MacLean L, *et al.* 2006 [23]	Tuberculosis [24,25], HIV [22]
CXCL10	Produced by glial cells and neurons [26]	Chemotactic effect on T lymphocytes for their recruitment to the site of inflammation [27,28]	Amin DN, *et al.* 2009 [29]	HIV, bacterial meningitis [26]
CXCL13	Produced by dendritic cells and macrophages, within the inflamed CSF [30,31]	Attraction and regulation of the recruitment of B and T CD4+ lymphocytes to the site of inflammation [30,31]	Courtioux B, *et al.* 2009 [32]	Lyme neuroborreliosis [33], multiple sclerosis [34,35]
B2MG	Expressed on the surface of all nucleated cells as part of the major histocompatibility complex I (MHC I) [36]	Involved in cellular immune responses against invading pathogens [36]	None	CMV infection [37], Alzheimer's disease [38,39], cancers [40]
IgM	Produced by B cells [41]	Involved in the host immune response [41]	Lejon V, *et al.* 2002 [42] Bisser S, *et al.* 2002 [43] Lejon V, *et al.* 2003 [44]	Many conditions involving immune system activation [41]

Table 2 Summary of the molecular functions of the 8 markers investigated during the validation phase. References to studies concerning their investigation in HAT or in other pathological conditions are provided.

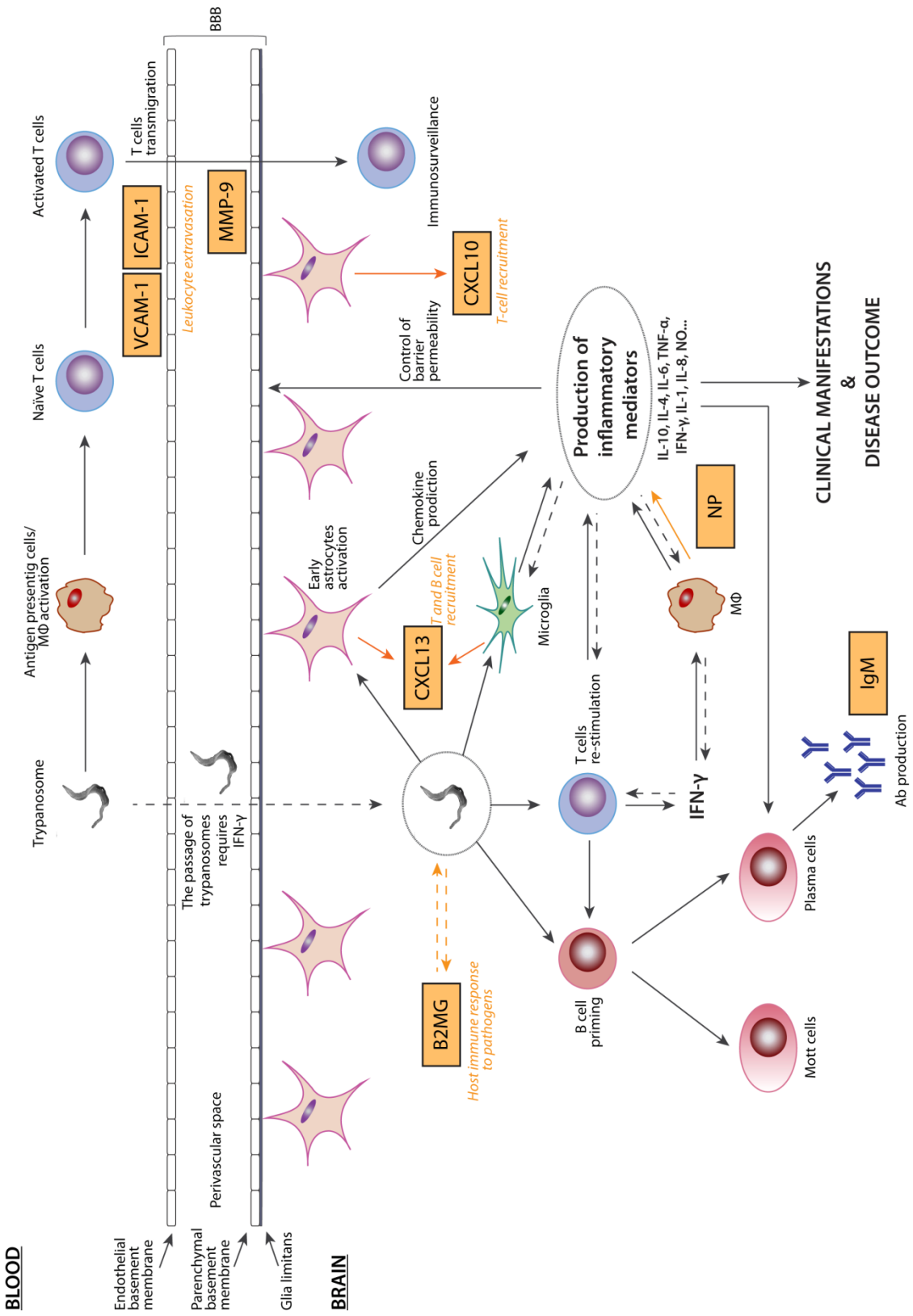


Figure 2 Potential involvement of the 8 markers investigated in the present study in the neuro-pathogenesis of HAT. MΦ = macrophage. Adapted from *Kennedy P, 2004* [45] and *Rodgers J, 2010* [9].

3. Can we really replace WBC counting?

The question of the real feasibility of replacing the counting of the leukocytes through the use of alternative CSF markers is a critical aspect in the evaluation of new HAT markers. The unsatisfactory performances of the counting of WBC for the detection of the CNS involvement is now well recognized.[1,46] However, it is a relatively simple tool, that can be easily performed on the field and which requires basic equipments already used for the parasitological confirmation of disease diagnosis, such as microscopes.

Here we proposed neopterin as a highly accurate alternative for stage determination and treatment outcome evaluation in *T. b. gambiense* patients. One of the main limitations of the validation approach adopted in the present study was the lack of a classification method that could be considered as a gold standard. The stratification of patients according to either their pre-treatment stage or their outcome after treatment was, in fact, partially based on the counting of CSF WBC, which is the method that we intend to replace. This means that our markers could never be more accurate than WBC counting, that we actually consider imperfect. This is a common issue in all studies focusing on the finding of new staging markers.[47] To partially overcome this problem, we classified patients based either on the presence of parasites in their CSF or on the neurological manifestations. These classifications, completely independent from WBC counting, allowed considering the number of leukocytes as a marker and comparing its performances to the other molecules.

Another important aspect that should be taken into account is the potential lack of specificity of these markers for sleeping sickness. As stated above, they are all involved in the host immune response and some of them have already been investigated for other clinical applications (Table 2). Even so, it should be considered that all patients undergoing stage determination had already a primary diagnosis of sleeping sickness either in blood or lymph. Furthermore, in both staging and follow-up cases the evaluation of new CSF biomarkers will be combined to the finding of trypanosomes in CSF, as it is currently done for WBC. Trypanosome visualization in CSF is, for the moment, the only HAT specific tool for the detection of the presence of parasites in the brain.

However, neopterin and CXCL13 were tested on a small number of healthy endemic control patients and on patients suffering from pathologies other than HAT, including cerebral malaria, HIV and meningitis. The concentration of both markers was significantly higher in the pre-treatment CSF of S2 patients and in the CSF of relapsing patients compared to both groups of controls (Chapter 8 supporting information, Figure S1 and Figure S2).

All these aspects, together with the utility of neopterin for both stage determination and treatment outcome evaluation, support the high potential for neopterin of being translated into clinical practice as alternative to the WBC counting.

The value of the markers that we proposed for the staging of *T. b. rhodesiense* still needs to be validated. In particular, the assessment of their concentration in the CSF of patients followed after treatment could help in clarifying their kinetics during disease progression and reappearance. However, the difficult recruitment and follow-up of *T. b. rhodesiense* patients may hamper this type of analysis.

4. Future perspectives

4.1 Translation of neopterin into clinical practice: POCT & TOC

The results presented in this manuscript indicate that neopterin has a strong potential of being translated into a rapid field test as a Point-Of-Care Test (POCT), for stage determination, and as Test-Of-Cure (TOC), for the assessment of the post-therapeutic outcome. The evaluation of the inclusion of this metabolite in a rapid field test for stage determination is currently ongoing towards a collaboration between the Foundation for Innovative New Diagnostics (FIND, Geneva, Switzerland) and Standard Diagnostics (Korea). A simple neopterin dipstick has already been proposed for the semi-quantitative detection of serum neopterin in patients suffering from infectious diseases[48] supporting the feasibility of such a test. A neopterin lateral flow assay for stage determination may help in further investigating and determining an accurate cut-off for the application of neopterin during the follow-up.

4.2 New insights in HAT pathophysiology

A very interesting result was the observation of different staging abilities of the markers when assessed on *T. b. gambiense* or *T. b. rhodesiense* patients. The two forms of disease are clinically different[46] and it has been supposed that the two parasites may adopt and elicit different mechanisms leading to the establishment of the disease.[23] The results presented in this manuscript further support this hypothesis. Deeper investigations should be performed to better determine the role of these markers in HAT neuro-pathogenesis using trypanosome infected animal models. Furthermore, the systematic comparison of samples taken from *T. b. gambiense* and *T. b. rhodesiense* patients, through proteomics and non-proteomics approaches, may help in better understanding the differences between the two diseases. Since the geographical distance between *T. b. gambiense* and *T. b. rhodesiense* foci in Uganda, the only country reporting both forms of disease, is reducing,[49,50] a better comprehension of the two diseases could have a major impact on HAT management and control.

4.3 From CSF markers to plasma markers

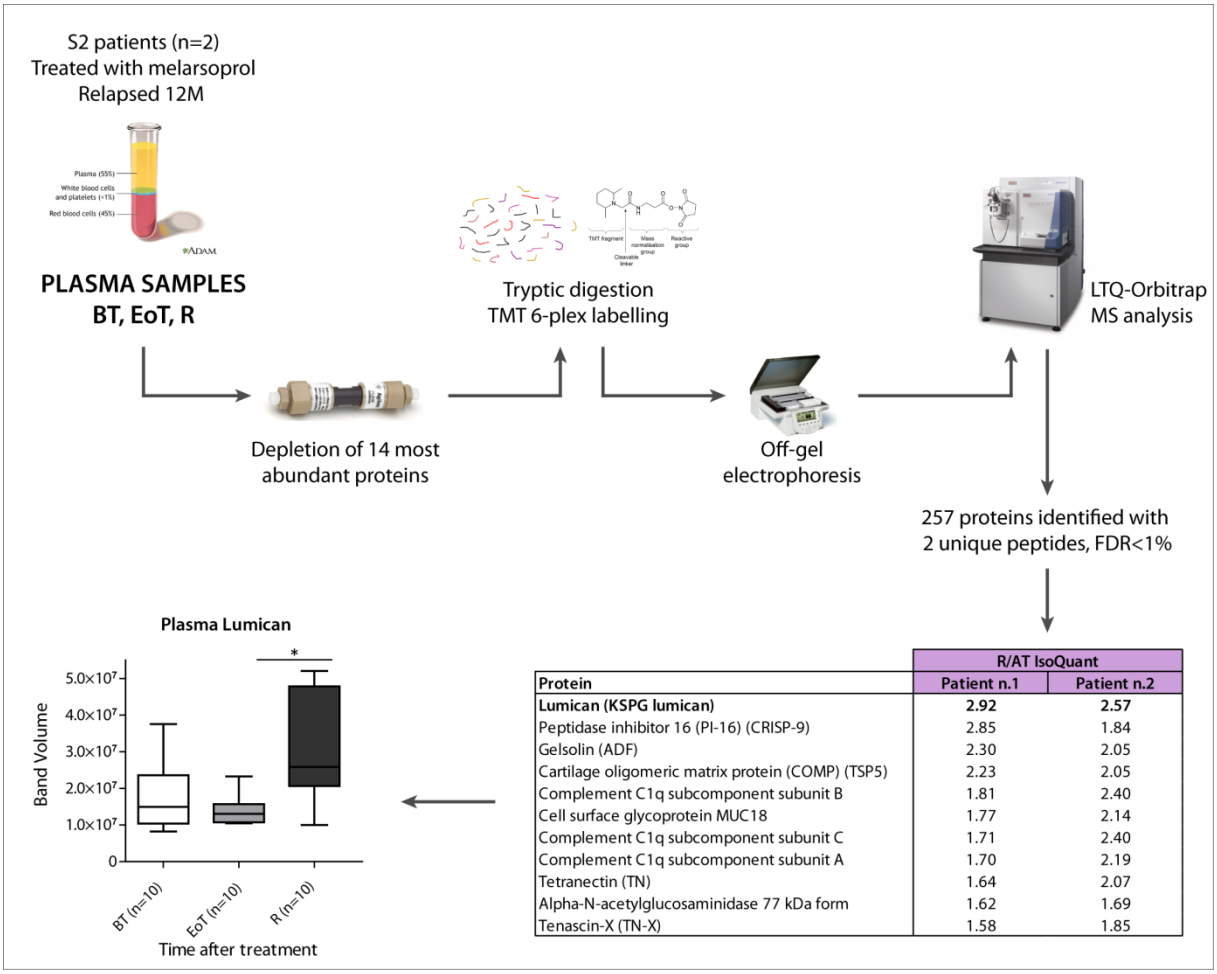
The whole project presented in this manuscript focused on cerebrospinal fluid markers. An important improvement for patients would be, however, the availability of plasma markers in order to limit the number of lumbar punctures. A plasma marker would find great utility in the post-therapeutic follow-up rather than in staging for two main reasons:

I. S2 patients experiencing a relapse have in most cases absence of parasites in blood but presence in CSF, increasing the likelihood of having a plasma protein increased only when a relapse occurs;
II. The high number of lumbar punctures performed during the follow-up is a cause of low attendance rate to the follow-up.

The 8 markers here investigated were all tested in the plasma of a small number of S1 (n=15) and S2 (n=15) patients, as well as in the plasma taken during the follow-up of relapsing patients

(n=10). However, significant differences in the plasma levels were highlighted neither for the staging nor for the follow-up (Chapter 8 supporting information, Figure S3 and Figure S4).

We thus hypothesized that proteins over-expressed in the plasma taken at the time of the relapse (R) compared to the plasma taken at the end of the treatment (EoT), could be identified using proteomics strategies. Similarly to what was done for the discovery of new CSF staging markers (Chapter 2), we set up a six-plex TMT quantitative MS workflow to analyze plasma samples obtained during the follow-up of two relapsing patients. The applied workflow is represented in Figure 3.



Protein	R/AT IsoQuant Patient n.1	R/AT IsoQuant Patient n.2
Lumican (KSPG lumican)	**2.92**	**2.57**
Peptidase inhibitor 16 (PI-16) (CRISP-9)	2.85	1.84
Gelsolin (ADF)	2.30	2.05
Cartilage oligomeric matrix protein (COMP) (TSP5)	2.23	2.05
Complement C1q subcomponent subunit B	1.81	2.40
Cell surface glycoprotein MUC18	1.77	2.14
Complement C1q subcomponent subunit C	1.71	2.40
Complement C1q subcomponent subunit A	1.70	2.19
Tetranectin (TN)	1.64	2.07
Alpha-N-acetylglucosaminidase 77 kDa form	1.62	1.69
Tenascin-X (TN-X)	1.58	1.85

Figure 3 Proteomics workflow applied for the investigation of plasma samples taken during the follow-up of 2 HAT relapsing patients. In the upper part is described the sample preparation; in the table are reported the proteins identified differentially expressed between R and EoT, the box-plot represents the verification of the over-expression of lumican by western blot. BT = before treatment; EoT = end of the treatment; R = relapse.

Eleven proteins were identified as over-expressed in the plasma taken at the time of the relapse compared to the sample taken at the end of the treatment with an R/EoT ratio ≥ 1.5 in both patients separately considered (Figure 3). Among these proteins, lumican, a small leucine-rich repeat proteoglycan of approximately 40KDa,[51] showed the highest fold-increased over-expression. Its expression has been documented in a variety of tissues, where it has been shown to be involved in the maintenance of the homeostasis of tissue structure and in cellular migration, adhesion and proliferation.[51] Recently, it has also been suggested a role of this protein in the host innate immune response and bacterial pathogens recognition.[52] The significant over-expression of this protein in the plasma at the time of the relapse was further confirmed using western blot on a larger number of patients (Figure 3).

These preliminary results pave the way for deeper investigations on plasma TOC biomarkers. Such a marker could have a major impact on the management of HAT patients during the follow-up. A plasma marker with a high negative predictive value could be used for patients' screening during the follow-up through a simple blood finger prick instead of a lumbar puncture. Only patients with a blood positive test would then be investigated by lumbar puncture for trypanosome detection and WBC counting or neopterin levels evaluation. A reduction in the number of lumbar punctures during the follow-up would have significant benefits for patients and would probably increase the attendance rate at interim visits.

Based on the promising preliminary results obtained, further verification of the value of lumican and of the other proteins identified as over-expressed are ongoing through the development of a selected reaction monitoring (SRM) assay for the investigation of larger cohorts.

5. Impact of the study and conclusions

The huge efforts made by WHO, NGOs and other research institutions during the last decade had a major impact on the control of HAT. The intensive control strategies and the introduction of safer, even if not optimal, drugs,[53,54] led to a considerable reduction in the number of reported cases.[3,54] Today, the incidence of sleeping sickness is considered under control and its elimination is

thought to be possible.[3] However, to reduce to zero the incidence of the disease, these control measures need to be kept and reinforced.

In this context, the identification of HAT biomarkers reported in the present manuscript may be particularly important. The use of neopterin for the stage determination of *T. b. gambiense* patients, together with the recent introduction of eflornithine,[54] would help in achieving a more reliable treatment of patients and, as a consequence, a decreased rate of treatment failures.

Furthermore, neopterin could also be used during the follow-up to early detect relapses and to reduce the number of lumbar punctures by shortening the follow-up from 24 months to, at maximum, 12 months. The possibility of using the same marker for the two applications would, in addition, have benefits in terms of costs of production and personnel training. With our results we showed that there are strong evidences for the replacement of WBC counting for *T. b. gambiense* HAT. Longitudinal prospective studies will define the clinical utility and the benefits for patients of neopterin.

The results presented here also highlight the problem of *T. b. rhodesiense* HAT, the neglected form of this neglected disease. Despite the difficulties in sample collection, due to the lower prevalence compared to *T. b. gambiense* and to the lack of an effective tool for the active case detection, efforts should be done to ameliorate the management of patients suffering from the acute form. Furthermore, the decrease in the number of *rhodesiense* cases reported by WHO was lower compared to the one observed for the *gambiense*,[53] indicating that even if accounting for less than 5% of reported cases, this disease is still an health problem and could play a central role in disease re-emergence.

REFERENCES

1. Kennedy PG. Diagnosing central nervous system trypanosomiasis: two stage or not to stage? Transactions of the Royal Society of Tropical Medicine and Hygiene 2008;102:306-7.
2. Kennedy PG. Difficulties in diagnostic staging of human African trypanosomiasis. J Neuroparasitol 2011;2.
3. Simarro PP, Jannin J, Cattand P. Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS medicine 2008;5:e55.
4. Moore AC. Prospects for improving African trypanosomiasis chemotherapy. The Journal of infectious diseases 2005;191:1793-5.
5. Simarro PP, Franco J, Diarra A, Postigo JA, Jannin J. Update on field use of the available drugs for the chemotherapy of human African trypanosomiasis. Parasitology 2012;139:842-6.
6. WHO. Recommendations of the informal counsultation on issues for clinical product development for human African trypanosomiasis. WHO/CDS/NTD/IDM/20071 2007.
7. Mumba Ngoyi D, Lejon V, Pyana P, et al. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. The Journal of infectious diseases 2010;201:453-63.
8. Kennedy PG. Diagnostic and neuropathogenesis issues in human African trypanosomiasis. International journal for parasitology 2006;36:505-12.
9. Rodgers J. Trypanosomiasis and the brain. Parasitology 2010;137:1995-2006.
10. Kristensson K, Nygard M, Bertini G, Bentivoglio M. African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. Progress in neurobiology 2010;91:152-71.
11. Man S, Ubogu EE, Ransohoff RM. Inflammatory cell migration into the central nervous system: a few new twists on an old tale. Brain pathology 2007;17:243-50.
12. Girard M, Giraud S, Courtioux B, Jauberteau-Marchan MO, Bouteille B. Endothelial cell activation in the presence of African trypanosomes. Molecular and biochemical parasitology 2005;139:41-9.
13. Masocha W, Rottenberg ME, Kristensson K. Migration of African trypanosomes across the blood-brain barrier. Physiology & behavior 2007;92:110-4.
14. Jaber SM, Hamed EA, Hamed SA. Adhesion molecule levels in serum and cerebrospinal fluid in children with bacterial meningitis and sepsis. Journal of pediatric neurosciences 2009;4:76-85.
15. Brown HC, Chau TT, Mai NT, et al. Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. Neurology 2000;55:104-11.
16. Green JA, Friedland JS. Astrocyte-leucocyte interactions and the mechanisms regulating matrix degradation in CNS tuberculosis. Biochemical Society transactions 2007;35:686-8.
17. Ramos-Fernandez M, Bellolio MF, Stead LG. Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association 2011;20:47-54.
18. Unsal Y, Kivilcim G, Aysegul A, et al. Matrix metalloproteinase-7 and matrix metalloproteinase-9 in pediatric multiple sclerosis. Pediatric neurology 2012;47:171-6.
19. Prato M, Giribaldi G. Matrix Metalloproteinase-9 and Haemozoin: Wedding Rings for Human Host and Plasmodium falciparum Parasite in Complicated Malaria. Journal of tropical medicine 2011;2011:628435.
20. Szklarczyk A, Stins M, Milward EA, et al. Glial activation and matrix metalloproteinase release in cerebral malaria. Journal of neurovirology 2007;13:2-10.
21. Sucher R, Schroecksnadel K, Weiss G, Margreiter R, Fuchs D, Brandacher G. Neopterin, a prognostic marker in human malignancies. Cancer letters 2010;287:13-22.
22. Berdowska A, Zwirska-Korczala K. Neopterin measurement in clinical diagnosis. Journal of clinical pharmacy and therapeutics 2001;26:319-29.
23. Maclean L, Odiit M, Sternberg JM. Intrathecal cytokine responses in Trypanosoma brucei rhodesiense sleeping sickness patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006;100:270-5.
24. Hagberg L, Cinque P, Gisslen M, et al. Cerebrospinal fluid neopterin: an informative biomarker of central nervous system immune activation in HIV-1 infection. AIDS research and therapy 2010;7:15.
25. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet 2006;368:1012-21.

26. Muller M, Carter S, Hofer MJ, Campbell IL. Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity--a tale of conflict and conundrum. Neuropathology and applied neurobiology 2010;36:368-87.
27. Rebenko-Moll NM, Liu L, Cardona A, Ransohoff RM. Chemokines, mononuclear cells and the nervous system: heaven (or hell) is in the details. Current opinion in immunology 2006;18:683-9.
28. Weng Y, Siciliano SJ, Waldburger KE, et al. Binding and functional properties of recombinant and endogenous CXCR3 chemokine receptors. The Journal of biological chemistry 1998;273:18288-91.
29. Amin DN, Rottenberg ME, Thomsen AR, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. The Journal of infectious diseases 2009;200:1556-65.
30. Lalor SJ, Segal BM. Lymphoid chemokines in the CNS. Journal of neuroimmunology 2010;224:56-61.
31. Rainey-Barger EK, Rumble JM, Lalor SJ, Esen N, Segal BM, Irani DN. The lymphoid chemokine, CXCL13, is dispensable for the initial recruitment of B cells to the acutely inflamed central nervous system. Brain, behavior, and immunity 2011;25:922-31.
32. Courtioux B, Pervieux L, Vatunga G, et al. Increased CXCL-13 levels in human African trypanosomiasis meningo-encephalitis. Tropical medicine & international health : TM & IH 2009;14:529-34.
33. Rupprecht TA, Plate A, Adam M, et al. The chemokine CXCL13 is a key regulator of B cell recruitment to the cerebrospinal fluid in acute Lyme neuroborreliosis. Journal of neuroinflammation 2009;6:42.
34. Sellebjerg F, Bornsen L, Khademi M, et al. Increased cerebrospinal fluid concentrations of the chemokine CXCL13 in active MS. Neurology 2009;73:2003-10.
35. Krumbholz M, Theil D, Cepok S, et al. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain : a journal of neurology 2006;129:200-11.
36. Cresswell P, Bangia N, Dick T, Diedrich G. The nature of the MHC class I peptide loading complex. Immunological reviews 1999;172:21-8.
37. Alarcon A, Garcia-Alix A, Cabanas F, et al. Beta2-microglobulin concentrations in cerebrospinal fluid correlate with neuroimaging findings in newborns with symptomatic congenital cytomegalovirus infection. European journal of pediatrics 2006;165:636-45.
38. Puchades M, Hansson SF, Nilsson CL, Andreasen N, Blennow K, Davidsson P. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. Brain research Molecular brain research 2003;118:140-6.
39. Zhang J, Keene CD, Pan C, Montine KS, Montine TJ. Proteomics of human neurodegenerative diseases. Journal of neuropathology and experimental neurology 2008;67:923-32.
40. Shi C, Zhu Y, Su Y, Chung LW, Cheng T. Beta2-microglobulin: emerging as a promising cancer therapeutic target. Drug discovery today 2009;14:25-30.
41. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. Nature reviews Immunology 2010;10:778-86.
42. Lejon V, Legros D, Richer M, et al. IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Tropical medicine & international health : TM & IH 2002;7:685-92.
43. Bisser S, Lejon V, Preux PM, et al. Blood-cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. Journal of the neurological sciences 2002;193:127-35.
44. Lejon V, Reiber H, Legros D, et al. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. The Journal of infectious diseases 2003;187:1475-83.
45. Kennedy PG. Human African trypanosomiasis of the CNS: current issues and challenges. The Journal of clinical investigation 2004;113:496-504.
46. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for field diagnosis of human african trypanosomiasis. Clinical microbiology reviews 2005;18:133-46.
47. Kennedy PG. Novel biomarkers for late-stage human African trypanosomiasis--the search goes on. The American journal of tropical medicine and hygiene 2010;82:981-2.
48. Buhrer-Sekula S, Hamerlinck FF, Out TA, Bordewijk LG, Klatser PR. Simple dipstick assay for semi-quantitative detection of neopterin in sera. Journal of immunological methods 2000;238:55-8.
49. Picozzi K, Fevre EM, Odiit M, et al. Sleeping sickness in Uganda: a thin line between two fatal diseases. Bmj 2005;331:1238-41.

50. Berrang-Ford L, Wamboga C, Kakembo AS. Trypanososma brucei rhodesiense Sleeping Sickness, Uganda. Emerging infectious diseases 2012;18:1686-7.
51. Nikitovic D, Katonis P, Tsatsakis A, Karamanos NK, Tzanakakis GN. Lumican, a small leucine-rich proteoglycan. IUBMB life 2008;60:818-23.
52. Wu F, Vij N, Roberts L, Lopez-Briones S, Joyce S, Chakravarti S. A novel role of the lumican core protein in bacterial lipopolysaccharide-induced innate immune response. The Journal of biological chemistry 2007;282:26409-17.
53. Burri C. Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitology 2010;137:1987-94.
54. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS neglected tropical diseases 2011;5:e1007.

LIST OF PUBLICATIONS

Tiberti N, Enock M, Hainard A, Enyaru JC, Lejon V, Robin X, Turck N, Mumba Ngoyi D, Krishna S, Bisser S, Courtioux B, Büscher P, Kristensson K, Ndung'u JM, Sanchez J-C (2013) New biomarkers for stage determination in patients affected by *Trypanosoma brucei rhodesiense* sleeping sickness. Clin Transl Med 2(1): 1

Tiberti N, Lejon V, Hainard A, Courtioux B, Robin X, Turck N, Kristensson K, Mumba Ngoyi D, Krishna S, Matovu E, Enyaru C, Bisser S, Ndung'u JM, Büscher P, Sanchez J-C (2013) Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness. PLoS NTD 7(2): e2088

Tiberti N, Hainard A, Lejon V, Courtioux B, Matovu E, Enyaru JC, Robin X, Turck N, Kristensson K, Mumba Ngoyi D, Vatunga G, Krishna S, Büscher P, Bisser S, Ndung'u JM, Sanchez J-C (2012) Cerebrospinal fluid neopterin as marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness. PloS ONE 7(7): e40909

Hainard A, **Tiberti N**, Robin X, Mumba Ngoyi D, Matovu E, Enyaru JC, Müller M, Turck N, Ndung'u JM, Lejon V, Sanchez J-C (2011) Matrix metalloproteinase-9 and intracellular adhesion moleculte 1 are pawerful staging markers for human African trypanosomiasis. Trop Med Int Health 16(1): 119-126

Robin X, Turck N, Hainard A, **Tiberti N**, Lisacek F, Sanchez J-C, Müller M (2011) pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics, 12: 77

Tiberti N, Hainard A, Lejon V, Robin X, Mumba Ngoyi D, Turck N, Matovu E, Enyaru JC, Ndung'u JM, Scherl A, Dayon L, Sanchez J-C (2010) Discovery and verification of osteopontin and beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Mol Cell Proteomics 9(12): 2783-2795

Hainard A, **Tiberti N**, Robin X, Lejon V, Mumba Ngoyi D, Matovu E, Enyaru JC, Fouda C, Ndung'u JM, Lisacek F, Müller M, Turck N, Sanchez J-C (2009) A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. PloS NTD 3(6): e459

PAPERS IN PREPARATION

Robin X, Turck N, Hainard A, **Tiberti N**, Lisacek F, Sanchez J-C, Müller M. PanelomiX: an algorithm to create panel of biomarkers based on thresholds.

Tiberti N, Hainard A, Sanchez J-C. Translation of human African trypanosomiasis biomarkers towards field application (review).

Bisser S, **Tiberti N**, Courtioux B, Gedeao V, Hainard A, Robin X, Tusck N, Krishna S, Josenando T, Sanchez JC, Ndung'u J. Neopterin and CXCL13 as test of cure in *Trypanosoma brucei gambiense* sleeping sickness: lessons from the field in Angola.