Université de Montpellier – Ecole Doctorale GAIA (584)
Habilitation à Diriger des Recherches
Dossier de Candidature

Human African trypanosomiasis: research along the diagnostic pipeline while moving towards elimination

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"A number of workers, who have experience with trypanosomiasis in Africa, believe that there are 2 types of sleeping sickness. The first type is sleeping sickness as it presents itself on conferences and in textbooks, and the second type is sleeping sickness as it presents itself in villages, rural health centres and rural hospitals."

Buyst H,

Ann. Soc. Belge Méd. Trop. (1975), 55, 551-557.

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1. Summary

Infection with the parasite *Trypanosoma brucei gambiense* causes human African trypanosomiasis (HAT) or sleeping sickness, a tropical neglected disease occurring in West and Central Africa. Control of HAT largely depends on case detection, followed by treatment. The majority of my research has focused on diagnostic aspects of gambiense HAT: stage determination, treatment outcome assessment, serodiagnosis, parasitological confirmation and external quality assessment. My research on diagnostics has, with time, been influenced by the improving treatment options for HAT, but also by the decreasing HAT prevalence.

In the context of choosing between pentamidine and melarsoprol for treatment of gambiense HAT, improved markers for diagnosis of neurological disease stage were considered a priority in 1994, when I started as a researcher. In order to identify better parameters for stage determination, I investigated the neuro-inflammatory response. The humoral immune response, in particular intrathecal immunoglobulin synthesis in the central nervous system was studied in detail, as well as some aspects of the cellular imune response. Our findings highlighted the presence of intrathecal IgM synthesis, the IgM concentration, the IL-10 concentration and the neopterin concentration in cerebrospinal fluid (CSF) as performant markers for stage determination in gambiense HAT. The relationship between treatment outcome and intrathecal IgM synthesis and the CSF IgM concentration before treatment was established. A semi-quantitative agglutination test for IgM in CSF, LATEX/IgM, was developed.

Taking into account that relapses after treatment are usually neurological, markers for neurological involvement in HAT are good candidates for assessing treatment outcome. Our approach to shorten and improve the post-therapeutic follow-up was double. On the one hand, we examined the evolution of the CSF white blood cell (WBC) count, the parameter which is most often used to assess treatment outcome. We could propose a 2-step algorithm, classifying two third of the patients at 6 months post-treatment, the rest at 12 months post-treatment. Application of this algorithm significantly reduces the follow-up time and the number of lumbar punctures. On the other hand, the markers already examined for stage determination were evaluated for their usefulness in treatment outcome assessment. The decrease of the IgM concentration in CSF was too slow to be an accurate marker for treatment outcome assessment. Due to the rapid normalization of neopterin in CSF immediately after treatment, and its increase in treatment failures, neopterin had high accuracy for disease outcome assessment. The observed lack of specificity of DNA detection in blood and CSF during follow-up was somewhat surprising, but confirmed other fragmentary observations. Detection of SL RNA in CSF or blood may represent a promising alternative for treatment outcome assessment, but needs confirmation.

Although CATT/*T.b. gambiense* played a crucial role in control of the last HAT epidemic, its format is not optimal. Serological testing can be improved by improving the antigen, and/or changing the test format. I was involved in the use of phage display to identify peptide mimotopes of variant surface glycoproteins. Although the selected peptides had potential for diagnosis of HAT, their accuracy remained lower than for native VSGs. We were able to publish the first papers on evaluation of HAT rapid diagnostic tests in phase 1 and 2 trials. Major limitations were the use of stored samples, or a selection bias caused through routine population screening with CATT. So far, no prospective phase 3 field studies have been performed on disease suspects presenting at health centres using all available rapid diagnostic tests in parallel. Also, data from West-Africa are largely missing.

Diagnostic accuracy studies of parasitological techniques and PCR on large patient series are rare. In the framework of HAT surveillance, the importance of remote molecular analysis might increase, but

prospective studies on reproducibility, repeatability and performance of PCR are lacking. We therefore compared the diagnostic accuracy of parasitological and molecular techniques for diagnosis of HAT. It was demonstrated that there is no benefit in performing the microhematocrite centrifugation when mAECT or mAECT on buffy coat are carried out. Combination of examination of the lymph node aspirate and mAECT on buffy coat examination reaches a sensitivity of 95%. Remote DNA detection did not offer more sensitive diagnosis of HAT than parasitology.

Finally, the results obtained by external quality assessment of interpretation of thick blood film microscopy for malaria and HAT diagnosis underlined the need of regular training, regular quality assessment, and establishment of clear SOPs to improve diagnostic practices, in particular in an elimination context.

With the HAT-polyB project, we examined if *T.b. gambiense* infection in humans destructs B-cell memory and abrogates vaccine induced protection, as is observed in experimental infections. We studied the protection level against measles; the numbers of circulating memory B- and T-cells; and the T-cell independent B cell activity. Overall, our results in gambiense HAT do not suggest massive memory cell destruction, or loss of antibody levels. If some degree of immunity loss may exist in *T.b. gambiense* HAT patients, it does not seem of clinical relevance.

In between the HAT prevalence has strongly decreased, and HAT has been targeted for elimination. Nevertheless, important challenges remain. At low prevalence, integration of case finding into routine activities of peripheral health centres becomes crucial. HAT case detection by the peripheral health system with limited resources requires adapted diagnostic tests and algorithms. Furthermore, continued post-elimination monitoring is required to ensure sustainability of zero transmission and to avoid re-emergence caused by remaining *T.b. gambiense* reservoirs. Last but not least, therapeutic trials are hampered by the long follow-up required to decide on treatment efficacy, slowing down the drug developmental process. Tests of cure for faster assessment of treatment outcome are needed. These challenges form the basis of the DiTECT-HAT project, a project supported by the European and Developing Countries Clinical Trial Partnership (EDCTP2, Horizon 2020 program) that I actually coordinate.

At the long term, the difficult treatment outcome assessment, existence of reservoirs, latent infections, and occurrence of multiple species, are shared by HAT with animal trypanosomosis, the leishmaniasis and Chagas disease. These phenomena represent major challenges for disease control and elimination, and may constitute a basis for new diagnostic research.

2. Curriculum Vitae

2.1 Generalities

Name, First name	LEJON, Veerle
Date of birth, nationality	01/05/1971, Belgian
Private address	2 Rue Beethoven, 34830 Jacou| Mob: +33645552298 | Mob: +32476383357
Professional address	Institut de Recherche pour le Développement, Unité Mixte de Recherche UMR 177 – Intertryp, Campus International de Baillarguet TA A-17/G, bureau F201, 34398 Montpellier Cedex 5, France Tel : +33467593950 | Fax: +33467593894 | veerle.lejon@ird.fr
Languages	Dutch (mother tongue), English, French, Spanish, German (passive)
ORCID ID, Publons link	orcid.org/0000-0002-6795-0962 | https://publons.com/a/322251
Websites	http://umr-intertryp.cirad.fr/ | http://www.ditect-hat.eu/

Actual positions

Since 01/01/2013	Directeur de Recherche grade 2, Institut de Recherche pour le Développement (IRD), Montpellier, France
Since 01/01/2011	Member WHO Expert Panel on Trypanosomiasis, Geneva, Switzerland
Since 22/06/2016	Member of Human African trypanosomiasis elimination Technical Advisory Group (HAT-e-TAG), WHO, Geneva, Switzerland

Previous positions

01/09/2011 - 31/12/2012	Post-doc Assistant, Dept. Clinical Sciences, Institute of Tropical Medicine (ITM), Antwerp, Belgium: Laboratory Quality Management, GCLP, Malaria Rapid Diagnostic Tests, Pulsed Field Gel Electrophoresis of *Salmonella* typhi
01/05/2006 - 01/09/2010	Foundation for Innovative New Diagnostics, Geneva, Switzerland: Consultant HAT diagnostics programme
01/01/2005 – 31/12/2005	Post-doc, National Reference Centre for Parasitology, McGill University Health Centre, Montréal, Canada: SELDI-TOF MS for disease related protein expression in HAT
01/01/2006 - 31/08/2011, 01/01/2003 – 31/12/2004	Post-doc Assistant, Dept. Parasitology, ITM, Antwerp, Belgium. Parasite Diagnostics Unit: Shortened post-treatment follow-up in HAT, peptide mimotopes of *T.b. gambiense* variable surface glycoprotein
01/01/1999 – 31/12/2002	Assisting Academic Personnel Dept. Parasitology, ITM, Antwerp, Belgium
01/01/1997 – 31/12/1998	Dehousse Fellowship, Dept. Biochemistry, University of Antwerp, Belgium
01/09/1994 – 31/12/1996	Scientific Collaborator, Dept. Parasitology, ITM, Antwerp, Belgium: Stage determination in HAT

Diplomas

PhD	Doctor in Sciences, 24/05/2002, University of Antwerp, Belgium. Thesis: "Neuro-inflammation in human West-African trypanosomiasis: a basis for improved stage determination"
Licentiate	Chemistry, direction biochemistry, 06/07/1993, University of Antwerp (UIA). Thesis: "Identification by micro-sequence analysis of microglial proteins purified by two-dimensional electrophoresis"

Candidate Sciences, group of chemistry, 10/07/1991, University of Antwerp (RUCA)

Summary of scientific production

Summary of publication record (journal impact factors 2016, according to InCites):

Journal name	JIF 2016	1 st author	2nd author	Last author	Other author	Total
N Engl J Med	72.4			1		1
Lancet Inf Dis	19.9	1			1	2
Lancet Glob Health	17.7			1		1
Clin Microbiol Rev	16.5				1	1
Clin Infect Dis	8.2	1				1
Emerg Infect Dis	8.2				1	1
Euro Surveill.	7.2			1		1
PLoS Pathog	6.6	1				1
Mol Cell Proteomics	6.5				1	1
J Infect Dis	6.3	1	1		2	4
Trends Parasitol	6.3				2	2
Clin Vaccine Immunol	5.4	1			1	2
Bull WHO	4.9	2	1	1		4
Eur J Neurol	4.0	1				1
PLOS Negl Trop Dis	3.8	1	4	4	10	19
Int J Parasitol	3.7	1				1
J Clin Microbiol	3.7	1	1			2
Trop Med Int Health	2.9	4	1	2	6	13
PLoS One	2.8		1		3	4
J Neuroimmunol	2.7	1				1
Am J Trop Med Hyg	2.5	2			2	4
Vet Parasitol	2.4	1			2	3
J Neurol Sci	2.3		1			1
Trans R Soc Trop Med	2.3	1			1	2
Acta Trop	2.2	1	2		2	5
Genet Mol Research	0.8				1	1
Bull Soc Pathol Exot (JIF 2003)	0.2	1				1
Clin Transl Med					1	1
Med Trop		1		1		2
Rev Elev Med Vet Pays Trop				1		1
Translational Proteomics			1			1
Total		**23**	**13**	**12**	**37**	**85**

Hirsh Index 27 (Web of Science, February 2018)

Citations 1381 citations in 740 citing articles (without self-citations)

Awards & prizes

Foundation Anne Maurer-Cecchini Award 2014. Büscher P., Gilleman Q, **Lejon V**. Rapid diagnostic test for sleeping sickness. Geneva Health Forum, Geneva Switzerland

Brabo Health Development Award 2002. Büscher P and **Lejon V**. Rotary Club Antwerpen-Schelde

Development Cooperation Prize 2002. **Lejon V**. Belgian State Secretary for Development Cooperation and Royal Museum for Central Africa

Expertise (since 2013)

23 reviews for 18 different journals (publons link https://publons.com/a/322251)

Referee for Fonds National de Recherche Scientifique, Belgium (2013)

Jury member “Habilitation à diriger des Recherches” Courtin D (2014), Université Paris 5-René Descartes, Faculté des Sciences et Biologiques

Admission jury IRD, Directeurs de Recherche (2013)

2.2 Articles in peer reviewed journals (A)

(students: PhD, Master, Bachelor)

A1. 2018 - Büscher P, Bart J-M, Boelaert M, Bucheton B, Cecchi G, Chitnis N, Courtin D, Figueiredo L, Franco J-R, Grébaut P, Hasker E, Ilboudo H, Jamonneau V, Koffi M, **Lejon V**, MacLeod A, Masumu J, Matovu E, Mattioli R, Noyes H, Picado A, Rock K, Rotureau B, simo G, Thévenon S, Trindade S, Truc P, Van Reet N; Informal expert group on gambiense HAT reservoirs. Do cryptic reservoirs threaten gambiense-sleeping sickness elimination? Trends in Parasitol. doi: 10.1016/j.pt.2017.11.008.

A2. 2017 - Kaboré JW, Ilboudo H, Noyes H, Camara O, Kaboré J, Camara M, Koffi M, **Lejon V**, Jamonneau V, MacLeod A, Hertz-Fowler C, Belem AMG, Matovu E, Bucheton B, Sidibe I; TrypanoGEN Research Group as members of The H3Africa Consortium. Candidate gene polymorphisms study between human African trypanosomiasis clinical phenotypes in Guinea. PLoS Negl Trop Dis. 2017 Aug 21;11(8):e0005833. doi: 10.1371/journal.pntd.0005833.

A3. 2017 - Ilboudo H, Noyes H, Mulindwa J, Kimuda MP, Koffi M, Kaboré JW, Ahouty B, Ngoyi DM, Fataki O, Simo G, Ofon E, Enyaru J, Chisi J, Kamoto K, Simuunza M, Alibu VP, **Lejon V**, Jamonneau V, Macleod A, Camara M, Bucheton B, Hertz-Fowler C, Sidibe I, Matovu E; TrypanoGEN Research Group as members of The H3Africa Consortium. Introducing the TrypanoGEN biobank: A valuable resource for the elimination of human African trypanosomiasis. PLoS Negl Trop Dis. 2017 Jun 1;11(6):e0005438. doi: 10.1371/journal.pntd.0005438.

A4. 2016 - Barbé B, Verdonck K, Mukendi D, **Lejon V**, Lilo Kalo JR, Alirol E, Gillet P, Horié N, Ravinetto R, Bottieau E, Yansouni C, Winkler AS, van Loen H, Boelaert M, Lutumba P, Jacobs J. The Art of Writing and Implementing Standard Operating Procedures (SOPs) for Laboratories in Low-Resource Settings: Review of Guidelines and Best Practices. PLoS Negl Trop Dis. 2016 Nov 3;10(11):e0005053. doi:10.1371/journal.pntd.0005053.

A5. 2016 - Alirol E, Horie NS, Barbé B, **Lejon V**, Verdonck K, Gillet P, Jacobs J, Büscher P, Kanal B, Bhattarai NR, El Safi S, Phe T, Lim K, Leng L, Lutumba P, Mukendi D, Bottieau E, Boelaert M, Rijal S, Chappuis F. Diagnosis of Persistent Fever in the Tropics: Set of Standard Operating Procedures Used in the NIDIAG Febrile Syndrome Study. PLoS Negl Trop Dis. 2016 Nov 3;10(11):e0004749. doi: 10.1371/journal.pntd.0004749.

A6. 2016 - Mukadi P, **Lejon V**, Barbé B, Gillet P, Nyembo C, Lukuka A, Likwela J, Lumbala C, Mbaruku J, Vander Veken W, Mumba D, Lutumba P, Muyembe JJ, Jacobs J. Performance of Microscopy for the Diagnosis of Malaria and Human African Trypanosomiasis by Diagnostic Laboratories in the Democratic Republic of the Congo: Results of a Nation-Wide External Quality Assessment. PLoS One. 2016 Jan 20;11(1):e0146450. doi: 10.1371/journal.pone.0146450.

A7. 2016 - Berthier D, Brenière SF, Bras-Gonçalves R, Lemesre JL, Jamonneau V, Solano P, **Lejon V**, Thévenon S, Bucheton B. Tolerance to Trypanosomatids: A Threat, or a Key for Disease Elimination? Trends Parasitol. 2016 Feb;32(2):157-68. doi:10.1016/j.pt.2015.11.001.

A8. 2015 - Ilboudo H, Camara O, Ravel S, Bucheton B, **Lejon V**, Camara M, Kaboré J, Jamonneau V, Deborggraeve S. Trypanosoma brucei gambiense Spliced Leader RNA Is a More Specific Marker for Cure of Human African Trypanosomiasis than *T. b. gambiense* DNA. J Infect Dis. 2015 Dec 15;212(12):1996-8. doi:10.1093/infdis/jiv337.

A9. 2015 - Jamonneau V, Camara O, Ilboudo H, Peylhard M, Koffi M, Sakande H, N'Dri L, Sanou D, Dama E, Camara M, **Lejon V**. Accuracy of individual rapid tests for serodiagnosis of gambiense sleeping sickness in West Africa. PLoS Negl Trop Dis. 2015 Feb 2;9(2):e0003480. doi: 10.1371/journal.pntd.0003480.

A10. 2015 - Tiberti N, **Lejon V**, Mumba Ngoyi D, Matovu E, Enyaru J, Walter N, Fouda C, Lutumba P, Kristensson K, Bisser S, Mathu Ndung'u J, Büscher P, Sanchez J-C. Increased acute immune response during the meningo-encephalitic stage of Trypanosoma brucei rhodesiense sleeping sickness compared

to Trypanosoma brucei gambiense. Translational Proteomics 2015; 6: 1–9. doi: 10.1016/j.trprot.2014.11.001.

A11. 2014 - Rogé S, Baelmans R, Claes F, **Lejon V**, Guisez Y, Jacquet D, Büscher P. Development of a latex agglutination test with recombinant variant surface glycoprotein for serodiagnosis of surra. Vet Parasitol. 2014 Oct 15;205(3-4):460-5. doi: 10.1016/j.vetpar.2014.08.026.

A12. 2014 - Büscher P, Mertens P, Leclipteux T, Gilleman Q, Jacquet D, Mumba-Ngoyi D, Pyana PP, Boelaert M, **Lejon V**. Sensitivity and specificity of HAT Sero-K-SeT, a rapid diagnostic test for serodiagnosis of sleeping sickness caused by *Trypanosoma brucei gambiense*: a case-control study. Lancet Glob Health. 2014 Jun;2(6):e359-63. doi: 10.1016/S2214-109X(14)70203-7.

A13. 2014 - Mumba Ngoyi D, Ali Ekangu R, Mumvemba Kodi MF, Pyana PP, Balharbi F, Decq M, Kande Betu V, Van der Veken W, Sese C, Menten J, Büscher P, **Lejon V**. Performance of parasitological and molecular techniques for the diagnosis and surveillance of gambiense sleeping sickness. PLoS Negl Trop Dis. 2014 Jun 12;8(6):e2954. doi:10.1371/journal.pntd.0002954.

A14. 2014 - Camara O, Camara M, **Lejon V**, Ilboudo H, Sakande H, Léno M, Büscher P, Bucheton B, Jamonneau V. Immune trypanolysis test with blood spotted on filter paper for epidemiological surveillance of sleeping sickness. Trop Med Int Health. 2014 Jul;19(7):828-31. doi: 10.1111/tmi.12316.

A15. 2014 - Ley B, Le Hello S, Lunguya O, **Lejon V**, Muyembe JJ, Weill FX, Jacobs J. Invasive *Salmonella* enterica serotype typhimurium infections, Democratic Republic of the Congo, 2007-2011. Emerg Infect Dis. 2014 Apr;20(4):701-4. doi:10.3201/eid2004.131488.

A16. 2014 - **Lejon V**, Mumba Ngoyi D, Kestens L, Boel L, Barbé B, Kande Betu V, van Griensven J, Bottieau E, Muyembe Tamfum JJ, Jacobs J, Büscher P. Gambiense human african trypanosomiasis and immunological memory: effect on phenotypic lymphocyte profiles and humoral immunity. PLoS Pathog. 2014 Mar 6;10(3):e1003947. doi:10.1371/journal.ppat.1003947

A17. 2014 - García C, **Lejon V**, Horna G, Astocondor L, Vanhoof R, Bertrand S, Jacobs J. Intermediate susceptibility to ciprofloxacin among *Salmonella enterica* serovar Typhi isolates in Lima, Peru. J Clin Microbiol. 2014 Mar;52(3):968-70. doi:10.1128/JCM.02663-13.

A18. 2013 - Abdulla MH, Bakhiet M, **Lejon V**, Andersson J, McKerrow J, Al-Obeed O, Harris RA. TLTF in cerebrospinal fluid for detection and staging of *T. b. gambiense* infection. PLoS One. 2013 Nov 19;8(11):e79281. doi: 10.1371/journal.pone.0079281.

A19. 2013 - Mitashi P, Hasker E, Ngoyi DM, Pyana PP, **Lejon V**, Van der Veken W, Lutumba P, Büscher P, Boelaert M, Deborggraeve S. Diagnostic accuracy of loopamp *Trypanosoma brucei* detection kit for diagnosis of human African trypanosomiasis in clinical samples. PLoS Negl Trop Dis. 2013 Oct 17;7(10):e2504. doi:10.1371/journal.pntd.0002504.

A20. 2013 - **Lejon V**, Jacobs J, Simarro PP. Elimination of sleeping sickness hindered by difficult diagnosis. Bull World Health Organ. 2013 Oct 1;91(10):718. doi:10.2471/BLT.13.126474.

A21. 2013 - Mukadi P, Gillet P, Lukuka A, Atua B, Sheshe N, Kanza A, Mayunda JB, Mongita B, Senga R, Ngoyi J, Muyembe JJ, Jacobs J, **Lejon V**. External quality assessment of Giemsa-stained blood film microscopy for the diagnosis of malaria and sleeping sickness in the Democratic Republic of the Congo. Bull World Health Organ. 2013 Jun 1;91(6):441-8. doi: 10.2471/BLT.12.112706.

A22. 2013 - Mukadi P, Gillet P, Lukuka A, Mbatshi J, Otshudiema J, Muyembe JJ, Buyze J, Jacobs J, **Lejon V**. External quality assessment of reading and interpretation of malaria rapid diagnostic tests among 1849 end-users in the Democratic Republic of the Congo through Short Message Service (SMS). PLoS One. 2013 Aug 13;8(8):e71442.doi: 10.1371/journal.pone.0071442.

A23. 2013 - Gillet P, Mumba Ngoyi D, Lukuka A, Kande V, Atua B, van Griensven J, Muyembe JJ, Jacobs J, **Lejon V**. False positivity of non-targeted infections in malaria rapid diagnostic tests: the case of human african trypanosomiasis. PLoS Negl Trop Dis. 2013 Apr 25;7(4):e2180. doi: 10.1371/journal.pntd.0002180.

A24. 2013 - Yansouni CP, Bottieau E, Lutumba P, Winkler AS, Lynen L, Büscher P, Jacobs J, Gillet P, **Lejon V**, Alirol E, Polman K, Utzinger J, Miles MA, Peeling RW, Muyembe JJ, Chappuis F, Boelaert M. Rapid

diagnostic tests for neurological infections in central Africa. Lancet Infect Dis. 2013 Jun;13(6):546-58. doi:10.1016/S1473-3099(13)70004-5.

A25. 2013 - Lunguya O, **Lejon V**, Phoba MF, Bertrand S, Vanhoof R, Glupczynski Y, Verhaegen J, Muyembe-Tamfum JJ, Jacobs J. Antimicrobial resistance in invasive non-typhoid *Salmonella* from the Democratic Republic of the Congo: emergence of decreased fluoroquinolone susceptibility and extended-spectrum beta lactamases. PLoS Negl Trop Dis. 2013;7(3):e2103. doi: 10.1371/journal.pntd.0002103.

A26. 2013 - Mumba Ngoyi D, Menten J, Pyana PP, Büscher P, **Lejon V**. Stage determination in sleeping sickness: comparison of two cell counting and two parasite detection techniques. Trop Med Int Health. 2013 Jun;18(6):778-82. doi: 10.1111/tmi.12102.

A27. 2013 - Büscher P, Gilleman Q, **Lejon V**. Rapid diagnostic test for sleeping sickness. N Engl J Med. 2013 Mar 14;368(11):1069-70. doi: 10.1056/NEJMc1210373.

A28. 2013 - Tiberti N, **Lejon V**, Hainard A, Courtioux B, Robin X, Turck N, Kristensson K, Matovu E, Enyaru JC, Mumba Ngoyi D, Krishna S, Bisser S, Ndung'u JM, Büscher P, Sanchez JC. Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness. PLoS Negl Trop Dis. 2013;7(2):e2088. doi: 10.1371/journal.pntd.0002088.

A29. 2013 - Tiberti N, Matovu E, Hainard A, Enyaru JC, **Lejon V**, Robin X, Turck N, Ngoyi DM, Krishna S, Bisser S, Courtioux B, Büscher P, Kristensson K, Ndung'u JM, Sanchez JC. New biomarkers for stage determination in *Trypanosoma brucei rhodesiense* sleeping sickness patients. Clin Transl Med. 2013 Jan 7;2(1):1. doi: 10.1186/2001-1326-2-1.

A30. 2013 - Van Nieuwenhove L, Büscher P, Balharbi F, Humbert M, Guisez Y**, Lejon V**. A LiTat 1.5 variant surface glycoprotein-derived peptide with diagnostic potential for *Trypanosoma brucei gambiense*. Trop Med Int Health. 2013 Apr;18(4):461-5. doi:10.1111/tmi.12058.

A31. 2012 - Mitashi P, Hasker E, **Lejon V**, Kande V, Muyembe JJ, Lutumba P, Boelaert M. Human african trypanosomiasis diagnosis in first-line health services of endemic countries, a systematic review. PLoS Negl Trop Dis. 2012;6(11):e1919. doi:10.1371/journal.pntd.0001919

A32. 2012 - Lunguya O, **Lejon V**, Phoba MF, Bertrand S, Vanhoof R, Verhaegen J, Smith AM, Keddy KH, Muyembe-Tamfum JJ, Jacobs J. *Salmonella* typhi in the Democratic Republic of the Congo: fluoroquinolone decreased susceptibility on the rise. PLoS Negl Trop Dis. 2012;6(11):e1921. doi: 10.1371/journal.pntd.0001921

A33. 2012 - Tiberti N, Hainard A, **Lejon V**, Courtioux B, Matovu E, Enyaru JC, Robin X, Turck N, Kristensson K, Ngoyi DM, Vatunga GM, Krishna S, Büscher P, Bisser S, Ndung'u JM, Sanchez JC. Cerebrospinal fluid neopterin as marker of the meningo-encephalitic stage of *Trypanosoma brucei gambiense* sleeping sickness. PLoS One. 2012;7(7):e40909. doi: 10.1371/journal.pone.0040909.

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2.3 Submitted articles (S)

S1. Hasker E, Kwete J, Inocencio Da Luz R, Mpanya A, Bebronne N, Makabuza J, Claeys Y, Ilunga J, **Lejon V**, Mumba Ngoyi D, Büscher P, Lutumba P, Boelaert M, Lumbala C. Quality assurance of diagnosis in the context of elimination of Human African Trypanosomiasis.

S2. **Lejon V**, Brenière SF, Desquesnes M, Thévenon S, Bras-Gonçalves R, Barnabé C, Truc P, Geiger A, Sereno D, Bucheton B, Ravel S, Bart, J-M, Berthier d, Bossard G, Herder S, De Meeûs T, Boulangé A, Vincendeau P, Lemesre J-L, Solano P, Jamonneau V. The diagnosis of trypanosomatid diseases in mammals: review and perspectives for elimination.

2.4 Book chapters (B)

B1. **Lejon V**, E Hasker E, Büscher P. Rapid Diagnostic Tests for Human African Trypanosomiasis. In: Revolutionizing the delivery of health care in the developing world: rapid diagnostic tests, cheap imaging and other innovative technologies together with work force task-shifting at the primary point-of-care". Eds K Atkinson & D Mabey. Wiley Publishers. In press.

B2. D Sudarshi, S Lawrence, W Mutombo Kalonji (2014). NeuroID: elearning module 8 Human African Trypanosomiasis. https://docs.google.com/presentation/d/15v4GCbSe5Hau7TGfa1YlwRFmhWcTF-ShXNeorxHqFgM/pub?start=false&loop=false&delayms=3000&slide=id.p Eds Kennedy P and **Lejon V**. University of Liverpool.

B3. **Lejon V**, Bentivoglio M, Franco JR (2013). Human African Trypanosomiasis. In: Handbook of Clinical Neurology, Neuroparasitology and Tropical Neurology. Volume 114, Eds Aminoff MJ, Boller F and Swaab DF. Elsevier. Pages 169-181.

B4. **Lejon V**, Büscher P. Parasitological diagnosis (2013). In: Sleeping sickness lectures. Eds Cattand P, Louis FJ, Simarro PP. Association contre la Trypanosomiase en Afrique. ISBN 978-2-9544171-0-3. Pages 183-197.

B5. **Lejon V**, Büscher P. Serological diagnosis (2013). In: Sleeping sickness lectures. Eds Cattand P, Louis FJ, Simarro PP. Association contre la Trypanosomiase en Afrique. ISBN 978-2-9544171-0-3. Pages 199-214.

B6. **Lejon V**, Büscher P. Stage determination and follow-up (2013). In: Sleeping sickness lectures. Eds P Cattand, FJ Louis, PP Simarro. Association contre la Trypanosomiase en Afrique. ISBN 978-2-9544171-0-3. Pages 215-234.

B7. **Lejon V**, Büscher P. Diagnosis: the future (2013). In: Sleeping sickness lectures. Eds Cattand P, Louis FJ, Simarro PP. Association contre la Trypanosomiase en Afrique. ISBN 978-2-9544171-0-3. Pages 235-255.

B8. Clerinx J, **Lejon V**, Sendid B. Trypanosoma species (2012). In: Cornaglia G, Courcol R, Herrmann JL, Kahlmeter G, Vila J, editors. European Manual of Clinical Microbiology. [London]: [ESCMID]; [London], [SFM];. p. 399-410.

B9. **Lejon V**, Franco JR & Simarro P. (2010) Human African Trypanosomiasis Monograph. BMJ Evidence Centre - Best Practice, http://bestpractice.bmj.com/best-practice/monograph/9999.html.

B10. Büscher P, **Lejon V**, Van den Ende J (2007). Diagnosis. In: Human African Trypanosomiasis. CD-ROM Topics in International Health. Wellcome Trust, London, UK.

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2.5 Communications (C) and other documents

2.5.1 Invited oral communications

C1. **Lejon V**. Drug resistance in human African trypanosomiasis: past fears, bright future. Sociedad Espanola de Medicina Tropical y Salud Internacional. 23-25/10/2017.

C2. **Lejon V**. Diagnostic tools for human African trypanosomiasis elimination and clinical trials. FIND symposium on NTD diagnostics. 10th European Congress on Tropical Medicine and International Health. 16-20/10/2017, Antwerp, Belgium.

C3. **Lejon V**. Development and evaluation of diagnostic tools for African trypanosomiasis. European Workshop on Label-free Particle Sorting. 6-09/2017. University of Lund, Lund, Sweden.

C4. **Lejon V**. Experiences in preparation of an EDCTP funded project: From the call for proposals to the year 1 report. Réunion d'information EDCTP. Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation (MESRI) et Aviesan-Sud. 30/06/2017, Paris, France.

C5. **Lejon V**. Situation of rHAT control tools: diagnostics. Second WHO stakeholders meeting on rhodesiense human African trypanosomiasis (r-HAT) elimination. 26-28/04/2017, Geneva. Switzerland.

C6. **Lejon V**. Capacity building for rHAT: lessons learned in previous experiences, needs. Second WHO stakeholders meeting on rhodesiense human African trypanosomiasis (r-HAT) elimination. 26-28/04/2017, Geneva. Switzerland.

C7. **Lejon V.** Diagnostic tools for human African trypanosomiasis elimination and clinical trials. Human African Trypanosomiasis Session, Neglected Tropical Disease Summit 21/04/2017, Geneva, Switzerland.

C8. **Lejon V**. Production de diagnostics en l'absence d'industriels, exemple de la THA : quelle pérennité? Réunion "accès au diagnostic des MTN" du réseau francophone des MTN, Fondation Mérieux, 03/2017

C9. **Lejon V**. Diagnosis of HAT : new tools in a changing context. Symposium 102: Approaches, advances and needs for the elimination of human African Trypanosomiasis. American Society of Tropical Medicine & Hygiene, 65th annual meeting. 13-17/11/2016. Atlanta, USA.

C10. **Lejon V**. Panorama des Maladies Tropicales Négligées: Stratégies d'intervention, outils et modalités, besoins. Réunion de lancement du réseau francophone sur les Maladies Tropicales Négligées. 7-8/04/2016.

C11. **Lejon V**. Diagnostic Tools for Human African Trypanosomiasis Elimination and Clinical Trials (DiTECT-HAT project). WHO stakeholders meeting on gambiense human African trypanosomiasis (g-HAT) elimination. Geneva, Switzerland 21-24/03/2016.

C12. **Lejon V**. Advances and challenges in current diagnostics for HAT. American Society of Tropical Medicine & Hygiene, 63rd annual meeting. 2-6/11/2014. New Orleans, USA.

C13. **Lejon V**. VIH et trypanosomiase humaine africaine. 7e Conférence Internationale Francophone VIH/Hépatites AFRAVIH 2014. 27-30/4/2014. MSF-IRD Symposium. Montpellier, France.

C14. **Lejon V.** Le diagnostic de la Trypanosomose Humaine Africaine : statu quo ou temps du changement ? Paludisme et Trypanosomose humaine Africaine: nouvelles stratégies de prévention et de contrôle. Cotonou, Benin 7-8/10/2010.

C15. **Lejon V.** Human African trypanosomiasis – an overview. Autumn symposium Dutch society for Parasitology: Exotes in The Netherlands. Rotterdam. 06/11/2009. National symposium.

C16. **Lejon V.** Markers of treatment efficacy: update and how can we improve? HATcap platform annual meeting. Brazzaville, PR Congo 17-22/11/08.

C17. **Lejon V.** Human African Trypanosomiasis – present situation in African countries. Infectious diseases of the nervous system - an African and European scourge. Joint meeting of the "Società Italiana di Medicina Tropicale" (Italian Society of Tropical Medicine) – SIMET – and the NEUROTRYP Consortium. Verona, 08/07/2008. International symposium.

C18. **Lejon V**. Need for, current status and perspectives for surrogate markers. Workshop on HAT clinical trial methodology. HATcap platform annual meeting. Sudan, Khartoum 27-29/11/07.

C19. **Lejon V**. Identification of patients for clinical trials. Workshop on HAT clinical trial methodology. HATcap platform annual meeting. Sudan, Khartoum 27-29/11/07.

C20. **Lejon V**, P. Büscher, J. Robays, M. Boelaert. Improved staging and post-treatment follow-up by IgM determination in field circumstances in Angola. Workshop Retaking Clinical Research on Human African Trypanosomiasis in Angola. Medical Mission Institute, Würzburg, Germany. 13-14/12/2004.

C21. **Lejon V**, F Claes, T Tran & P Büscher. The basis of biological diagnosis of human African trypanosomiasis. Premier Congrès International de Brazzaville sur la Mouche tsé-tsé et les trypanosomoses. Brazzaville, République du Congo. 23-25/3/2004.

C22. **Lejon V**, F Claes, T Tran & P Büscher. Progress in diagnosis of human African trypanosomiasisPremier Congrès International de Brazzaville sur la Mouche tsé-tsé et les trypanosomoses. Brazzaville, République du Congo. 23-25/3/2004.

C23. **Lejon V**. & Büscher P. Immunoglobulin patterns & applications for field diagnosis of CNS involvement in human African trypanosomiasis. International Colloquium "Cerebrospinal Fluid Analysis in Tropical Neurology", Institute of Tropical Medicine, Antwerp, 26-29 November 2003. International conference.

C24. **Lejon V** & Büscher P. CSF in the diagnosis of infectious diseases: the case of Human African Trypanosomiasis. EKN Satellite symposium: CSF in clinical practice and basic research. 16/10/03. Alden Biesen. Belgium.

C25. **Lejon V** & Büscher P. Introduction to open Research Questions in Sleeping Sickness diagnosis I. State of the Art Workshop & Roundtable on diagnosis & treatment of human African Trypanosomiasis. Satellite workshop to the Meeting of International Scientific Council for Trypanosomiasis Research and Control (ISCTRC). Pretoria, South-Africa, 2/10/03.

C26. Büscher P & **Lejon V**. Introduction to open Research Questions in Sleeping Sickness diagnosis II. State of the Art Workshop & Roundtable on diagnosis & treatment of human African Trypanosomiasis. Satellite workshop to the Meeting of International Scientific Council for Trypanosomiasis Research and Control (ISCTRC). Pretoria, South-Africa, 2/10/03.

C27. **Lejon V** & Büscher P. Immuno-magnetic isolation of trypanosomes from blood. Second international meeting on the diagnostic applications of magnetic microspheres. Paris, France, June 12-13 2003.

C28. **Lejon V**. Le diagnostic de phase dans la maladie du sommeil, vers une nouvelle approche. Journée en hommage au Méd. Général Lapeyssonnie. Société de Pathologie Exotique, IMTSSA Le Pharo, Marseille, 20/3/02.

C29. **Lejon V**, Büscher P, Bisser S. New tools for stage determination and follow-up in sleeping sickness International Colloquium Sleeping Sickness Rediscovered, ITG, Antwerp: 14-18/12/98. International conference.

2.5.2 Oral communications in conferences

C30. **Lejon V**, Koné M, Makabuza J, Ngay I, Camara O, Kaba D, Lumbala C, Mumba D, Camara M, Ilboudo H, Dama E, Fèvre E, Jamonneau V, Bucheton B, Büscher P.. Diagnostic tools for human African trypanosomiasis elimination and clinical trials (DITECT-HAT). 34th Meeting of the ISCTRC, Livingstone, Zambia: 11-15/09/2017.

C31. Büscher P, **Lejon V**, Hasker E. Digital recording of DITECT-HAT study participant data, including macroscopic and microscopic images. 34th Meeting of the ISCTRC, Livingstone, Zambia: 11-15/09/2017.

C32. **Lejon V**, Ilboudo H, Mumba D, Camara M, Kaba D, Lumbala C, Fèvre E, Jamonneau V, Bucheton B, Büscher P. Diagnostic tools for human African trypanosomiasis elimination and clinical trials: the DiTECT-HAT project. Eight EDCTP forum. 6-9/11/2016, Lusaka, Zambia.

C33. Dama E, Camara O, Koffi M, Dayo G-K, Büscher P, Sakande H, Somda MB, Ilboudo H, Courtin F, Kaba D, Ouédraogo E, Lingue K, Camara M, Bucheton B, **Lejon V** & Jamonneau V. The immune Trypanolysis test: an accurate serological marker to manage elimination of gambiense human African trypanosomiasis. Eight EDCTP forum. 6-9/11/2016, Lusaka, Zambia.

C34. **Lejon V**, Camara M, Kaba D, Lumbala C, Ilboudo H, Mumba D, Fèvre E, Jamonneau V, Bucheton B, Büscher P. Diagnostic tools for HAT elimination and clinical trials: DiTECT-HAT. 4th joint meeting of the HAT-platform and EANETT. 17-19/09/2016, Conakry, Guinea.

C35. Mukadi P, **Lejon V**, Barbé B, Gillet P, Nyembo C, Lukuka A, Likwela J, , Lumbala C, Mbaruku J, Van der Veken W, Mumba D, Lutumba P, Muyembe JJ, Jacobs J .External quality assessment of Giemsa - stained blood film microscopy for the diagnosis of malaria and human african trypanosomiasis in Democratic Republic of the Congo. 33th ISCTRC meeting/14th PATTEC meeting, Ndjaména, Tchad, 14-18/09/2015.

C36. Jamonneau V, Koffi M, Ilboudo H, Kaboré J, Kaba D, Garcia A, Courtin D, Courtin F, Solano P, Laveissière C, Coulibaly B, N'Dri L, Sakande H, Lingue K, Büscher P, **Lejon V**, Bucheton B. Human Trypanotolerance: description, definition and consequences for the targeted elimination of sleeping sickness. 33th ISCTRC meeting/14th PATTEC meeting, Ndjaména, Tchad, 14-18/09/2015.

C37. Dama E, Camara O, Koffi M, **Lejon V**, Sanou D, Compaoré C, Sakande H, Somda MB, Ilboudo H, Courtin F, Kaba D, Ouédraogo E, Lingue K, Camara M, Bucheton B, Jamonneau V. The immune trypanolysis test: an accurate serological marker to manage elimination of gambiense human african trypanosomiasis. 33th ISCTRC meeting/14th PATTEC meeting, Ndjaména, Tchad, 14-18/09/2015.

C38. Gillet P, Mumba Ngoyi D, Lukuka A, Kande V, Atua B, van Griensven J, Muyembe JJ, Jacobs J, **Lejon V**. Human African trypanosomiasis may cause false positive reactions in malaria rapid diagnostic tests. 32nd meeting of the International Scientific Council for Trypanosomiasis Research and Control, 8-12/9/2013. African Union. Khartoum, Sudan.

C39. Büscher P, Mertens P, Leclipteux T, Gilleman Q, Jacquet D, Mumba Ngoyi D, Pyana PP, Menten J, **Lejon V**. Diagnostic accuracy under field conditions of HAT Sero-K-SeT, a rapid diagnostic test for sleeping sickness. 32nd meeting of the International Scientific Council for Trypanosomiasis Research and Control, 8-12/9/2013. African Union. Khartoum, Sudan. International conference.

C40. **Lejon V**, Mumba Ngoyi D, Kestens L, Boel L, Barbé B, Kande V, van Griensven J, Bottieau E, Muyembe JJ, Jacobs J, Büscher P. Influence of human African trypanosomiasis on memory B-and T- cells and on acquired immunity. 6ième Congrès International de Pathologies Infectieuses et Parasitaires (CIPIP6) Kinshasa 12-15/03/2013. Ann. Afr. Med. Mars 2013, Vol 6, N°2 Suppl, p50.

C41. Mumba Ngoyi D, Gilleman Q, Pyana P, **Lejon V**, Menten J, Büscher P. Les nouveaux tests de diagnostic rapide pour la THA à *T.b. gambiense*. 6ième Congrès International de Pathologies Infectieuses et Parasitaires (CIPIP6) Kinshasa 12-15/03/2013. Ann. Afr. Med., Mars 2013.Vol 6, N°2 Suppl, p47

C42. Büscher P, Ali Ekangu R, Mumba Ngoyi D, Muvemba Kodi MF, Kande Betu V, Van der Veken W, Sese C, Menten J, **Lejon V**. Performance of parasitological and molecular techniques for the diagnosis and surveillance of sleeping sickness. 6ième Congrès International de Pathologies Infectieuses et Parasitaires (CIPIP6) Kinshasa 12-15/03/2013. Ann. Afr. Med., Mars 2013 Vol 6, N°2 Suppl, p47.

C43. Tiberti N, **Lejon V**, Hainard A, Courtioux B, Matovu E, Enyaru JC, Robin X, Turck N, Kristensson K, Mumba Ngoyi D, Krishna S, Bisser S, JM Ndung'u, P Büscher, JC Sanchez. Neopterin for stage determination and treatment outcome evaluation in patients affected by T.b. gambiense human African trypanosomiasis. 6ième Congrès International de Pathologies Infectieuses et Parasitaires (CIPIP6) Kinshasa 12-15/03/2013. Ann. Afr. Med. Mars 2013, Vol 6, N°2 Suppl, p49.

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C45. Deborggraeve S, **Lejon V**, Ali Ekangu R, Mumba Ngoyi D, Pyana PP, Ilunga M, Mulunda JP, Büscher P. How reliable is PCR for diagnosis, staging and follow-up of gambiense sleeping sickness? 31th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 12-16/9/2011. African Union. Bamako, Mali.

C46. Büscher P, Mumba Ngoyi D, Balharbi F, Kande Betu V, Van der Veken W, Sese C, **Lejon V**. Improved parasitological and molecular techniques for the diagnosis and surveillance of sleeping sickness. 31th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 12-16/9/2011. African Union. Bamako, Mali.

C47. Van Nieuwenhove L, Rogé S, Balharbi F, Dieltjens T, Guisez Y, Büscher P, **Lejon V**. Identification of peptides that mimic variant surface glycoprotein epitopes for diagnosis of *Trypanosoma brucei gambiense* HAT. 31th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 12-16/9/2011. African Union. Bamako, Mali.

C48. Tiberti N, Hainard A, **Lejon V**, Courtioux B, Matovu E, Enyaru JC, Robin X, Turck N, Kristensson K, Mumba Ngoyi D, Vatunga GML, Krishna S, Büscher P, Bisser S, Ndung'u JM, Sanchez J-C. Neopterin for the staging and follow-up of sleeping sickness patients: evidence from a multi-centric cohort. 31th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 12-16/9/2011. African Union. Bamako, Mali.

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C51. Hasker E, Mitashi P, Baelmans R, Lutumba P, Jacquet D, **Lejon V**, Kande V, Declercq J, Boelaert M. A new format of the CATT test for the detection of human African trypanosomiasis, designed for use in peripheral health facilities. 5è congrès International de Pathologies Infectieuse et Parasitaire, 4-6/11/2009, Kinshasa, RD Congo. Annales Africaines de Médedine, Vol 2 suppl. I, Novembre 2009.

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C55. 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, and Sanchez J-C. A combined CXCL10, CXCL8 and H-FABP panel for the staging of human African trypanosomiasis patients. 30th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 21-25/9/2009. Kampala, Uganda.

C56. Mumba Ngoyi D, **Lejon V**, Pyana P, Boelaert M, Muyembe Tamfum JJ, Ilunga M, Mulunda JP, Van Nieuwenhove S, and Büscher P. How to shorten patient follow-up after treatment for *Trypanosoma*

gambiense sleeping sickness. 30th meeting of the International Scientific Council for Trypanosomiasis Research and Control, 21-25/9/2009. African Union. Kampala, Uganda.

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C67. Jamonneau V, Solano P, Garcia A, **Lejon V**, Djé N, Miezan TW, N'Guessan P, Cuny G & Büscher P. Application of PCR/CSF for stage determination and therapeutic decision in Human African Trypanosomiasis in Côte d'Ivoire. 2nd FAO/IAEA Research Coordination Meeting on "developing, validating, and standardising PCR and PCR ELISA for the diagnosis of trypanosomiasis. Rio de Janeiro, Brazil, 7-11/4/2003.

C68. **Lejon V**, & Büscher P. Sleeping sickness: from intrathecal IgM synthesis to card agglutination. 3d European Congres on Tropical Medicine and International Health. Lisboa, Portugal, September 8-12/2002, Acta Tropica (2002), 83, S70.

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2.5.3 Poster communications

C82. Büscher P, Hasker E, **Lejon V**. The development of an application for digital recording of DiTECT-HAT study participant data, including macroscopic and microscopic images. 10th European Congress on Tropical Medicine and International Health. 16-20/10/2017, Antwerp, Belgium.

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C107. Pansaerts R, **Lejon V**, Verloo D, Wouters I, Van Raemdonck C, Brandt J, Büscher P. Improved detection of trypanosomes in blood by a direct immunofluorescence assay. International Colloquium sleeping sickness rediscovered, Antwerp, Belgium, 14-18/12/98.

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2.5.4 Other communications

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C116. **Lejon V**. How to write scientific articles. HAT Platform Newsletter n° 17, June 2016, p20-21. http://www.dndi.org/wp-content/uploads/2016/06/HATplatform_newsletter17_June2016_EN.pdf.

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C118. **Lejon V**. Mission de recherche sur la trypanosomiase humaine Africaine en RD Congo. Site web IRD Cameroun. http://www.cameroun.ird.fr/toute-l-actualite/l-actualite/mission-de-recherche-sur-la-trypanosomiase-humaine-africaine-en-rd-congo.

C119. **Lejon V**. Recherches sur la maladie du sommeil en RDC. Carrefour sciences, le Bulletin d'informations de l'IRD-Afrique Centrale, n°009, Mars 2015. p9

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Maputo, Mozambique, 1997. Nairobi: Organization of African Unity (OAU), Scientific and Technical Research Council (STRC), 1999:122-128. (OAU/STRC Publications; 119).

C126. Verloo D, Van Meirvenne N, **Lejon V**, Magnus E, Büscher P. (1999) Evaluation of serological tests in rabbits, experimentally infected with T. evansi stocks and clones from different origin. In: 24th Meeting of the International Scientific Council for Trypanosomiasis Research & Control (ISCTRC); Maputo, Mozambique, 1997. Nairobi: Organization of African Unity (OAU), Scientific and Technical Research Council (STRC), 1999:152-159. (OAU/STRC Publications; 119).

C127. **Lejon V**, Moons A, Büscher P, Magnus E and Van Meirvenne N. (1997) Trypanosome specific antibody profile in serum and cerebrospinal fluid of T.b. gambiense patients. In: 23th Meeting of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC). Banjul, The Gambia, 1995. Nairobi: Organization of African Unity (OAU), Scientific and Technical Research Council (STRC), 1997:78-91. (OAU/STRC Publications; 118).

2.6 Student supervision (T)

2.6.1 PhD students

T1. Ngay Ipos, INRB, DR Congo. New markers outcome assessment after treatment for human African trypanosomiasis in a therapeutic trial (DiTECT-HAT-WP4). 01/02/2017-31/01/2020. University of Kinshasa, promotor Mumba D. Ongoing, financed by DiTECT-HAT.

T2. Koné Minayégninrin IPR-INSP Côte d'Ivoire. Performance of different diagnostic techniques for elimination of human African trypanosomias (DiTECT-HAT-WP2 et WP3). 01/01/2017- 31/12/2019. Promotors Kaba D and Koffi M. Ongoing, financed by DiTECT-HAT.

T3. Makabuza Mukabela Jacquies, PNLTHA-DRC DR Congo. Performance of different diagnostic techniques for elimination of human African trypanosomiasis in the Democratic Republic of Congo (DiTECT-HAT-WP2 and WP3). 01/02/2017- 31/01/2020. University of Kinshasa, promotors Lutumba P & Lejon V. Ongoing, financed by DiTECT-HAT.

T4. Camara Oumou, Ministry of Health Guinea. Passive detection of human African trypanosomias patients in health centres in Guinea (DiTECT-HAT-WP2). 01/02/2016-31/01/2020. University of Bouaké. Promotors Camara M / Bucheton B. Ongoing, financed by DiTECT-HAT. A2, A8, A9, A14

T5. Mukadi Kaninga Pierre Patrick, INRB, DR Congo (2017). Evaluation externe de la Qualité du diagnostic biologique du Paludisme et de la Trypanosomiase Humaine Africaine en République Démocratique du Congo. University of Lubumbashi. Promotors Nyembo Mukena C / Jacobs J, Muyembe Tamfum JJ. A6, A21, A22

T6. Lunguya Octavie, INRB, DR Congo (2013). Microbiological and diagnostic aspects of invasive salmonellosis in the Democratic Republic of the Congo. University of Leuven. Promotors Jacobs J /Verhaegen J. A15, A25, A32

T7. Van Nieuwenhove Lies, IMT Antwerpen, Belgium (2012). Identification of peptide mimotopes of *Trypanosoma brucei gambiense* variable surface glycoproteins. Doctoral thesis, University of Antwerp. Promotors Büscher P / Guissez Y. A30, A34, A35, A38

T8. Mumba Ngoyi Dieudonné, INRB, DR Congo (2010) Shortening of the treatment follow-up in gambiense human African trypanosomiasis. University of Antwerp. Promotors Büscher P / Muyembe JJ / Cras P. A3, A6, A12, A13, A16 A19, A23, A26, A28, A33, A39, A40, A44, A45, A46, A47, A48, A50, A51, A52, A53, A55, A57, S1

2.6.2 Master students

T9. Mwamba Miaka E (2016). Association entre le polymorphisme rs73885319 du gène APOL1 et la résistance/susceptibilité à l'infection à *Trypanosoma brucei gambiense* en République Démocratique du Congo. Master 2 Génétique épidémiologique et biomarqueurs. Université de Paris Sud.

T10. Achiambo VN. (2000) Development of an inhibition ELISA for antibody detection in *T.b. gambiense* sleeping sickness patients. MSc thesis. Free University Brussels.
T11. Baeten K. (2000) Identification of agglutinogens of *Leishmania donovani* in the DAT test. MSc thesis. Free University Brussels.
T12. Groeninckx B. (2000) Indirect plate agglutination for antibody detection in *Trypanosoma brucei gambiense* sleeping sickness. MSc thesis. Free University Brussels.
T13. Lardon J. (1999) Preparational methodological research into cytokine determination in sleeping sickness patients. MSc thesis. University of Antwerp. A71
T14. Mataa L. (1997) Experimental infection of *Bos indicus* with *Trypanosoma brucei brucei*: diagnostic aspects. MSc thesis. Tropical Animal Health, Institute of tropical Medicine, Antwerp.
T15. Moons, A. (1995) Serological research into rationalization of stage determination in sleeping sickness. MSc. thesis. Free University Brussels. Co-publication. A84

2.6.3 Other diploma students

T16. Maes, I. (2008) Risk factors for relapse after treatment for sleeping sickness. Graduate thesis, KAHO Sint-Lieven, Gent.
T17. Ceusters, A. (2007) Selection of peptides for diagnosis of sleeping sickness from phage display libraries. Graduate thesis, KAHO Sint-Lieven Gent.
T18. Wierckx, A. (2005) Antibody detection in saliva for diagnosis of sleeping sickness. Graduate thesis, KAHO Sint-Lieven Gent. A57
T19. Ameel, V. (2003) Development of a direct immunofluorescence test for diagnosis of African trypanosomiasis. Industrial Engineer Chemistry: Hogeschool West-Vlaanderen, Kortrijk.
T20. Balharbi, F. (2003) Diagnosis of sleeping sickness trough direct immunofluorescence. Graduate thesis, KAHO Sint-Lieven Gent. A38, A57
T21. De Winter, T. (2002) Isolation of trypanosomes from blood using immuno-magnetic particles. Graduate thesis, Hogeschool Vesalius Gent.
T22. Anné, N. (1996) Determination of IgM antibodies in cerebrospinal fluid of sleeping sickness patients. Graduate thesis, KAHO Sint-Lieven, Gent.

2.7 Teaching & training

Diplôme Interuniversitaire Capacité Médecine Tropicale et Méditerranéenne (DIU/CAPA).

Trypanosomiase Humaine Africaine. Réseau Francophone sur les Maladies Tropicales Négligées. Lejon V & Solano P. Montpellier (Institut Buisson Bertrand). 09/11/2017.

Maladies Tropicales Négligées et les Trypanosomoses. Lejon V & Solano P. Institut Buisson Bertrand, Montpellier. 08/12/2016.

Master in Tropical Neurosciences and Parasitology, University of Limoges

Module “Parasites du système nerveux”. Diagnostic trypanosomiase (travaux pratiques). Belgium, Antwerpen (ITM). 11-22/10/2010

Module “Parasites du système nerveux”. Biologie et epidémiologie Trypanosomiase. Diagnostic, clinique et traitement Trypanosomiase. Diagnostic trypanosomiase (travaux pratiques). Belgium, Antwerpen (ITM). 19-30/10/2009.

Module “Parasites du système nerveux”. Biologie, epidémiologie, diagnostic Trypanosomiase. Diagnostic trypanosomiase (travaux pratiques). Belgium, Antwerpen (ITM). 20-31/10/2008.

International Course on African Trypanosomes (ICAT) - WHO

7th International Course on African Trypanosomes - World Health Organisation Association against African Trypanosomes, Makerere University, IRD. Course co-organization (7-26/8/2017). Modules antigenic variation, serological and parasitological diagnosis of sleeping sickness, disease stage diagnosis, practical training on diagnosis of sleeping sickness (10-12/8/2017). Makerere University, Kampala, Uganda.

6th International Course on African Trypanosomes - World Health Organisation, Association against African Trypanosomes, INRB, IRD. Course co-organization (9-27/6/2014). Modules antigenic variation, serological and parasitological diagnosis of sleeping sickness, disease stage diagnosis, practical training on diagnosis of sleeping sickness. INRB, Kinshasa, 11-13/6/2014.

5th International Course on African Trypanosomes - World Health Organisation. Modules antigenic variation, serological and parasitological diagnosis of sleeping sickness, disease stage diagnosis, practical training on diagnosis of sleeping sickness. ICIPE, Nairobi, 13-14/10/2009.

4th International Course on African Trypanosomes - World Health Organisation. Modules antigenic variation, serological and parasitological diagnosis of sleeping sickness, disease stage diagnosis, practical training on diagnosis of sleeping sickness. Institut Pasteur, Tunis, 21-22/10/2005.

3rd International Course on African Trypanosomes - World Health Organisation. Modules disease stage diagnosis, Practical exercises. Institute of Tropical Medicine and Hygiene, Lisbon, 14-15/05/03.

2nd International Course on African Trypanosomes - World Health Organisation. Modules disease stage diagnosis, Practical exercises. WHO, Lyon, 20-21/11/2001.

1st International Course on African Trypanosomes - World Health Organisation. Modules disease stage diagnosis, Practical exercises. Le Pharo, Marseille, 1-2/11/2000.

Capacity building in HAT endemic countries (for WHO)

Training on HAT surveillance in Rwanda (WHO & MoH). Rwanda, Kayonza & Rwamagana Hospital 02-05/08/2016.

Human African Trypanosomiasis (HAT) capacity building in Zambia (WHO & MoH). Zambia, Mpika & Mambwe. 30/10-15/11/2015.

Human African Trypanosomiasis (HAT) capacity building in Zambia (WHO & MoH). Zambia, Mpika & Chipata 18/06-09/07/2009.

Control and surveillance of HAT in Serengeti National Park (United Republic of Tanzania). Tanzania, Mugugu district hospital. 24-30/04/2009.

WHO HAT Specimen Bank: Training in diagnosis of human African trypanosomiasis and biological specimen collection. Tanzania, Dar Es Salaam (WHO, NIMR), Tabora (NIMR-Tabora), Kaliua (Kaliua Health Centre). 12-23/01/08.

Capacity building for human African trypanosomiasis (HAT) case detection in Malawi. Malawi, Lilongwe, Nkotakota and Rumphi. 19/05-06/06/2007.

Prevention and control of HAT in 3 endemic foci in Nigeria (WHO & FMoH-Nigeria). Nigeria, Abuja, Delta State & Kaduna. 2/9-10/9/2006.

Capacity building for clinical trials

Training DiTECT-HAT-WP3. Burkina Faso, Bobo-Dioulasso (CIRDES). 16-17/03/2018.

Training DiTECT-HAT-WP2. Cote d'Ivoire, Bonon, Sinfra. 12-15/03/2018.

Training DiTECT-HAT-WP2. DR Congo, Kinshasa, Bandundu, Masamuna (PNLTHA). 14-21/07/2017.

Training DiTECT-HAT-WP2. DR Congo, Kinshasa (PNLTHA, INRB). 14-19/05/2017.

Training DiTECT-HAT-WP2. Cote d'Ivoire, Bouaké (Institut Pierre Richet). 13-17/03/2017.

Training DiTECT-HAT-WP2. Guinea, Conakry (PNLTHA). 20-24/03/2017.

Training DiTECT-HAT-WP4. RD Congo. Kinshasa & Masi Manimba (PNLTHA, INRB). 19-25/02/2017.

Formation sur le diagnostic de la trypanosomiase humaine africaine aux investigateurs DNDi-OXA-02-HAT. DR Congo, Kinshasa (DNDi, IRD, INRB). 22-28/05/2016

DNDi workshop on clinical development of fexinidazole. Teaching and practical training of medical personnel in diagnosis and stage determination of sleeping sickness. DR Congo, Kinshasa. 18-21/01/2012.

Development of DB289 Phase III – Investigators meeting. Practical exercises on diagnosis and disease staging of sleeping sickness. DR Congo, Kinshasa (CAP & INRB). 4-7/04/2005.

DB289 phase IIb trial for the treatment of stage I African trypanosomiasis. Teaching and practical training of medical personnel in diagnosis and stage determination of sleeping sickness. DR Congo, Vanga, Bandundu (Immtech International & Swiss Tropical Institute). 20-31/3/2003.

DB289 phase IIa trial for the treatment of stage I African trypanosomiasis. Teaching and practical training of medical personnel in diagnosis and stage determination of sleeping sickness. DR Congo, Maluku, Kinshasa (Immtech International & Swiss Tropical Institute). 2-23/2/2002.

DB289 phase IIa trial for the treatment of stage I African trypanosomiasis. Teaching and practical training of medical personnel in diagnosis and stage determination of sleeping sickness. Angola, Vianna (Immtech International & Swiss Tropical Institute). 21/8-11/9/2001.

Other training activities

Comment écrire un article scientifique? Cote d'Ivoire, Bouaké (Institut Pierre Richet). 18/03/2017.

Formation sur le diagnostic de la trypanosomiase humaine africaine, le paludisme et les filarioses. DR Congo, Kinshasa (PNLTHA, DNDi, IMT, IRD). 26/01-11/03/2016 (+- 120 hours).

Comment écrire un article scientifique? DR Congo, Kinshasa (INRB). 01/10/2015.

Buenas prácticas de laboratorio en estudios clínicos. Quality assessment and external quality assessment. Peru, Lima (Universidad Peruana Cayetano Heredia). 24-26/10/2012.

Good Clinical Laboratory Practice Workshop. Specimen labeling. Belgium, Antwerpen (Institute of Tropical Medicine). 27/2-2/03/2012

Implementation of Pulsed Field Gel Electrophoresis of *Salmonella*. Peru, Lima (Instituto de Medicina Tropical Alexander Von Humboldt). 20/22-2/12/2012.

3ième Rencontre Cercle de Réflexion de Techniques de Laboratoire (CRTL) . Les tests rapides pour le diagnostic de malaria, erreurs fréquentes (Mukadi Kaningu PP & Lejon V). Hygiène des mains (Lejon V & De Boeck H). DR Congo, Kinshasa. 27-29/10/2011.

1er atelier de formation des personnels de santé des foyers d'endémie d'Afrique Centrale aux techniques de diagnostic et de lutte contre la Trypanosomiase Humaine Africaine. Teaching and practical training in diagnosis and stage determination. Cameroun, Yaoundé (Organisation de Coordination pour la lutte contre les endémies en Afrique Centrale). 2-9/11/07.

Postgraduate course Tropical Medicine and International Health, Block Vector Borne Diseases: HAT basic concepts: staging, drugs, resistance, follow-up. Belgium, Antwerpen (Institute of Tropical Medicine). 5 & 16/10/2006.

Scientists@work - Flemish Institute for Biotechnology: organisation of sessions on sleeping sickness in 2010, 2009 and 2008. The 2009 group was selected as top-10 laureates of the event.

2.8 Research funding

2.8.1 Funding as coordinator/principal investigator

2016-2020 EDCTP2 (European Union, Horizon 2020), DRIA-2014-306. 2999006 €.
Diagnostic tools for human African trypanosomiasis elimination and clinical trials (DiTECT-HAT). [S1]
Coordinator: IRD. Partners: PNLTHA (RD Congo), INRB (RD Congo), IPR (Côte d'Ivoire), PNLTHA (Guinea), CIRDES (Burkina Faso), University of Liverpool (UK), ITM (Belgium). In collaboration with DNDi (Switzerland)

2014 Actions Thématiques Structurantes (ATS), IRD & AIRD. 4000€.
Organization « International Course on African Trypanosomiasis". Kinshasa 09-27/06/2014.
Coordinator : IRD. Partners : WHO (Switzerland), Association against Trypanosomes in Africa (France), INRB (DR Congo)

2014 Actions Incitatives IRD. 1300 €.
Mise en place du test trypanolyse pour le diagnostic de la THA en RD Congo.
Coordinator : IRD. Partners : ITM (Belgium), CIRDES (Burkina Faso), INRB (DR Congo)

2009-2011 Secondary Research Funding ITM, Spearhead Project Scheme SOFI-B. 350 000€.
Polyclonal B-cell activation in human African trypanosomiasis: impact on acquired immunity and on rapid diagnostic tests (HAT Poly-B). [A16, A23]
Coordinator: ITM. Partners: VIB (Belgium), PNLTHA (DR Congo), INRB (DR Congo)

2008-2009 ITM Interdepartmental Group "Neglected Diseases" (IDND). 75000€.
Aptamer selection for antigen detection in visceral leishmaniasis (VL-aptamer)
Coordinator: ITM. Partners: Darmstadt University of Technology (Germany)

2007-2008 Foundation for Innovative New Diagnostics. 58 625$.
Development of a single format test for IgM quantification in CSF of sleeping sickness patients (7772-HAT staging markers)
Coordinator: ITM. Partners: KIT (The Netherlands)

2006 WHO-TDR. 73262$.
Improved staging and post-treatment follow-up by IgM determination in field circumstances in Angola
Coordinator: ITM. Partners: ICCT (Angola)

2005 WHO/CDS HAT-net. 35000$.
Improved staging and post-treatment follow-up by IgM determination in field circumstances in Angola
Coordinator: ITM. Partners: ICCT (Angola)

2004 Fund for Scientific Research Flanders. Krediet aan navorsers 1.5062.04N. 20.000€.
Evaluation of saliva tests for diagnosis of human African trypanosomiasis and of leishmaniasis. [A57]
Coordinator: ITM. Partners: Koirala Institute of Health Sciences (Nepal), Institut Pasteur (Tunisia), PNLTHA (DR Congo), INRB (DR Congo)

2003 Fund for Scientific Research Flanders, Organisation of a scientific meeting. 3500€.
Organization: International Colloquium on Cerebrospinal Fluid Analysis in Tropical Neurology. [A58]
Coordinator: ITM. Partners: University of Göttingen (Germany)

2.8.2 Funding as partner

2018-2023 EDCTP2 (European Union, Horizon 2020), RIA2017NCT-1846 — HAT-R-AC. 3769845 €.
Towards an arsenic-free oral treatment for human African trypanosomiasis due to *Tb rhodesiense* as a tool for disease elimination (HAT-R-ACC).
WP2 leader (To ensure proper execution of the clinical trial through strengthening capacity of treatment and care)
Coordinator: DNDi. Partners: IRD (France), Ministry of Health (Uganda), Epicentre (France), IHMT (Portugal), Ministry of Health (Malawi), Makerere University Uganda, Swiss TPH (Switzerland)

2014-2015 WHO-NTD APW 2014/432972-0. 380000 US$.
Innovative approach in the case detection and management for sustainable elimination of gambiense HAT. [A9]
Coordinator : IRD/CIRAD. Partners: CIRDES (Burkina Faso), PNLTHA (Guinea), PNLTHA (RD Congo)

2012-2017 Wellcome Trust, H3Africa. 3012463 £.
TrypanoGEN: An integrated approach to the identification of genetic determinants of susceptibility to trypanosomiasis. [A2, A3]
Coordinator: Makerere University (E Matovu). Partners: University of Glasgow (UK), IRD (France), ITM (Belgium), University of Liverpool (UK), University of Zambia (Zambia), CIRDES (Burkina Faso), University of Dschang (Cameroon), INRB (DR Congo), University of Malawi (Malawi), The University of Abobo-Adjame (Côte d'Ivoire)

2010-2015 EU, FP-7 theme Health 2010.2.3.4-2. 5000000€.
Syndromic approach to neglected infectious diseases (NID) at primary health care level: an international collaboration on integrated diagnosis-treatment platforms (NIDIAG). WP6 leader (Quality assurance). [A4, A5, A12, A24, A27]
Coordinator: ITM (M Boelaert). Partners: Inserm-Transfert (France), Université de Genève (Switzerland), London School of Hygiene & Tropical Medicine (UK), INRB (DR Congo), Gadjah Mada University (Indonesia), INSP (Mali), Swiss TPH (Switzerland), Koirala Institute of Health Sciences (Nepal), University of Khartoum (Sudan), Sihanouk Hospital Corporation (US), Coris Bioconcept (Belgium)

2004-2007 Belgian Ministry of Foreign Affairs, Directorate General for Development Co-operation
Etude prospective sur le raccourcissement du suivi des patients traités pour la trypanosomiase humaine africaine (THARSAT). [A8, A26, A39, A46, A47, A48, A52]
Coordinator: ITM (Belgium). Partners: INRB (DR Congo), PNLTHA (DR Congo)

2.9 Patent application

Van Nieuwenhove L, **Lejon V**, Büscher P. Diagnosis of *Trypanosoma brucei*. International Patent application PCT/EP2013/052962 filed on 14/02/2013 claiming priority of GB1202460.0 filed on 14/02/2012, in the name of Institute of Tropical Medicine: the use of a combination of *Trypanosoma brucei* variant surface glycoproteins-derived mimotope peptides. [A30, A34, A35, A38]

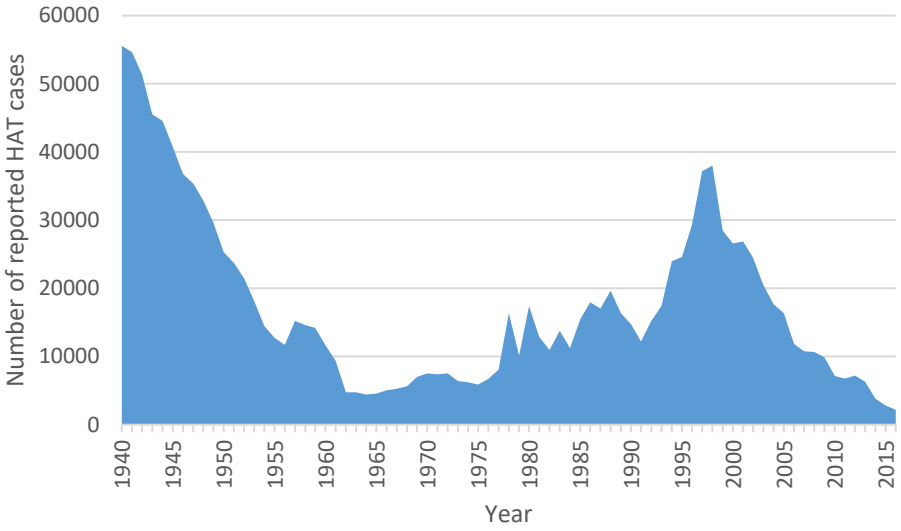
3. Research context

References to my own work are formatted as [An°] in the order of chapter 2.

3.1 Setting the scene

I started my research career in 1994 at the laboratory of Parasite Diagnostics (formerly Serology) at the Institute of Tropical Medicine (ITM) in Antwerpen, Belgium. At the wake of a new sleeping sickness epidemic (Figure 1), a number of specialists had already sounded the alarm on the rising prevalences [1,2]. Due to political and social instability, and for economic reasons, control of human African trypanosomiasis (HAT) had been ignored.

Figure 1: Reported number of sleeping sickness cases in function of time (1940-2016).



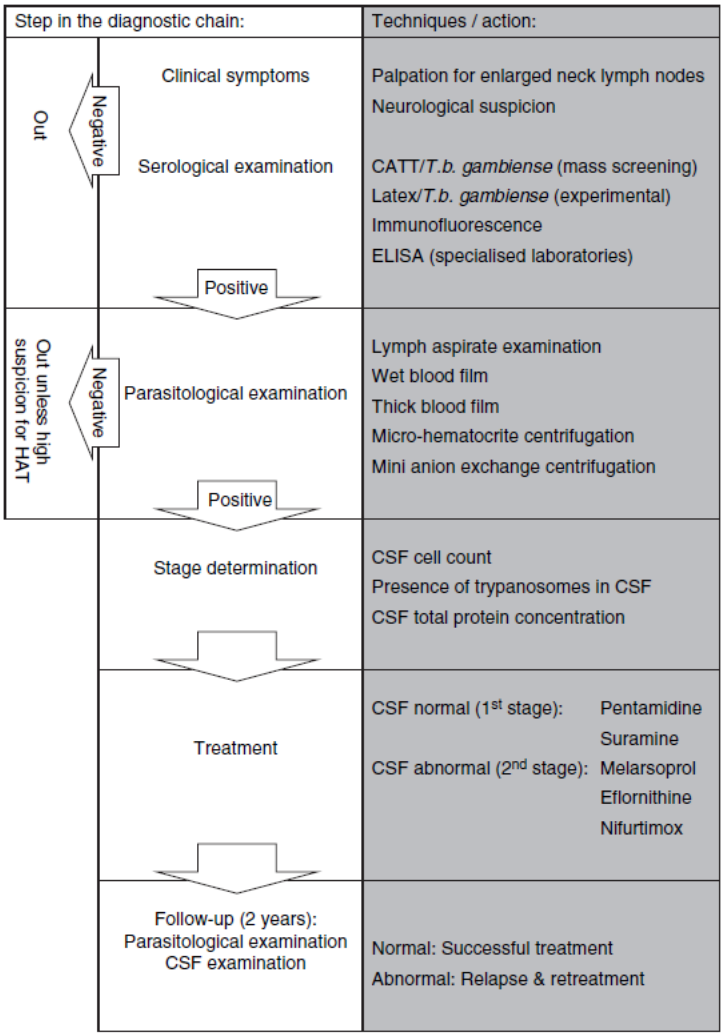
As it is still the case, control of HAT relied mainly on diagnosis and treatment.

For diagnosis of HAT provoked by *Trypanosoma brucei* (*T.b.*) *gambiense*, serological screening using CATT/*T.b. gambiense* was being progressively introduced in the field. CATT/*T.b. gambiense*, the predecessor of the rapid diagnostic test (RDT), had been developed at the Serology lab at ITM [3], which conducted research on diagnosis of HAT. It allowed mobile teams to screen considerably more people, and limit parasitological examinations to CATT positives (Figure 2). Treatment options for HAT in 1994 were limited [4]. First line treatment for first stage (cerebrospinal fluid (CSF) white blood cell count (WBC) ≤5/µl and no trypanosomes in CSF) was pentamidine for gambiense HAT, suramin for rhodesiense HAT. Second stage HAT (CSF WBC >5/µl or trypanosomes in CSF) was treated exclusively with melarsoprol, feared for provoking reactive encephalopathy in 5-10% of *T.b. gambiense* patients, and fatal for 10-50% of these cases [5]. Clinical trials with eflornithine had been promising [6-8], but the drug was not available for routine and very soon its production would be discontinued. Nifurtimox had only been used to a very limited extent for compassionate treatment of melarsoprol refractive infections [9,10].

3.2 Summary of activities

The majority of my research has focused on different aspects of diagnosis of gambiense HAT, summarized in Figure 2.

Figure 2: A general flowchart for diagnosis, stage determination, treatment and follow-up of sleeping sickness patients in 2005. [A58]



In the context of choosing between pentamidine and melarsoprol for treatment of gambiense HAT, improved markers for diagnosis of neurological involvement were considered a research priority in 1994, when I was recruited at ITM. **Stage determination** therefore became the topic of my PhD and part of my post-doctoral research.

To assess treatment outcome, patients had to be followed-up for 2 years after treatment: at 3 and 6 months, and at 6 monthly intervals thereafter [5]. Besides evaluating the clinical condition, the blood and CSF should be examined for presence of trypanosomes. The CSF WBC number and protein concentration

had to be determined, but no clear guidelines existed for interpretation of the results in the absence of trypanosomes. Out of fear for the repeated lumbar punctures, many patients did not return to the hospital for check ups. Furthermore, around 2000, several reports on high relapse rates after melarsoprol treatment started to emerge [11-14]. Taking into account that relapses after treatment are usually neurological, markers for neurological involvement in HAT are good candidates for assessing treatment outcome. The venue of Dieudonné Mumba as a PhD[T8], and the set up of a diagnostic trial in Mbuji-Mayi (RD Congo), a gambiense HAT focus with a high melarsoprol treatment failure rate, allowed to initiate research on improved and shortened **follow-up after treatment**.

Although CATT/*T.b. gambiense* played a crucial role in control of the last HAT epidemic, its format is not optimal. Disadvantages include: 1° suited for mass screening but not for individual testing; 2° invasive (require blood sample); 3° limited sensitivity and specificity. Serological testing can be improved by improving the antigen, and/or changing the test format. Working in a research laboratory specialized in serodiagnosis of HAT, I became involved in **development and evaluation of diagnostic tests**.

Research on improved antigens for serodiagnosis included discovery of peptide mimotopes of *T.b. gambiense* variant surface glycoprotein for serodiagnosis of HAT, within the framework of the PhD of Lies van Nieuwenhove [T7].

My contributions to improve test formats include 1° the adaptation of CATT, LATEX, ELISA and trypanolysis for testing of dried blood on filter paper, allowing these tests to be applicable for surveillance [A14, A45,A69]; 2° Adaptation of the CATT format for use in peripheral health centres [A49]; 3° The use of saliva for non-invasive antibody detection [A66, A57]; 4° Evaluation of rapid diagnostic tests (RDT) [A9, A12, A24, A27].

Based on my experience in development of ELISA and agglutination tests, I also developed tests for animal trypanosomiasis (Surra and nagana [A11, A59, A64, A70, A85]) and contributed to the design of an rK39 based ELISA for antibody detection in visceral leishmaniasis [A41, A42]. This work is not discussed in this document.

Tests for parasite detection suffer from limited sensitivity. I have contributed to increased sensitivity by enhanced visualization of the parasite, for example by fluorescent labeling of microscopic slides [C66], or by examining larger volumes of body fluids [A50, A80] in combination with a preceding concentration step.

After a promising publication on proteomic fingerprinting for HAT diagnosis using surface-enhanced laser desorption-ionisation time-of-flight mass spectrometry (SELDI-ToF) [15], I was invited in 2005 at McGill University (Montreal, Canada) to perform SELDI-ToF for discovery of diagnostic disease markers for HAT [C95]. This research was soon abandoned due to the low diagnostic performance of the discovered markers (inferior to antibody detection) and the failure of the technique to identify disease specific markers (up-regulation of similar peaks in other infections).

I was also involved in evaluation of the diagnostic performance of parasitological and molecular techniques for diagnosis of HAT [A13, A19].

With the HAT-polyB project, a new research line was initiated in 2010. Between 2008 and 2010, experimental models in mice had shown that *T. brucei* infections give rise to memory B cell destruction with a potential negative impact on persistence of vaccine induced responses and protection [16,17]. We wanted to know if *T.b. gambiense* infection in humans also destructs B-cell memory and abrogates vaccine induced protection and studied 1° the protection level against measles; 2° the numbers of circulating memory B- and T-cells; and 3° the T-cell independent B cell activity [A16]. Within the framework of this project and polyclonal B-cell stimulation, we also examined false positivity of HAT patients in malaria RDTs [A23], after

having discovered by coincidende the low specifity of HIV tests performed on HAT patients from the Mbuji-May cohort [A46].

In 2011, I joined the Unit of Tropical Laboratory Medicine of ITM. The research on *Salmonella* conducted with Octavie Lunguya (T6) had nothing in common with my activities in HAT and is not further discussed in this document. However, I learned that I was able to integrate and publish in a completely new field of research quickly [A15, A17, A25]. The link between quality assessment of malaria diagnosis and HAT was easy to make [A22, A21, A20, A6], by including trypanosomes in the microscopy slides for external quality assessment (PhD Pierre Mukadi [T5]). Experiences in evaluation of malaria RDTs [A23] were translated to HAT RDTs [A9]. At the same time I was responsible for the work package on quality management of the European FP-7 project "NiDiag" [A4, A5, A24].

3.3 Ongoing and future research

In 2013, I was recruited by the Institut de Recherche pour le Développement (IRD), which allowed me to fully focus on HAT again. By that time, the sleeping sickness epidemic had been controlled and HAT had been targetted for elimination by WHO [18]. This new epidemiological situation poses some **diagnostic challenges**, which are dealt with in the **EDCTP project "DiTECT-HAT"** of which I am coordinator [S1, T1, T2, T3, T4]. Furthermore, questions emerge about the impact of potential animal reservoirs for *T.b. gambiense* [A1] and latent infections [A2, A3, A7, A43, A56, A79, TrypanoGEN project]. Similarities in latent infections and the diagnostic situation, open perspectives for performing (diagnostic) research on the other *Trypanosomatidae* [S2] -animal trypanosomosis, the leishmaniasis and Chagas diseases-, which is further facilitated by working in the Intertryp unit.

4. Stage determination

Accurate determination of the disease stage is an essential step to select an adapted treatment with minimal risk for the patient, in particular when toxic drugs such as melarsoprol are used to treat second stage HAT. Stage determination is performed by examination of the CSF (Table 1). Although in practice rarely applied, the total protein concentration in CSF was also recommended by WHO for staging [5].

Table 1: Criteria for disease stage determination in HAT

	CSF WBC ≤ 5/µl	CSF WBC >5/µl
No trypanosomes in CSF	1st stage (early/ haemato-lymphatic)	2nd stage (late, meningo-encephalitic)
Trypanosomes in CSF	2nd stage (late, meningo-encephalitic)	2nd stage (late, meningo-encephalitic)

In order to identify better parameters for stage determination gambiense HAT, I investigated the neuro-inflammatory response. Both the **humoral and cellular immune response** in the central nervous system were studied, although the former more thoroughly. Subsequently, I developed a field-adapted staging test, **LATEX/IgM**. Finally, the relationship between **treatment outcome** and some of the disease stage markers was assessed.

In 2007, research on staging of HAT was taken up by the **University of Geneva**, with the study in CSF of different chemokines, adhesion molecules, proteomics and neopterin [A10, A29, A33, A40, A44, A51].

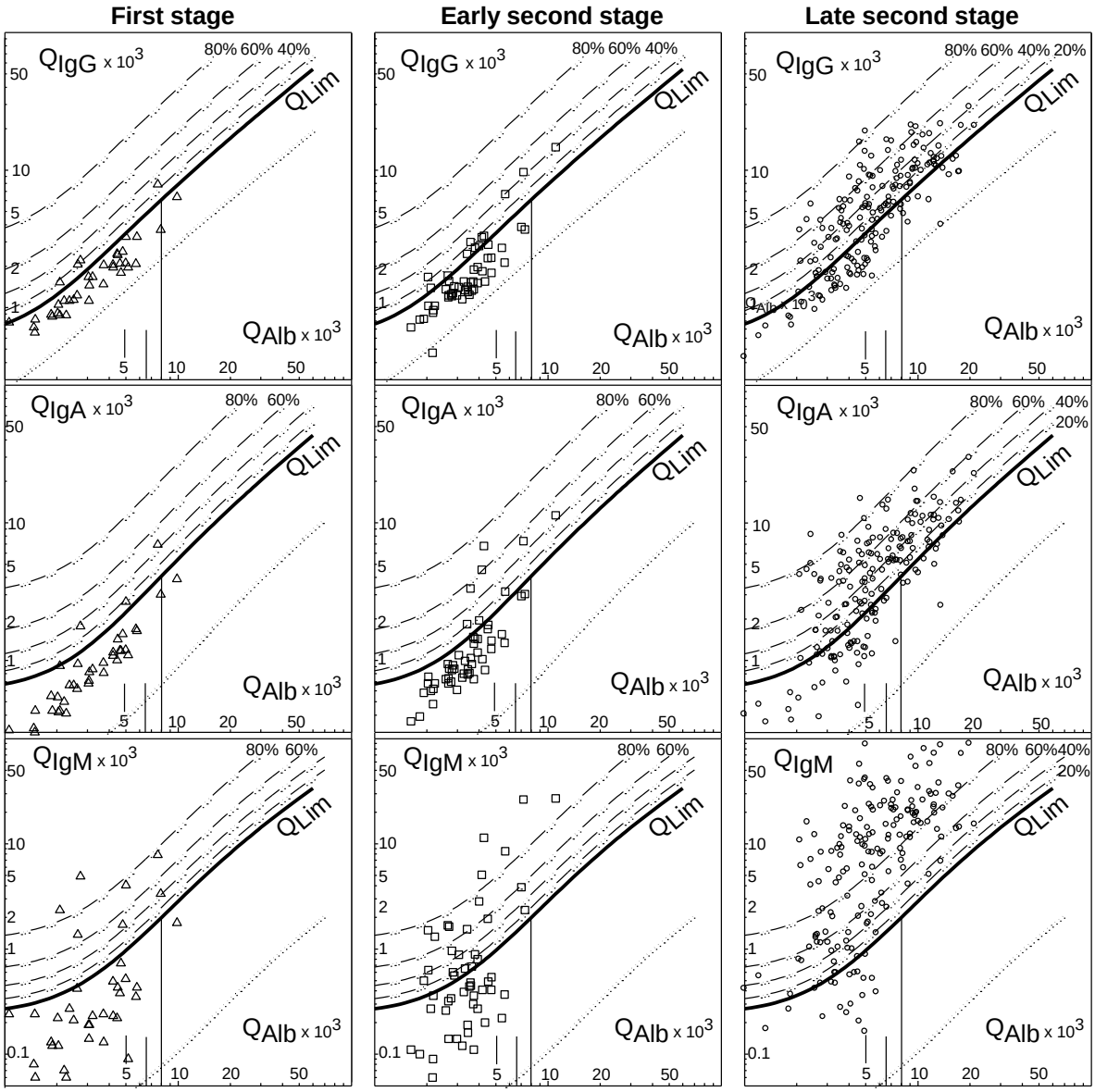
4.1 Humoral neuro-immune response in HAT

As a first step, I developed a semi-quantitative ELISA for the detection of antibodies of different immunoglobulin (Ig) isotypes in serum and CSF of *T.b. gambiense* sleeping sickness patients. The antibody profiles of paired serum and CSF samples were studied and total concentrations of various Ig isotypes were determined [A84]. In serum and CSF, a drastic increase in IgG, basically IgG1, as well as in IgM levels was observed. The concentration of IgA remained relatively normal. The anti-trypanosomal antibodies detected in serum and CSF were mainly of the IgG (IgG1 and IgG3) and IgM isotypes.

Measurement of immunoglobulin, albumin and trypanosome specific antibody concentrations in serum and CSF allows quantification of the humoral immune response in the brain [A67, A74].

The intrathecal immunoglobulin response can be quantified using Reibers hyperbolic discrimination line $Q_{Lim} = (a/b) \times (Q_{Alb}^2 + b^2)^{1/2} - c$ (Figure 3) with different values for a, b and c for IgG, IgA and IgM. Q_{Alb} is the CSF/serum quotient of the albumin concentration, characterizing blood-CSF barrier function [19]. Q_{Lim} describes the normal relationship between Q_{Alb} and the immunoglobulin CSF/serum quotient Q_{Ig}. If the CSF/serum quotient Q_{Ig} of IgG, IgA or IgM concentration is higher than their respective Q_{Lim} (or above the bold reference line in Figure 3), intrathecal synthesis of immunoglobin is present and the intrathecal fraction (IF) can be calculated. Contrary to the peripheral immune response which changes from IgM to IgG in function of time, the obtained intrathecal immunoglobulin pattern is disease dependent. The intrathecal trypanosome specific antibody response is calculated through the antibody index $AI = Q_{sp}/Q_{Ig}$ when $Q_{Ig} < Q_{Lim}$, or $AI = Q_{sp}/Q_{Lim}$ when $Q_{Ig} > Q_{Lim}$ [20], with Q_{sp} being the CSF/serum quotient of trypanosome specific antibody concentrations. The AI is considered pathological if $AI \geq 1.5$.

Figure 3: Quotient diagrams for IgG, IgA and IgM [19] with data from 272 *T.b. gambiense* patients in first stage (left diagrams, n=38), early second stage (WBC of 6-20/µl, no trypanosomes in CSF, middle diagrams, n=53) and late second stage (WBC of >20/µl, or trypanosomes in CSF, right diagrams, n=181). The reference ranges of blood derived IgG, IgA and IgM fractions in CSF are between the upper discrimination line (Q_{Lim}, bold line) and lower discrimination line (dotted line). The upper hyperbolic line, Q_{Lim} represents the discrimination line between brain derived and blood derived immunoglobulin fractions in function of increasing Q_{Alb}. Values above Q_{Lim} indicate intrathecal fractions, which can be read from the diagrams (dashed lines for 20, 40, 60, 80% intrathecal synthesis). The line Q_{Lim} represents 0% synthesis. The age dependent vertical lines indicate the upper limit of the age-related normal blood-CSF barrier function Q_{Alb}=5, 6.5 and 8 x 10^{-3} for ages up to 15, 40 and 60 years respectively [A67].



Blood-CSF barrier dysfunction in sleeping sickness patients is infrequent and late, and when present, moderate. In addition, as immunoglobulin levels in serum and consequently also in CSF, are high in all stages of the disease, determination of the CSF total protein concentration is irrelevant for stage determination.

Neurological stage gambiense HAT is characterized by a two to three class immunoglobulin response (Figure 3), with predominant IgM class response. The predominant intrathecal IgM response, expressed as intrathecal fraction IF_{IgM}, exceeds the IgG and IgA response in frequency and strength:

- The frequency of intrathecal IgM synthesis is higher than that of other immunoglobulins. For example, for the late second stage group, (right column in Figure 3), the frequencies are respectively 87%, 65% and 60% for IgM, IgA and IgG synthesis (98%, 80% and 73% for those with WBC>20/µl)
- The IgM class response is predominant: intrathecal IgG or IgA synthesis does not occur without IgM synthesis and the intrathecal fraction of IgM is higher than of IgA or IgG ($IF_{IgM} > IF_{IgA}$ or IF_{IgG}).

The intrathecal humoral immune response of gambiense HAT patients, assessed as intrathecal immunoglobulin synthesis and specific Antibody Index, starts early and can already occur during the first stage (respectively 31 and 18% in 2 separate studies [A74, A67]). Such "misclassified" first stage patients subsequently may receive an inadequate pentamidine treatment. Pentamidine relapse rates are between 7% and 16%, and are mainly attributed to wrong staging [21]. However, for both studies [A74, A67], the treatment outcome of the patients was not known.

Besides quantitatively, using Reibers' formulae and the antibody index, the total and specific intrathecal immune response can be detected qualitatively, using iso-electric focusing in agarose gels followed by blotting and detection of total or specific oligoclonal Ig (OC_{Ig} or OC_{Igsp}). We compared quantitative (IF and AI) and qualitative techniques (OC_{Ig} and OC_{Igsp}) [A62]. The sensitivity for detection of an intrathecal humoral immune response was $OC_{IgGsp} > IF_{IgM} > AI_{IgMsp} > AI_{IgGsp} > OC_{IgG} > IF_{IgG}$. Detection of oligoclonal IgM and trypanosome specific oligoclonal IgM failed. The sensitivity of both IF_{IgG} and OC_{IgG} for detection of neuro-inflammation in sleeping sickness patients was limited. Trypanosome specific antibody synthesis, assessed by AI_{IgGsp}, was confirmed by OC_{IgGsp} detection, but the latter method was more sensitive. Although oligoclonal IgM detection failed, we confirmed again by IF_{IgM} and AI_{IgMsp} that the meningo-encephalitic stage of *T.b. gambiense* human African trypanosomiasis is characterised by a dominant intrathecal IgM immune response.

So all of our findings point to a strong intrathecal IgM synthesis in the central nervous system and stressed the interest of IgM detection in CSF as a marker for stage determination in gambiense HAT.

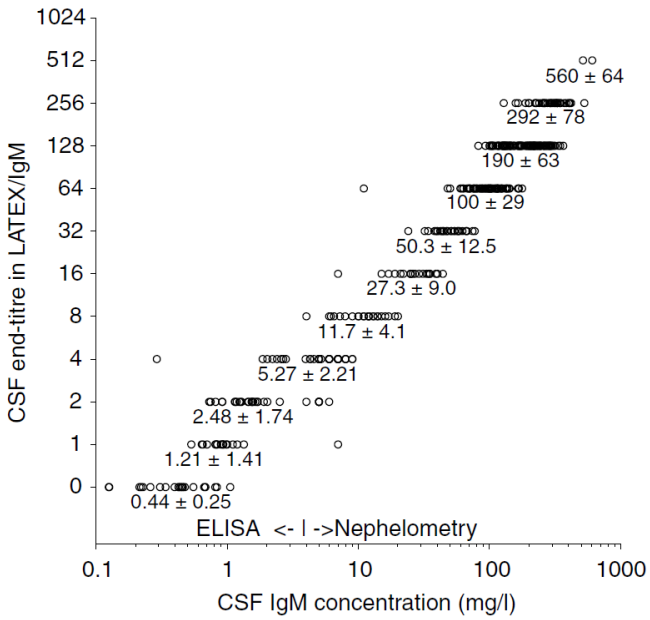
4.2 LATEX/IgM, a field test for stage determination in HAT

Long before the start of my research, it was known that high CSF IgM concentrations are a common feature of human African trypanosomiasis in the meningo-encephalitic stage but not in first stage [22,23] and that concentrations could be as high as 500 mg/l [24,25], compared to a normal upper limit of about 0.4 mg/l [26]. These extremely elevated CSF IgM concentrations, the intrathecal origin of IgM and the interest of IgM detection in CSF of sleeping sickness patients for stage determination were confirmed in our studies [A67, A74]. However, in spite of its relevance, IgM detection in CSF was not carried out in practice for stage determination in HAT. This is explained by the lack of simple and robust tests that are applicable in African rural regions where the disease prevails.

Two simple commercial tests, radial immunodiffusion and Rapi Tex IgM, (Behring, Germany), a particle agglutination test originally developed for detection of IgM in the serum of neonates, were evaluated for IgM quantification in CSF samples [A83]. Implementation of radial immunodiffusion for stage determination was impractical: 1° the detection limit of 3.5 mg/l was too high; 2° the incubation time (72 hours) was too long and; 3° the faint precipitation rings were difficult to measure. However, also Rapi Tex IgM was considered unsuited for staging in HAT as 1° the detection limit of around 20 mg/l IgM was too high, and 2° the reagent had to be stored at 4°C and had a relatively short shelf live. Based on the technology of latex agglutination tests for antibody detection in trypanosomiasis [27, A81], a card latex agglutination test for IgM detection, LATEX/IgM, was developed and evaluated. The newly developed LATEX/IgM had a detection limit lower than 5 mg/l and the freeze-dried reagent was stable for at least 1 year, even at 45°C. However, the use of different batches of polyclonal anti-IgM antibodies for coupling onto latex beads caused batch-to-batch reactivity variations.

In order to eliminate batch-to-batch variation, the polyclonal anti-IgM was replaced by monoclonal antibodies. The resulting LATEX/IgM test [A72] was simple and fast and the lyophilised reagent remained stable at 45°C. The CSF end titre or highest dilution still causing an agglutination reaction in LATEX/IgM, corresponded well with the total IgM concentration in the sample and the detection limit was around 1mg/l (Figure 4).

Figure 4: End titre in LATEX/IgM of 435 CSF samples in function of the CSF IgM concentration measured by nephelometry (concentrations > 4mg/l) and ELISA (concentrations < 4mg/l). For each end titre, the mean IgM concentration ± standard deviation is indicated [A72].



At a cut-off ≥8, LATEX/IgM end titres showed 92.7% specificity and 89.4% sensitivity for intrathecal IgM synthesis, reflecting CNS pathology. Using a cut-off value of ≥8, the number of positive and negative samples in a total of 937 *T.b. gambiense* patients were determined (Table 2). Among these, respectively 342 and 27 CSF samples had been analysed with LATEX/IgM in sleeping sickness control programs in Southern Sudan and Central African Republic, showing the practical feasibility of the test.

Table 2: Number of positive and negative samples in 4 groups of *T.b. gambiense* patients using a cut-off LATEX/IgM CSF end titres of ≥ 8 [A72]

LATEX/IgM end titre	0-5 cells/µl, no trypanosomes (n=191)	6-20 cells/µl, no trypanosomes (n=198)	≤20 cells/µl, trypanosomes (n= 51)	>20 cells/µl (n=497)
LATEX/IgM <8	169 (88.5%)	133 (67.2%)	31 (60.8%)	34 (6.8%)
LATEX/IgM ≥8	22 (11.5%)	65 (32.8%)	20 (39.2%)	463 (93.2%)

Of the late 2nd stage samples, 88% (483/548) were positive in LATEX/IgM (93% with WBC>20, and 39% with WBC –20 and trypanosomes in CSF). Among the first stage patients, 11.5% were positive at a cut-off ≥ 8 in LATEX/IgM. Such patients should be considered at risk for relapse when treated with pentamidine, but their outcome was not known to us.

Although applicable in the field, a major constraint for LATEX/IgM remained its stability. Once resuspended, LATEX/IgM had to be stored frozen. This problem can be solved by spotting and drying the reagent onto the agglutination card (dri-dots) which are wrapped individually and can be stored at ambient temperature. The technology had previously been applied by the Royal Tropical Institute (KIT) of Amsterdam on latex card agglutination tests for human leptospirosis, brucellosis and typhoid fever [28-30]. In collaboration with KIT, we therefore aimed to transform LATEX/IgM test into a dri-dot test (FIND funded project [C90]). Optimal conditions for producing and executing LATEX/IgM dri-dots were established. The reactivities of the developed LATEX/IgM dri-dot test and the standard LATEX/IgM test were comparable, and the end-titres reflected the IgM concentration of the CSF sample. The LATEX/IgM dri-dot test was stable for at least 6 months if stored at 4°C or ambient temperature. However, at higher temperatures, stability became borderline.

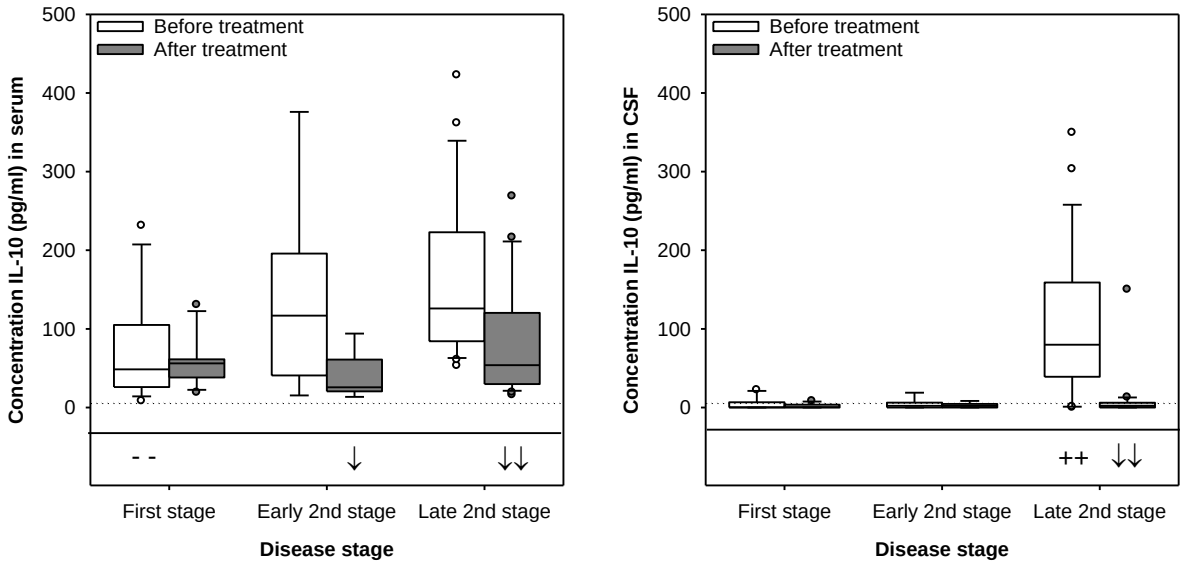
4.3 Cellular neuro-immune response in HAT

In experimental trypanosomiasis animal models, meningo-encephalitis is accompanied by activation of microglia and astrocytes and by infiltration and activation of plasma cells, Mott cells and T-cells [31-33]. Cytokine and chemokine production in the CNS, including IL-1, IL-6, TNF-α, IFN-γ, and macrophage inflammatory protein-1 and -2 are early events. Post-mortem histological examinations of the brain of HAT patients revealed microglial and astrocytal activation in the periventricular areas, white matter and basal ganglia. Tissue necrosis, mild demyelination and neurodegeneration have been occasionally reported [34-36]. Little data on cytokines in meningo-encephalitic stage sleeping sickness patients, in particular in CSF, were available. Normal IL-1α and increased prostaglandin levels had been detected in the CSF of *T.b. gambiense* infected patients [37]. In *T.b. rhodesiense* infections, elevated plasma IFN-γ had been observed which remained high after treatment, but IFN-γ could not be detected in the CSF [38].

Glial fibrillary acidic protein (GFAp) and light subunit neurofilament are markers of astrogliosis and neuronal degeneration and can be detected in the CSF by ELISA. Increased GFAp and neurofilament concentrations had been observed in other neuro-infectious diseases such as Lyme neuroborreliosis, bacterial meningitis and viral encephalitis [39-41]. We performed two studies on GFAp and neurofilament in gambiense HAT patients [A78, A82]. In the first study [A82], increased GFAP levels in CSF confirmed the astrogliosis observed in HAT and experimental models of HAT, while abnormal neurofilament CSF levels indicated neurodegeneration in one third of the second stage patients studied. Next [A78], the relationship between CSF GFAp and neurofilament levels and the presence of trypanosomes in the lymph node aspirate, presence of trypanosomes in CSF, CSF WBC, CSF total protein concentration, and some clinical signs was examined. Both astrogliosis and neurodegeneration correlated better to increased CSF WBC count and total protein concentration than to the clinical symptoms. As they were mainly abnormal in patients with very high WBC counts and total protein concentrations in CSF, GFAp and neurofilament in CSF were however not retained as staging markers.

In order to explore the alterations in cytokines during human *T.b. gambiense* trypanosomiasis infection, we measured the IL-6, IL-8, IL-10, TNF-α and IFN-γ concentrations in paired serum and CSF samples of 46 *T.b. gambiense* HAT patients, before and immediately after treatment [A71]. TNF-α and IFN-γ were increased only sporadically. In serum, high concentrations of IL-6, IL-8 and IL-10 were observed in all patients. Compared to first stage patients, higher serum IL-10 concentrations were detected in second stage patients, which decreased after treatment. In CSF, elevated concentrations of IL-6, IL-8 and especially of IL-10 were observed in late 2nd stage *T.b. gambiense* patients (WBC>20/µl), which fell below the detection limit immediately after treatment (Figure 5). Due to its highly elevated levels in CSF of late 2nd stage patients compared to first stage patients, CSF IL-10 was retained as an interesting parameter for stage determination.

Figure 5: Box and whisker plot of IL-10 concentrations in serum (left) and CSF (right) of 46 *T.b. gambiense* patients before and after treatment, grouped according to disease stage. …..: detection limit of ELISA. -- and ++: Significantly (p<0.05) lower and higher IL-10 concentration in this patient group than in other groups. ↓ and ↓↓: Significant (p<0.1 and p<0.05) decrease in IL-10 concentration after treatment [A71].



4.4 Intrathecal immune response and treatment outcome

From our previous studies, it was clear that an intrathecal immune response could occur in first stage patients, and that a large fraction of the patients with 6-20 WBC/µl, irrespective of presence of trypanosomes, had no intrathecal immune response. Successful pentamidine treatment of second stage patients with up to 20 cells/µl had been reported [4,42].

We were able to study the relationship between the intrathecal immune response and treatment failures on 2 gambiense HAT patient groups [A63, A55]. For both studies, we hypothesised that unsuccessful treatment with either pentamidine or suramin was related to uncured central nervous system infection.

A first study was performed on gambiense HAT patients with WBC ≤20/µl, irrespective of the presence of trypanosomes in the CSF, who participated to an equivalence trial organised by Médecins sans Frontières and Epicentre in Uganda, comparing the efficacy of pentamidine with melarsoprol [A63]. In this study, respectively 21/49 and 16/49 pentamidine and melarsoprol treated patients relapsed. We were able to demonstrate that CSF cell counts of 11-20 cells/µl, presence of intrathecal IgM synthesis, CSF end titres in LATEX/IgM ≥4, ≥8 and ≥16 or LATEX/*T.b. gambiense* positive CSF were associated with increased risk of pentamidine treatment failure among second stage patients with ≤20 cells/µl (Table 3). Such relationship could not be identified in the melarsoprol treated patient group. This confirmed that those parameters may be useful for assessment of central nervous system involvement, which is incurable by pentamidine.

Table 3: Proportion of treatment failures in function of CSF cell count, presence of trypanosomes, total protein, blood CSF-barrier function, intrathecal IgG and IgM synthesis, the antibody index for IgG (AI_{IgG}), LATEX/IgM at different cut-off end titres, and LATEX/*T.b. gambiense*. * Significant difference in proportion of treatment failures. OR: odds ratio, CI: 95%confidence interval [A63].

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

The second study was carried out on first stage gambiense HAT patients (WBC ≤5/µl, no trypanosomes in CSF) who were enrolled during routine screening and treatment in Bwamanda (Equateur, DR Congo) [A55]. Due to a shortage in the supply of pentamidine these had been treated with suramin, and 14 out of 60 (23%) patients relapsed. Again, presence of intrathecal IgM synthesis, elevated CSF IgM and elevated CSF IL-10 concentrations were associated with treatment failure (Table 4). In contrast with our findings in early second stage patients [A63], no association between trypanosome specific antibodies in CSF and treatment failure was observed. Absence of any detectable relationship between the total CSF protein concentration and relapse, and the limited impact of CSF total protein determination for stage determination, was confirmed [A67,A63]. The irrelevance of clinical signs and symptoms for staging of human African trypanosomiasis was corroborated [43].

Taken together, the interest of intrathecal IgM synthesis, CSF IgM and IL-10 as markers for stage determination was demonstrated again in these studies [A63,A55].

Table 4: Number of cured and relapsed patients after suramin treatment in function of pre-treatment test results, the *p* value (calculated by Fisher exact test), odds ratio (OR) with confidence interval (95% CI) for association of test result with occurrence of relapse, area under the curve (AUC) and negative and positive predictive values (NPV, PPV). CSF cerebrospinal fluid, IL interleukin [A55].

	Cured	Relapsed	*P* OR (CI)	AUC	NPV PPV
Intrathecal IgM synthesis (n=57)			0.000002	0.86	
Absent	41	3	46 (8.0-260)		93%
Present (IgM_{IF}>0%)	3	10			77%
CSF IgM concentration (n=60)			0.0004	0.81	
<1.9 mg/l	35	3	11.7 (2.7-50)		92%
≥1.9 mg/l	11	11			50%
CSF LATEX/IgM end titer (n=60)			0.001	0.81	
<2	34	3	10.4 (2.5-44)		92%
≥2	12	11			48%
CSF LATEX/IgM end titer (n=60)			0.024	0.81	
<4	40	8	5.0 (1.3-20)		83%
≥4	6	6			50%
CSF LATEX/IgM end titer (n=60)			0.014	0.81	
<8	41	8	6.2 (1.5-25)		84%
≥8	5	6			55%
CSF IL-10 (n=60)			0.024	0.70	
≤10 pg/ml	40	8	5.0 (1.3-20)		83%
>10 pg/ml	6	6			50%

4.5 Collaboration with the University of Geneva

After the above studies, I concentrated mainly on treatment outcome assessment (chapter 5), but collaborated for staging research with the Biomedical Proteomics Group at the University of Geneva. The approach applied by the University of Geneva was double. On the one hand discovery research was carried out on CSF using proteomics, the speciality of the Biomedical Proteomics Group. On the other hand, known markers for neuro-inflammation, brain dammage and cell adhesion were tested for stage determination in HAT. The idea of proteomics research for stage determination was not entirely new. Already in 1995, two-dimensional gel electrophoresis of CSF of *T.b.gambiense* patients had been carried out in collaboration with the University of Antwerp, but we stopped this research due to lack of funding. In 2007, proteomic techniques had improved, and the University of Geneva was better equipped and more experienced.

Using two-dimensional gel electrophoresis and sixplex tandem mass tag isobaric labelling quantitative mass spectrometry, 73 proteins were found overexpressed in 2nd stage gambiense HAT patients [A44]. Two of these proteins, osteopontin and β-2-microglobulin, were confirmed as potential staging markers by western blot and ELISA.

In parallel, heart-fatty acid binding protein, GSTP-1 and S100β protein, known markers of brain damage in stroke, Creutzfeldt-Jakob disease, traumatic brain injury, blood-brain barrier and neuronal damage [44-46] and thirteen inflammation-related proteins (IL-1rα, IL-1b, IL-6, IL-9, IL-10, G-CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 and CXCL10) were quantified in ELISA and bead suspension arrays [A51]. This study identified CXCL10 as a biomarker for staging of gambiense HAT patients. The diagnostic accuracy could be improved by combining CXCL10 with heart-fatty acid binding protein and CXCL8 in a panel. In a next step, matrix metallo-proteinases and cell adhesion molecules (MMP-2, MMP-9, ICAM-1, VCAM-1 and E-selectin) were tested in CSF and compared with CXCL10, CXCL8 and heart-fatty acid binding protein in CSF for staging [A40]. This study identified ICAM-1 and MMP-9 as most powerful CSF staging markers of HAT. A panel consisting of ICAM-1, MMP-9 and heart-fatty acid binding protein had higher diagnostic accuracy than the previously identified CLCX10, CXCL8 and heart-fatty acid binding protein panel.

Finally, the potential of CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, β-2-microglobulin, IgM and neopterin was assessed on 512 patients from Angola, Chad and DR Congo [A33]. CXCL13 or B-cell-attracting chemokine 1 had been identified at the University of Limoges as a potential staging marker of HAT [47]. Neopterin, an indicator of activation of the Th1 immune response, had previously been investigated in the staging of *T.b. rhodesiense* HAT [48]. Among the markers tested, IgM and neopterin had the highest and similar diagnostic accuracies for stage determination in gambiense HAT (Table 5).

Table 5: Results obtained for IgM and neopterin on the validation cohort after application of the cut-off calculated on the training cohort. 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. AUC: area under the curve. CI: 95% confidence interval [A33].

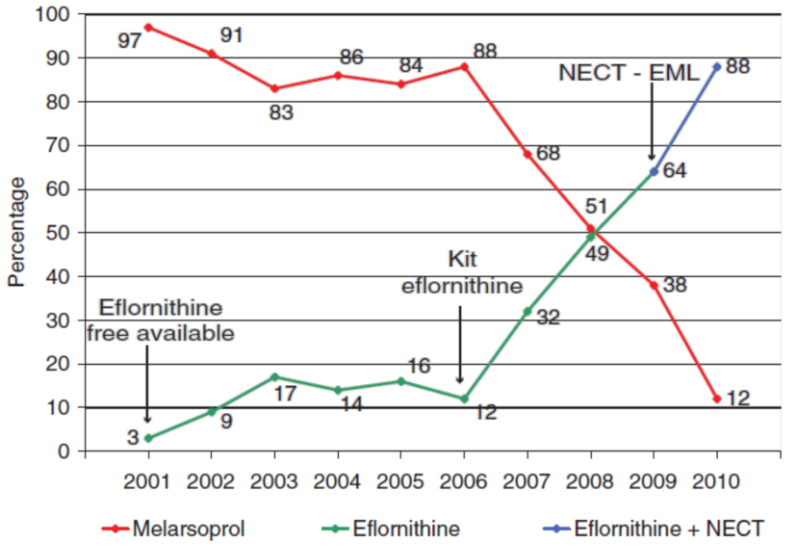
Marker	[S2/S1]	AUC% (CI)	Cut-off	Specificity (CI)	Sensitivity (CI)	p value*
IgM [µg/ml]	76.5	96 (94-98)	3.4	86% (81-91%)	92% (88-95%)	< 0.0001
Neopterin [nmol/l]	14.8	95 (93-97)	14.3	87% (83-92%)	88% (84-92%)	< 0.0001

4.6 Impact in the field and perspectives

Our findings highlighted the presence of intrathecal IgM synthesis, the IgM concentration, the IL-10 concentration and the neopterin concentration in CSF as performant markers for stage determination in gambiense HAT. These markers were taken up in subsequent research on treatment outcome assessment (chapter 5). The interest of neopterin in CSF for disease staging was confirmed recently by metabolomics discovery research [49]. For IgM, LATEX/IgM, a semi-quantitative field test, was available. As a consequence of our findings, WHO stopped to recommend determination of the total protein concentration in CSF for staging of HAT [50].

In function of time, the impact of stage determination diminished progressively, as melarsoprol was replaced by less toxic first line treatments for gambiense HAT [51] (Figure 6). From 2001 on, eflornithine became freely available through WHO for treatment of gambiense HAT. Although far safer than melarsoprol [52,53], the cost and the difficulties in logistics with eflornithine meant that the melarsoprol was continued to be used, despite an increasing number of reports on melarsoprol treatment failures. Around 2007, most control programs finally switched, when WHO released a medical kit containing all the materials needed to use eflornithine and had trained sufficient technicians in eflornithine administration. In between, a number of clinical trials [A54,54,55] had shown the efficacy and safety of combination therapy, in particular of nifurtimox-eflornithine combination therapy (NECT). NECT being safer and easier to administer than eflornithine, was included in the Essential Medicines List in 2009 and became first line treatment for 2nd stage *T.b. gambiense* HAT.

Figure 6: Rates of drug use for treatment of second stage gambiense HAT [51]



Given the relative safety of NECT, the question of choosing between pentamidine and NECT for treatment became far less important, although pentamidine can be given ambulatory while NECT administration still requires hospitalisation and remains cumbersome.

In the near future, stage determination for gambiense HAT will probably become redundant. Fexinidazole, an oral drug, has been shown recently to be effective, safe and simple for treatment of both stages of *T.b. gambiense* HAT [56]. With the introduction of a 10 days fexinidazole treatment that can be given at primary health facilities, the need for staging and lumbar puncture will be eliminated. Acoziborole, a new single

dose oral drug, is currently being tested for all stages of *T.b. gambiense* HAT in a phase II/III therapeutic trial, and may further simplify *T.b. gambiense* HAT treatment.

In contrast to the therapeutic improvements for *T.b. gambiense* HAT in the past decade, not much has changed in the treatment of *T.b. rhodesiense* HAT. Melarsoprol is still the drug of choice for 2nd stage rhodesiense treatment, suramin for 1st stage treatment.

Due to the low prevalence and different epidemiology of *T.b. rhodesiense* HAT, which is zoonotic and causes an acute disease in humans, biological specimens are more difficult to obtain and stage determination has attracted less attention. Similar to *T.b. gambiense*, it was demonstrated that IL-6, IL-10 and neopterin are significantly increased in the CSF of 2nd stage patients [48,57,58]. Different from gambiense HAT, the intrathecal inflammatory process in rhodesiense HAT patients seems mainly a single class IgM response. IgM was considered not to offer sufficient sensitivity or specificity for disease stage diagnosis [59]. When assessing IgM, MMP-9, CXCL13, CXCL10, ICAM-1, VCAM-1, neopterin and β-2-microglobulin for their diagnostic performance for stage determination in rhodesiense HAT [A29], these markers behaved differently compared to gambiense HAT patients. IgM, MMP-9 and CXCL13 individually or in combination with CXCL-10 were the most accurate for staging. Using mass spectrometry quantitative proteomics to compare *T.b. gambiense* and *T.b. rhodesiense* 2nd stage patients, C-reactive protein and orosomucoid 1 were found overexpressed in *T.b. rhodesiense* HAT patients [A10].

Also for rhodesiense HAT, it can be questioned how long staging will remain relevant. Theoretically, fexinidazole and acoziborole should be effective against rhodesiense HAT. Clinical trials are however difficult to perform and to get financed due to the limited number of *T.b. rhodesiense* patients (52 in 2016) and their dispersal over different foci and countries.

5. Follow-up after treatment

As none of the drugs to treat HAT is 100% efficacious, treated HAT patients need to be followed up after treatment. Until recently, to assess treatment outcome, patients had to be followed-up for 2 years after treatment: at 3 and 6 months, and at 6 monthly intervals thereafter [5]. A HAT patient was declared cured if no trypanosomes were detected and the CSF WBC returned to normal [5]. Detection of trypanosomes during follow-up in any body fluid is an absolute proof for treatment failure. However, due to the low numbers of circulating parasites and limited sensitivity of the parasitological techniques, the detection of trypanosomes may occur late. Thus, diagnosis of treatment failure is often based on clinical symptoms and the WBC count, although no precise guidelines existed for interpretation of the latter. Furthermore, a complete follow-up is rarely achieved, because of the multiple lumbar punctures and the burden for patients and their families [60-62]. This situation also hampers development of new drug regimens and therapeutic trials [63,64].

Because of the increase of treatment failures observed with melarsoprol[11-14], the infectiousness of relapses, the risk of neurological sequelae, and the fatal outcome, early and accurate detection of treatment failure is important.

Our approach to shorten and improve the post-therapeutic follow-up was double. In order to propose cut-off values and algorithms for its interpretation, we examined the evolution of the **CSF WBC count**, the parameter which is most often used to assess treatment outcome [A52, A48]. On the other hand, the **markers already examined for stage determination** were evaluated for their usefulness in treatment outcome assessment: CSF IgM, the total protein concentration, trypanosome specific antibodies and IL-10 [A53, A48] as well as CSF neopterin, CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, and β-2-microglobulin [A28]. We also examined some additional markers: the **CATT** on serum [A47], and **DNA and RNA detection** [A39, A8].

For these studies, data and biological specimens before treatment and during post-therapeutic follow-up of *T.b. gambiense* patients were available from 2 cohorts. A first cohort (Bwamanda cohort) consisted of serum and CSF samples from 278 late 2nd stage patients participating to an equivalence trial of melarsoprol and nifurtimox monotherapy and combination treatment [A54] in Bwamanda (Equateur, DR Congo) in 1998. This cohort was supplemented with biological specimens from 73 first stage patients [A55] and 60 early 2nd stage patients who had been excluded from the therapeutic trial. A second cohort (Mbuji-Mayi cohort) consisted of biological speciments of 360 HAT patients, included between May 2005 and February 2006 into a longitudinal study (THARSAT) to evaluate biomarkers for monitoring the clinical outcome of patients treated for HAT, carried out in Mbuji-Mayi (East Kasai, DR Congo) . This focus turned out to have a 59% treatment failure rate with melarsoprol [A48].

5.1 White blood cell count for treatment outcome assessment

In a first attempt to establish guidelines for the interpretation of the WBC count in CSF for treatment outcome assessment [A52], 10 existing criteria, extracted from literature, were evaluated on the Bwamanda cohort (Table 6).

Table 6: Ten operational criteria for HAT relapse evaluated in the study [A52].

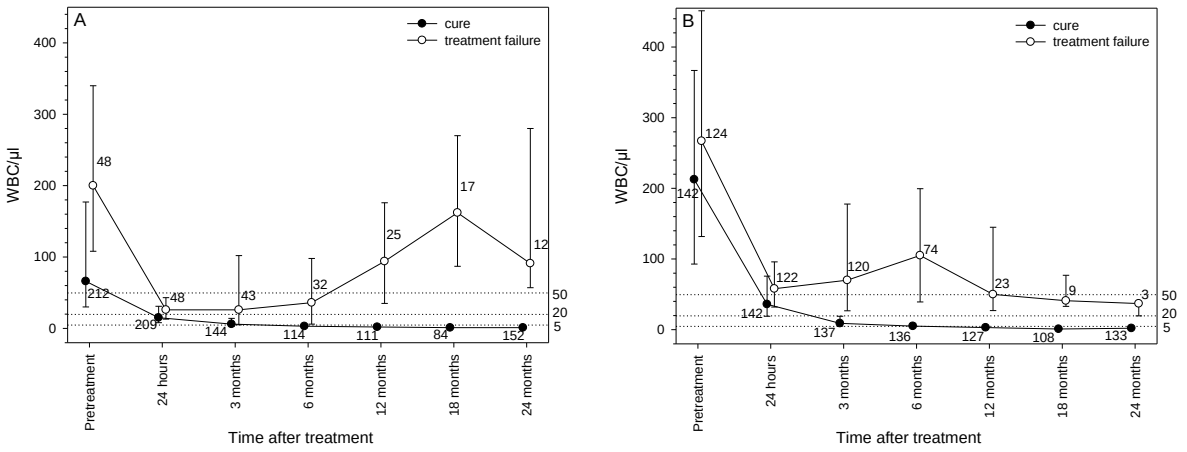
	Criterion for relapse
A	Presence of trypanosomes in blood, lymph or CSF
B	Presence of trypanosomes in blood, lymph or CSF OR; CSF WBC counts increase twice consecutively by at least 20 WBC/µl any time after completion of treatment OR; at the 18 month examination, a CSF WBC count ≥ 20 WBC/µl, regardless of previous counts. [65]
C	Presence of trypanosomes in blood, lymph or CSF OR; CSF WBC count increased to ≥ 50 WBC/µl. [8]
D	Presence of trypanosomes in blood, lymph or CSF OR; CSF WBC count higher than previous determination and ≥ 50 WBC/µl. [4,66]
E	Presence of trypanosomes in blood, lymph or CSF OR; CSF WBC count > 50 WBC/µl, which has at least doubled since the previous examination. [67-70]
F	Presence of trypanosomes in blood, lymph or CSF OR ; WBC count has increased with >30 WBC compared to the minimum count observed at any previous examination at <24 months OR; WBC count >20/µl at ≥ 24 months. (Mumba D. personal communication)
G	Presence of trypanosomes in blood, lymph or CSF OR; a CSF WBC count > 20 WBC/µl and at least twice as high as the previous follow-up result OR; a CSF WBC count > 20 WBC/µl at ≥ 24 months after treatment [A54].
H	Presence of trypanosomes in blood, lymph or CSF OR ; >20 WBC/µl at <24 months and higher than the previous two counts OR; >20/µl at ≥ 24 months after treatment. [64]
I	Presence of trypanosomes in blood, lymph or CSF OR; >20 WBC/µl and 20% increase compared to the previous count at <24 months follow-up OR; >20 WBC/µl at ≥ 24 months after treatment. [71]
J	Presence of trypanosomes in blood, lymph or CSF OR; >20 WBC/µl and either higher than WBC at the end of treatment or increased twice consecutively. [54]

Based on the results, 3 groups of criteria could be discerned. Criteria A and B had the highest specificity but were the least sensitive to detect relapses: after 24 months A and B detected respectively only 87% and 71% of all relapses, versus 100% for all other criteria. Criteria G-J had the lowest specificities (90-96%) and carried the highest risk for false positives and unnecessary toxic retreatment. For post-treatment follow-up in HAT, criteria C, D, E, and F presented the highest diagnostic accuracy. In particular criterion C, "Presence of trypanosomes in blood, lymph or CSF OR; CSF WBC count increased to ≥ 50 WBC/µl" [8] was retained as it does not refer to previous WBC count values. Its sensitivity increased in function of time from 56% at 6 months, 64% at 12 months, to 78% at 18 months. Criterion C always was >97% specific.

We also observed that interpretation of the CSF WBC count at 3 months led to false positives and should be avoided. This observation questions the benefit of a lumbar puncture 3 months after treatment.

When further analysing the WBC count in CSF of the late 2nd stage patients of the Bwamanda cohort [A53], the CSF WBC count had acceptable diagnostic accuracy from 6 months post-treatment on. Cut-offs between 8 and 10 WBC/µl provided the best trade-off between specificity and sensitivity. Importantly, in the group of cured patients, the median WBC count already normalized 6 months after treatment (Figure 7A). A diagnostic marker for cure, enabling early discharge of patients at low relapse risk, would allow more intensive follow-up of the smaller group of high risk patients [72]. A WBC count ≤5/µl criterion at 6 months seemed promising in this regard, as in our data it categorized a subgroup of 63% (92/146) of patients as 'low risk of relapse', leaving a 37% subgroup of patients who would require intensive monitoring. The latter group included 81% (26/32) of all relapses (PPV 48%, NPV 93%).

Figure 7: Evolution of CSF WBC count in cured and relapsed patients during follow up (A) in the Bwamanda cohort [A53] and (B) in the Mbuji-Mayi cohort [A48]. Medians and interquartile ranges are shown, with the number of patients next to the data point.



In the Mbuji-Mayi cohort [A48], the CSF WBC count was found to have acceptable diagnostic accuracy from 3 months post-treatment on (Figure 7B). In this cohort, cut-offs between 23 and 36 WBC/µl provided the best trade-offs between specificity and sensitivity. A cut-off of >20 WBC/µl, already applied in the majority of existing criteria (Table 6), had similar sensitivities and specificities.

Based on these observations, we constructed 4 algorithms with variable durations of follow-up (Table 7):

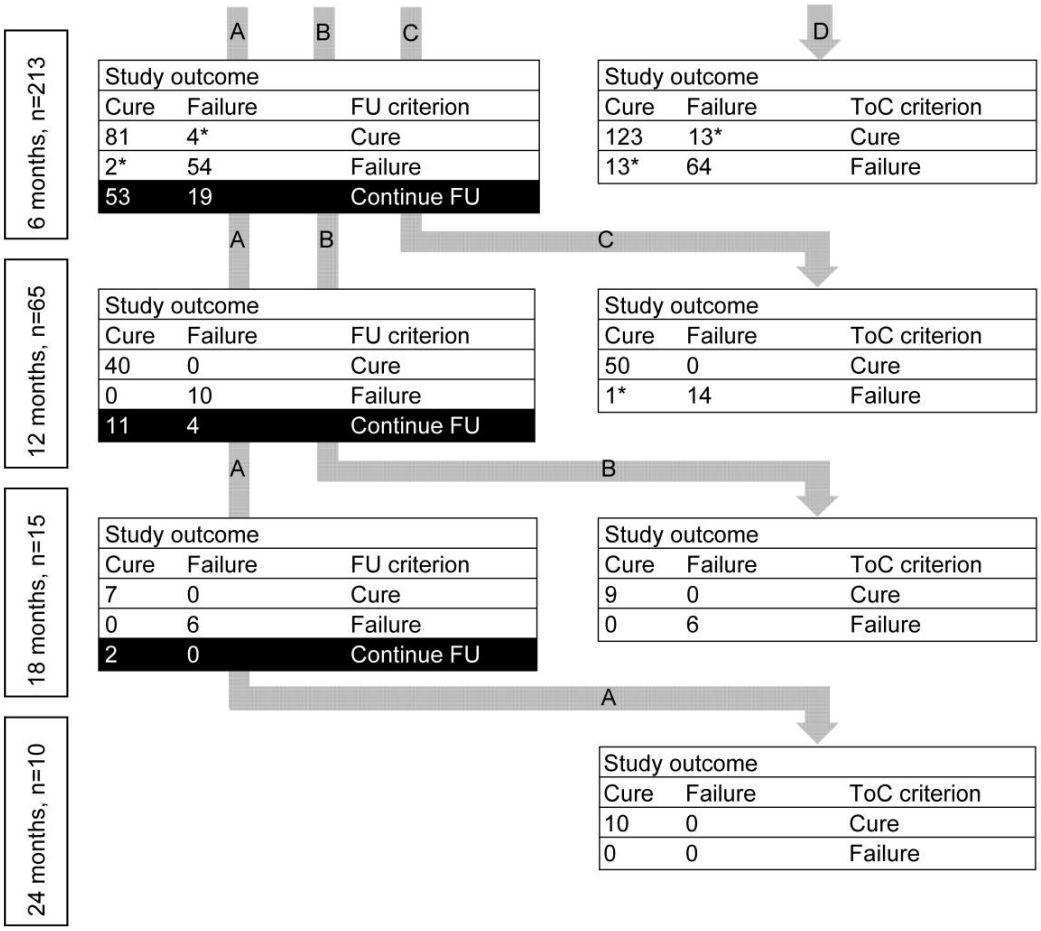
- At any follow-up visit, patients with ≤5 WBC/µl were considered 'cured' (stop follow-up). Patients with ≥50 WBC/µl or trypanosomes were considered 'failure' (rescue treatment). Patients with 6-49 WBC/µl were considered as "uncertain evolution" (continue follow-up).
- At the final test-of-cure (6, 12, 18 and 24 months post treatment for algorithms D, C, B and A), patients with presence of trypanosomes or >20 WBC/µl were classified as 'treatment failure', those with ≤20 WBC/µl were considered cured.

The accuracies of these algorithms were checked against the data of 2nd stage patients of the Mbuji-Mayi cohort (Figure 8, Table 7) and Bwamanda cohort (Table 7).

Table 7: Sensitivity and specificity of WBC count for follow-up and test-of-cure in 4 new algorithms

	Algorithm A	**Algorithm B**	**Algorithm C**	**Algorithm D**
Mbuji-Mayi 2nd stage cohort:				
Sensitivity	94.6%	94.6%	94.4%	83.1%
Specificity	98.5%	98.5%	97.8%	90.4%
Bwamanda 2nd stage cohort:				
Sensitivity	85.4%	84.2%	79.4%	
Specificity	99.5%	98.9%	98.8%	

Figure 8: Effect of 4 new algorithms on classification and the duration of follow-up of 2nd stage patients of the Mbuji-Mayi cohort [A48]. * wrongly classified outcome, not corresponding with real treatment outcome



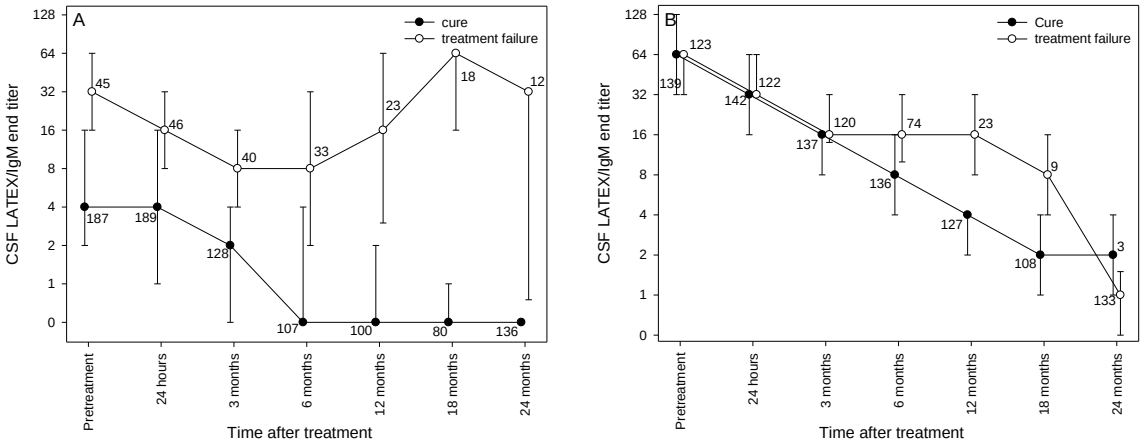
Algorithm B and C resulted in a considerable reduction of follow-up after treatment from 24 to respectively 18 and 12 months. Because the difference between their sensitivities and specificities was not significant, algorithm C with a test-of cure assessment at 12 months (and 74% reduction in lumbar punctures) seemed the most appropriate. Its specificity was around 98%, its sensitivity was respectively 94 and 79% in the Mbuji-Mayi and Bwamanda cohort. Thus, we retained 2-step algorithm C for follow-up:

- At 6 months post-treatment, patients with ≤5 WBC/µl are considered 'cured' and stop follow-up. Patients with ≥50 WBC/µl or trypanosomes are 'treatment failures' and receive rescue treatment. Patients with 6-49 WBC/µl have an "uncertain evolution" and continue follow-up.
- At 12 months post-treatment, patients with presence of trypanosomes or >20 WBC/µl are 'treatment failures' and should be retreated, patients with ≤20 WBC/µl are cured.

5.2 Staging markers for treatment outcome assessment

On the Bwamanda cohort, the IL-10 concentrations, the LATEX/IgM end titre, the LATEX/*T.b. gambiense* end titre and the total protein concentration were determined in CSF [A53]. Contrary to the CSF WBC count, which was accurate for treatment outcome from 6 months post-treatment on, the IL-10 concentrations, the LATEX/IgM end titre and total protein concentration had sufficient diagnostic accuracy only from 12 months on, LATEX/*T.b. gambiense* from 18 months on. Findings from the Bwamanda cohort were confirmed in the Mbuji Mayi cohort [A48]: only from 12 months post-treatment on, accuracy of LATEX/IgM for correct diagnosis of treatment outcome was sufficient (sensitivity 69%, specificity 97% at cut off ≥1:16). The lack of accuracy for treatment outcome assessment of LATEX/IgM was mainly caused by a slow decrease of IgM in cured patients (Figure 9). The intrathecal response may take years to normalize, as observed for other central nervous system infections [20].

Figure 9: Evolution of CSF LATEX/IgM end titre in cured and relapsed patients during follow up (A) in the Bwamanda cohort [A53] and (B) in the Mbuji-Mayi cohort [A48]. Medians and interquartile ranges are shown, with the number of patients next to the data point.



In collaboration with the University of Geneva, CSF neopterin, CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, β-2-microglobulin and IgM were determined on a subset of the Mbuji-Mayi cohort (n=97 [A28]). Together with the WBC count, neopterin followed by CXCL13 had the highest accuracies for treatment outcome. These were further verified on the rest of the Mbuji-Mayi cohort (n=242). At 3 months post-treatment, diagnostic accuracies of neopterin, CXCL13 and of the WBC count were similar. 6 and 12 months post treatment, CSF neopterin was more accurate than the CSF WBC count (Table 8). Neopterin was therefore retained as a promising marker for early treatment outcome assessment.

Table 8: Sensitivity and specificity of CSF neopterin and WBC count for follow-up and test-of-cure at 6 and 12 months post treatment, in a subset of the Mbuji-Mayi cohort (n=242) [A28].

Marker	AUC%	Cut-off	Specificity (CI)	Sensitivity (CI)
Neopterin [nmol/l]				
6 months	93.9	27.9	87 (81-93)	92 (84-98)
12 months	98.4	41.4	97 (94-100)	94 (83-100)
WBC/µl				
6 months	93.3	11.5	81 (74-91)	91 (83-97)
12 months	94.4	9.5	93 (87-97)	94 (83-100)

5.3 CATT, DNA and RNA detection for treatment outcome assessment

No blood markers for HAT treatment outcome assessment have been identified so far. Trypanosome specific antibodies in blood, detectable by CATT, have been demonstrated even 24 months after successful treatment [61,73,74]. In routine practice and to avoid lumbar punctures during follow-up, a negative CATT result after treatment is often considered as a sign of cure. We examined the value of a normalising CATT as a marker for treatment outcome on the Mbuji-Mayi cohort [A47]. We observed that CATT titres decreased after treatment both in patients who experienced treatment failure as in cured patients. A post-treatment negative CATT result does not indicate cure. Moreover, the sensitivity of CATT in relapse cases included in the Mbuji-Mayi cohort was only 78% versus 98% in the cases that had never been treated for HAT before. We concluded that CATT is unreliable for monitoring treatment outcome.

PCR has been considered as promising in HAT patient diagnosis, staging and post-treatment follow-up. However, very few PCRs have been evaluated on a large number of clinical specimens and none had been evaluated for treatment outcome assessment in a prospective longitudinal study. We therefore evaluated PCR for diagnosis, staging and post therapeutic follow-up on the Mbuji-Mayi cohort [A39]. We used the M18S-II Tb RNA gene primers, targeting the multi-copy 18S ribosomal RNA gene [75]. PCR on blood had 99.2% specificity (CI 97.7-100%) for diagnosis of HAT, while only 88.4% (84.4-92.5) sensitivity, similar to the 89.5% found in a later study with the same primers [A13]. For stage determination, PCR on CSF had 88.4% (CI 84.8-91.9%) sensitivity and 82.9% (CI 71.2-94.7%) specificity. Sensitivity of PCR for staging was slightly lower than observed in previous studies applying the more sensitive TBR1-2 primers [A5176,77]. With one exception, the PCR positive stage 1 patients had been cured with pentamidine in the Mbuji-Mayi cohort. For treatment outcome assessment, PCR on blood was neither sensitive (12.5-50%) nor specific (80.5-92.4%). These observations support the hypothesis that relapses originate from the central nervous system [78], and that trypanosomes are not likely to be found in blood of relapsing patients [61]. Lack of specificity , i.e. detectable parasite DNA in blood of some cured patients up to 24 months after treatment, corroborates earlier observations in mice where DNA was detected for a significant time beyond successful melarsoprol treatment [79]. Also PCR on CSF had limited diagnostic value with sensitivities ranging 40.0-72.2% and specificities 56.3-83.7%. With these findings we confirmed -on a larger scale- earlier observations of PCR positivity in CSF of 2 successfully cured *T.b. gambiense* infections [76,80,81]. Potential explanations include persistence of DNA from killed trypanosomes, for example in the form of immune complexes [82], or integration of DNA sequences in host genomes, as also observed with kinetoplast minicircle DNA from *Trypanosoma cruzi* [83]. Alternatively, living trypanosomes may persist as silent infections in pharmacologically privileged sites such as cells, organs or tissues, although apparent clinical cure has been obtained. Whatever the source of PCR positivity, a role of PCR or other DNA detection techniques for post-treatment follow-up in HAT remains questionable.

Detection of messenger RNA, in particular spliced leader (SL) RNA, is considered as one of the best surrogate markers for viable organisms [84] and was proposed for assessment of treatment efficacy [85]. We therefore assessed the accuracy of SL RNA detection compared with DNA detection in blood of 61 *T.b. gambiense* patients as markers for cure [A8]. The low specificity of PCR on blood was confirmed (77%), while detection of SL RNA had specificities of 100 and 98.4% in the first and second run. The detection of SL RNA might therefore be an accurate marker for treatment outcome assessment.

5.4 Impact in the field and perspectives

Our observations highlighted the CSF WBC count and neopterin as markers for treatment outcome assessment in HAT, while a proof of principle of the potential of SL RNA detection was obtained.

For interpretation of the CSF WBC count for treatment outcome assessment in 2nd stage HAT patients, we recommend the following 2-step algorithm:

- At 6 months post-treatment, patients with ≤5 WBC/µl are considered 'cured' and stop follow-up. Patients with ≥50 WBC/µl or trypanosomes are 'treatment failures' and receive rescue treatment. Patients with 6-49 WBC/µl have an "uncertain evolution" and continue follow-up.
- At 12 months post-treatment, patients with presence of trypanosomes or >20 WBC/µl are 'treatment failure' and should be retreated, patients with ≤20 WBC/µl are cured.

The interest of our 2-step algorithm was confirmed by epicentre and Médecins sans Frontières on data from Uganda, Sudan, Angola, Central African Republic, Republic of Congo and DR Congo corresponding to 2190 2nd stage gambiense HAT patients [86]. Out of 8 different 2-step algorithms, our 2-step algorithm had the highest specificity (97.7%) while its sensitivity of 87.4% was not significantly different from other algorithms. Again, it allowed to classify two third of the patients already 6 months after treatment.

Taking into account the recent observations that the relapse rate with NECT is ≤2% [55,56,87] and with pentamidine is <5% [70,88], that in routine the follow-up rate is extremely low, and that treated patients who become symptomatic return to the hospital, WHO adapted its recommendations for follow-up [50]. For routine, no systematic follow-up is recommended anymore, in order for follow-up and CSF examinations to focus on symptomatic patients. For cases where a follow-up examination is carried out, our recommended 2-step algorithm was adopted for 2nd stage HAT, including our recommendation not to interpret WBC counts at 3 months. In the absence of reliable data for rhodesiense HAT, the same guidelines apply.

The above WHO recommendation does not apply for new treatment regimens or clinical trials, for which accurate and early treatment outcome assessment remains important. For clinical trials, a minimal follow-up of 18 months has been defined. The limit of 50 WBC/µl has been retained as a criterion for probable relapse at interim follow-up visits, while the limit of 20 WBC/µl is used at the test-of-cure visit at 18 months to differentiate between cure and probable relapse [89].

Due to the rapid normalization of neopterin in CSF immediately after treatment, and its increase in treatment failures, neopterin had high accuracy for disease outcome assessment, and has the potential of being an earlier and more accurate marker than the WBC count in CSF. So far, neopterin for follow-up in HAT has been measured only on the Mbuji-Mayi cohort. Confirmation on other cohorts is indicated. As neopterin is quantified by ELISA, its determination is technically not adapted to routine practice. Development of a rapid diagnostic inhibition test for detecting neopterin in the CSF has been initiated [90].

The lack of specificity of PCR during follow-up was somewhat surprising, but confirmed other fragmentary observations. Detection of SL RNA may represent a promising alternative, but needs further confirmation. The need of sampling biological specimens on RNA stabilisation buffer has hampered research. Previous studies have not collected CSF samples on stabilisation buffer, nor any biological specimens from treatment failures.

6. Development and evaluation of diagnostics

Diagnosis of gambiense HAT is usually performed stepwise (Figure 2). In a first step, the blood is screened for the presence of antibodies, next, antibody positive persons are examined for the presence of trypanosomes. Antibody negative persons are not considered for parasitological examinations, unless clinical symptoms indicative for HAT are present.

Until 2013, only one antibody detection test was available for field use, CATT/*T.b. gambiense*, the Card Agglutination Test for Trypanosomiasis. In order to improve serodiagnosis, our laboratory invested in discovery of improved antigens. I was particularly involved in the use of phage display to identify **peptide mimotopes of variant surface glycoproteins** of diagnostic interest, as supervisor of PhD student Lies Van Nieuwenhove [A34, A35, A38]. Furthermore, within the NiDiag project, ITM collaborated with a commercial company for the development of RDTs, which are better suited for individual testing. I contributed to evaluations of the **diagnostic accuracy of this RDT** [A12, A27], but after my move to IRD also to other tests [A9].

After serodiagnosis, antibody positive persons undergo parasitological examinations. Diagnostic accuracy studies of parasitological techniques and PCR on large patient series are rare. In the framework of HAT surveillance, the importance of remote molecular analysis might increase but prospective studies on reproducibility, repeatability and performance of PCR are lacking. We therefore compared the **diagnostic accuracy of parasitological and molecular techniques** for diagnosis of HAT (Figure 10) [A13].

Finally, the results obtained by **external quality assessment** of interpretation of RDT results for malaria [A22] (not discussed here) or of thick blood film microscopy for malaria and HAT diagnosis [A21, A6] underline the need of regular training, regular quality assessment, and establishment of clear SOPs [A4, A5] to improve diagnostic practices, in particular in an elimination context [A20].

Figure 10: Field work for the study of diagnostic accuracy of parasitological and molecular techniques for diagnosis of HAT [A13].

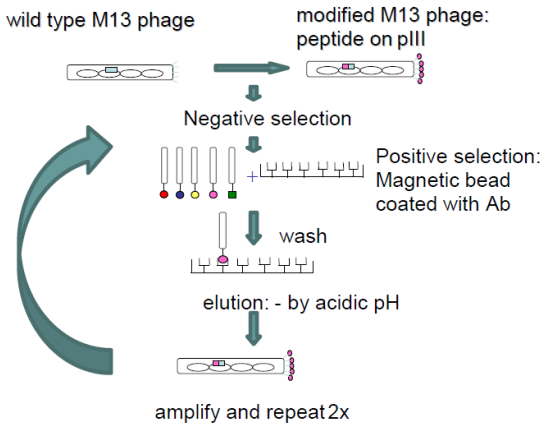
6.1 Peptide VSG mimotopes

Research on improved antigens was based on 1° identifying a synthetic alternative for native variant surface glycoproteins (VSG) LiTat 1.3 and 1.5; and 2° the use of phage display to discover VSG mimotopes.

All antibody detection tests for gambiense HAT are based on VSGs. These VSGs cover the surface of bloodstream trypanosomes and determine the variable antigen type (VAT) [91]. The VATs LiTat 1.3, LiTat 1.5 and LiTat 1.6 of *T.b. gambiense* are predominant: they are expressed by most trypanosomes and appear early during infection. Most patients have antibodies against these VATs [91]. The CATT/*T.b. gambiense* [3], consists of whole lyophilised trypanosomes of VAT LiTat 1.3. LiTat 1.3 VSG is not expressed in all endemic HAT foci [92,93], LATEX/*T.b. gambiense* and ELISA/*T.b. gambiense* therefore combine the VSGs LiTat 1.3, 1.5 and 1.6 as antigen [A66,A81]. The use of native VSGs however has disadvantages. Non-specific epitopes may cause cross-reactions and VSG production relies on culture of infective *T.b. gambiense* parasites in laboratory rodents, posing a risk of infection to the staff [94]. The production of synthetic peptides is standardised, does not require laboratory animals and is without infection risk [95].

Phage display is a selection technique based on DNA recombination (Figure 11). Short random DNA sequences are inserted in the genome of the phage virions, which express the corresponding peptide as a fusion peptide on their surface. After rounds of antibody mediated affinity selection (panning), the phage DNA is sequenced and the corresponding peptide mimotope sequence is deduced. Phage display is a powerful tool to identify mimotopes, small peptides that mimic linear, discontinuous and/or non-protein epitopes [96-98] and has been successfully applied for Lyme disease [99], hepatitis C [95,100], typhoid fever [101], tuberculosis [102] and leishmaniasis [103]. Some mimotopes have been patented to become incorporated in commercially available tests, for example for neurocysticercosis [104].

Figure 11: Principle of phage display.



We applied 2 different approaches for mimotope discovery.

In a first step, we used monoclonal antibodies against LiTat 1.3 and LiTat 1.5 VSG, which were fixed on magnetic particles for the panning [A38].
For LiTat 1.5 VSG, all but one of the 37 identified sequences were mapped against a linear region (amino acid 268-281) in the N-terminal domain. A selection of 10 peptides was synthesized, and inhibited binding of the monoclonal to LiTat 1.5 VSG. For LiTat 1.3 VSG, among the 16 sequences identified, 10 were mapped

against amino acid 196-210 and 2 against amino acids 338-351 of the LiTaT 1.3 VSG. The remaining peptides could not be mapped. Out of 12 peptides synthesized, 7 inhibited monoclonal binding to LiTat 1.3 VSG. Next, the diagnostic potential of the peptides was assessed. HAT sera significantly inhibited binding of the monoclonal antibodies to respectively 6 and 3 synthetic mimotope peptides of LiTat 1.5 and LiTat 1.3. This confirmed that the identified mimotopes had diagnostic potential for HAT.

Taking into account that monoclonal antibodies could have missed some mimotopes with important diagnostic potential, and that the mouse and human immune system might recognise different epitopes, we also used human antibodies for phage display [A34]. LiTat 1.3 and 1.5 VSG specific antibodies were affinity purified from human sera, and coated onto magnetic particles for phage display.
For LiTat 1.5 VSG, the 20 obtained sequences could be aligned against 6 LiTat 1.5 stretches (amino acids 33-47, 81-119, 145-166, 245-281, 341-368 and 468-489). 18 sequences mapped against amino acids 81-119, mainly 81-109. Seven peptides were synthesised, plus the LiTat 1.5 VSG amino acid 81-109 stretch. After screening in indirect ELISA using synthetic peptides as antigens and the panning sera, 6 peptides including the Li1.5/81-109 stretch were retained for further evaluation.
For LiTaT 1.3, 18 sequences could be aligned against 3 LiTat 1.3 VSG stretches (amino acids 72-116 containing an internal repeat, 180-196 and 404-443). All sequences aligned against amino acids 72-116, in particular amino acids 78-110. Seven peptides were synthesised, plus the LiTat 1.3 VSG amino acids 78-110 (Li1.3/78-110) and 424-439. After screening in indirect ELISA using the synthetic peptides as antigens and the panning sera used for affinity purification, 7 peptides including the Li1.3/78-110 stretch were retained for further evaluation.

The diagnostic performance of all selected peptides and native VSG as antigen was tested with 102 HAT and 102 control sera (Table 9). Taking into account that in the monoclonal antibody experiment, so many mimotope sequences mapped against amino acid 268-281 of LiTat 1.5 VSG [A38], we decided to synthesize this VSG stretch as well (peptide 1.5/268-281) [A30]. We confirmed the diagnostic potential of some synthetic peptides, that may replace the native full length LiTat 1.3 and LiTat 1.5 VSG, although they were less accurate.

Table 9: Diagnostic performance of synthetic peptides in indirect ELISA. The area under the receiver operator characteristics curve (AUC), the sensitivity and specificity at maximum Youden index [A34, *A30].

Antigen type	Name	AUC (95 % CI)	sensitivity (95 % CI)	specificity (95 % CI)
LiTat 1.3	1.3/78-110	0.95 (0.91-0.98)	0.96 (0.90-0.99)	0.85 (0.77-0.92)
LiTat 1.3	3-2-G10	0.95 (0.91-0.97)	0.90 (0.83-0.95)	0.93 (0.86-0.97)
LiTat 1.3	3-2-G5	0.93 (0.89-0.96)	0.85 (0.77-0.92)	0.94 (0.88-0.98)
LiTat 1.3	3-3-E3	0.89 (0.84-0.93)	0.96 (0.90-0.99)	0.76 (0.67-0.84)
LiTat 1.3	3-3-F6	0.89 (0.84-0.93)	0.82 (0.74-0.89)	0.86 (0.78-0.92)
LiTat 1.3	3-2-C5	0.89 (0.84-0.93)	0.90 (0.83-0.95)	0.82 (0.74-0.89)
LiTat 1.3	3-2-D10	0.86 (0.81-0.91)	0.86 (0.78-0.92)	0.81 (0.72-0.88)
LiTat 1.5*	1.5/268-281	0.95 (0.92-0.98)	0.89 (0.82-0.95)	0.95 (0.89-0.98)
LiTat 1.5	5-1-F9	0.95 (0.91-0.97)	0.94 (0.88-0.98)	0.95 (0.89-0.98)
LiTat 1.5	5-2-D3	0.94 (0.90-0.97)	0.92 (0.85-0.97)	0.89 (0.82-0.94)
LiTat 1.5	5-2-H2	0.88 (0.82-0.92)	0.82 (0.74-0.89)	0.81 (0.72-0.88)
LiTat 1.5	5-3-C1	0.87 (0.82-0.92)	0.86 (0.78-0.92)	0.79 (0.70-0.87)
LiTat 1.5	5-3-B9	0.85 (0.79-0.89)	0.79 (0.70-0.87)	0.83 (0.75-0.90)
LiTat 1.5	1.5/81-109	0.79 (0.73-0.85)	0.81 (0.72-0.88)	0.75 (0.65-0.83)
Native VSG	LiTat 1.3	1.000 (0.98-1.00)	1.000 (0.96-1.00)	1.000 (0.96-1.00)
Native VSG	LiTat 1.5	0.997 (0.97-1.00)	1.000 (0.96-1.00)	0.990 (0.95-1.00)

6.2 Diagnostic accuracy of RDTs

Within the NIDIAG project, I contributed to evaluation of the first generation RDT HAT Sero *K*-SeT which has been commercialized by Coris Bioconcept, Belgium. This RDT uses LiTaT 1.3 and LiTaT 1.5 VSGs as antigens, in a single test line. The proof of principle (phase 1 study), performed on 99 plasma samples of Congolese HAT patients and 99 Congolese controls which were mixed with red blood cells of a healthy volunteer, showed a test sensitivity of 93.9% (95% CI 87.9-99.9%) and specificity of 99.0% (96.5-100%) [A27].

The diagnostic accuracy of HAT Sero *K*-SeT was further assessed in the field (RD Congo, phase 2 study) on fresh blood of 134 HAT cases and 356 endemic negative controls [A12] recruited at treatment centres and during active screening. HAT Sero *K*-SeT had a test sensitivity and specificity of respectively 98.5% (94.7-99.6%) and 98.6% (96.8-99.4%). There was no significant difference with diagnostic accuracy of CATT or trypanolysis. The major limitation of this study was the fact that the confirmed HAT patients had been detected by previous CATT screening, implying a risk of overestimating the test sensitivity.

Another phase 2 evaluation was done on both commercially available first generation RDTs, HAT Sero *K*-SeT and SD Bioline HAT (Standard Diagnostics, Korea). This retrospective study used for the first time samples originating from West-Africa, including 231 confirmed HAT patients and 257 endemic controls [A9]. Tests were evaluated using 3 independent readers blinded to all other results, who scored the test line intensity compared to the control line intensity (faint, weak, medium or strong, Figure 12). This methodology was the one I had previously applied for evaluation of malaria RDTs at ITM [A23]. We observed no significant difference in sensitivity between HAT Sero *K*-SeT (99.1%, 96.9-99.9%) and SD Bioline HAT (99.6%, 97.6-100%). However, the specificity on endemic controls of both tests, respectively 87.9% (83.3-91.7%) and 88.3% (83.8-92.0%) was unexpectedly low. Combination of the 2 RDTs, a principle also applied for diagnosis of HIV [105], increased specificity to 93.4% (CI 89.6-96.1%), while maintaining sensitivity. Unfortunately also for this study, selection bias caused by previous screening with CATT, could not be excluded. Another limitation was the use of stored plasma instead of fresh blood.

Figure 12: Evaluation of test line intensity compared to the control line in RDTs. This reader scored the test lines for this sample 222 in SD Bioline HAT "L" for line 2 (middle line, L=leger=weaker than the left control line) and "M" for line 1 (right line, M=medium= equal to the control line).

6.3 Diagnostic accuracy of parasitological and molecular techniques

This study on performance of parasitological tests and PCR [A13] included 237 persons that were either CATT seropositive (n=214, CATT positive on ¼ blood dilution) or had clinical symptoms suggestive of HAT (n=165). One hundred forty three persons were confirmed parasitologically (Table 10).

Table 10: Parasitological results in 143 HAT patients. (LNA Lymph node aspirate, mHCT micro-hematocrite centrifugation, mAECT mini Anion Exchange Centrifugation, BC buffy coat). *5 missing results [A13].

LNA	mHCT*	mAECT	mAECT-BC	CSF	Frequency
-	-	-	-	+	6
-	-	-	+	-	11
-	-	-	+	+	2
-	-	+	-	-	1
-	-	+	+	-	21
-	-	+	+	+	11
-	+	+	+	-	32
-	+	+	+	+	3
+	-	-	-	-	6
+	-	-	+	-	3
+	-	+	+	-	14
+	-	+	+	+	2
+	+	+	+	-	29
+	+	+	+	+	2
56+ (39.2%)	66+ (47.5%)	115+ (80.4%)	130+ (90.9%)	26+ (18.2%)	143

No participant was micro-hematocrite centrifugation (mHCT) positive only, showing that there is no benefit in performing mHCT when mAECT or mAECT-BC are carried out. mAECT-BC was significantly more sensitive than mAECT (90.9% versus 80.4%, p<0.001), but more difficult to carry out. Trypanosome detection in the lymph node aspirate was 39% positive, but picked up 4% of cases that otherwise would have been missed. Combination of lymph node aspirate and mAECT-BC examination reached a sensitivity of 95%.

For PCR, blood was conserved on filter paper (FP) and guanidine-EDTA (GE) buffer. On the parasitologically confirmed cases, sensitivity of PCR-GE (87.4%) was similar to mAECT-BC, PCR-FP was less sensitive (80.4%). Remote DNA detection does therefore not offer more sensitive diagnosis of HAT than parasitology. Repeatability was modest with kappa's of 0.74 for PCR-GE and 0.65 for PCR-FP, inter laboratory reproducibility was modest for PCR-GE (kappa 0.72) and poor for PCR-FP (kappa 0.38). From our results, collection of blood on GE buffer therefore seems to be preferred. Reproducibility problems were worse in aparasitemic serological suspects, which confirms earlier observations [A56].

In a separate study, LAMP was performed on the same samples [A19]. On 142 confirmed HAT patients from which blood on GE buffer was still available, sensitivities of 93.0% (95% CI 87.5%–96.1%) in the first run and 87.3% (80.9–91.8%) in the second run were observed with LAMP, similar to PCR. Specificity in healthy controls was 92.8% (86.4–96.3%) in the first run and 96.4% (91.1–98.6%) in the second run. Reproducibility was excellent with a kappa value of 0.81.

6.4 External quality assessment (EQA)

Although sensitivity of thick blood films for diagnosis of HAT is low (around 25%), the technique is adequate for non-specialised health centres that do not have electricity and cannot apply concentration techniques. Reading of thick blood films for HAT diagnosis appears simple, but the observations from two EQAs in DR Congo were alarming [A21, A6].

A first experience was done in the context of an EQA for malaria [A21]. An EQA panel consisting of 4 slides was delivered to 277 diagnostic laboratories in all provinces of DR Congo. Out of 4 slides, 2 contained malaria parasites (results not discussed), one slide contained *Trypanosoma brucei* and another was parasite negative. Of the participating laboratories, 50.4% missed the diagnosis of trypanosomiasis. Laboratories in provinces with high numbers of sleeping sickness cases recognized trypanosomes "more frequently" (57.0% versus 31.2%, $p<0.001$).

Alarmed by these results, it was decided to add trypanosomes again in the next EQA in DR Congo, which addressed 445 diagnostic laboratories[A6]. Out of 5 slides, one slide contained trypanosomes and another slide contained both *Plasmodium falciparum* and trypanosomes. The slide containing only trypanosomes was correctly red by 44.9% participants. However, 84% missed trypanosomes in the malaria-trypanosomiasis co-infection slide. Only 13% of all participants recognized trypanosomes in both slides. Although the response rate to an accompanying questionnaire was low (51%), 94% of the responding labs stated to use thick blood films for HAT diagnosis, 33% used serological tests, only 15% used mHCT and 6% mAECT.

6.5 Impact in the field and perspectives

6.5.1 Peptide VSG mimotopes

Our choice for LiTaT 1.3 and 1.5 VSGs as a basis for the panning experiments was confirmed by an independent evaluation of 35 antigens for diagnosis of HAT, in which native LiTaT 1.3 and 1.5 VSGs ranked among the most promising [106]. Both panning strategies seemed to give complementary results and selected other mimotopes. Although the selected peptide mimotopes had potential for diagnosis of HAT, their accuracy remained lower than for native VSGs.

In between, other methods than phage display have been used to produce antigens for HAT serodiagnostic tests, such as expression of the N terminal part of LiTat 1.3 and 1.5 VSG in yeast cells [107], expression of recombinant VSG in *Leishmania tarentolae* [108] or in *Escherichia coli*. The native VSGs LiTat 1.3 and LiTat 1.5 have been incorporated in both first generation RDTs for HAT diagnosis, SD Bioline HAT [109] and HAT Sero *K*-SeT [A27]. For diagnosis of Surra, provoked by infection with *Trypanosoma evansi*, Surra Sero *K*-SeT makes use of recombinant VSG RoTat 1.2 produced in the yeast *Pichia pastoris* [110]. Invariant surface glycoprotein (ISG)-65 was selected by proteomics as a promising antigen for HAT diagnosis, expressed in *Escherichia coli* and incorporated in a prototype RDT [111]. When expressed in *Leishmania tarentolae*, the diagnostic accuracy for ISG-65 was not significantly different from native nor recombinant LiTat 1.3 and LiTat 1.5 VSG [108]. Theoretically, ISG-65 has the potential to complement LiTat 1.3 and LiTat 1.5 VSG as an antigen, as it might be able to react with HAT sera that do not react with the VSGs. The new second generation tests SD Bioline HAT 2.0 and rHAT Sero-strip are therefore based on the combination of recombinant LiTat 1.5 VSG and recombinant ISG65, expressed in *Escherichia coli* or in *Pichia pastoris*.

6.5.2 Diagnostic accuracy of RDTs

We were able to publish the first papers on HAT RDTs and their diagnostic accuracy evaluated in phase 1 and 2 trials. Major limitations were the use of stored samples, or a selection bias caused through routine population screening with CATT.

In between, 3 prospective phase 3 evaluations have been published. These papers illustrate the difficulty of prospective test evaluation at diminishing HAT prevalences: 2 studies needed to screen 14000-15000 people to detect sufficient HAT cases, the third one screened 58000 people. In a first study [109], the sensitivity of prototype SD Bioline HAT in 149 HAT cases was 89.3% (95% CI 83.3-93.3%). Sensitivity of CATT was higher at 94.0% (88.9-96.8%). The prototype SD Bioline HAT specificity was 94.58% (94.20–94.94%), similar to CATT. A subsequent study enrolled 131 HAT cases and found 92.0% (86.1-95.5%) sensitivity and 97.1% (96.8-97.4%) specificity for SD Bioline HAT, and 69.1% (60.7-76.4%) sensitivity and 98.0% (97.8 - 98.2%) specificity for CATT [112]. The most recent study found surprisingly low sensitivities of respectively 71.2%, 62.5% and 59.0% for SD Bioline HAT 2.0, CATT and SD Bioline HAT [113]. Specificities ranged 98.1-99.2%. Based on these results it was suggested to screen with 2 RDTs in parallel, SD Bioline HAT 2.0 and SD Bioline HAT, to reach a more acceptable sensitivity of 90.1% at 97.3% specificity.

Finally, a recent phase 3 field study, also in the framework of NIDIAG, evaluated HAT Sero *K*-SeT in patients presenting in a hospital with a neurological problem [114]. The sensitivity of the HAT Sero *K*-SeT was 100% (67.6-100.0%) and the specificity was 97.0% (94.2-98.5%). As parasitology was carried out on all included disease suspects irrespective of the serological result, this study was not biased by CATT, and provides a good estimate of RDT specificity. Its main drawback was the number of HAT patients included, 8 only.

So far, no prospective phase 3 field studies have been performed on disease suspects presenting at health centres using all available RDTs in parallel (HAT Sero *K*-SeT, SD Bioline HAT, SD Bioline HAT 2.0 and rHAT Sero-strip). Observed sensitivities and specificities remain to be confirmed, and the best screening strategy to be determined. Also, data from West-Africa are still missing.

6.5.3 Diagnostic accuracy of parasitological and molecular techniques

The superior sensitivity of mAECT-BC compared to mAECT was recently confirmed [113]. Although mAECT-BC is the most sensitive parasitological technique available so far, we only recommend it for experienced technicians. For routine we promote combination of lymph node examination and mAECT, which is simpler.

Despite PCR-GE being superior to PCR-FP, conservation of blood in guanidine-EDTA buffer has not found its way to the field. In the field and for HAT surveillance, blood samples are conserved on filter paper.

Although in our hands, results of LAMP and PCR were comparable, DNA extraction had been performed in a reference lab on blood conserved in GE buffer. LAMP in the field is performed on blood conserved on Whatman nr1 filter paper, but phase 2 and 3 diagnostic accuracy studies using this protocol are missing.

6.5.4 External quality assessment

The limited availability of specialised trypanosome concentration techniques in routine labs was confirmed in the EQA. Despite its low sensitivity for gambiense trypanosomiasis, giemsa stained slide microscopy could represent a widely distributed alternative to detect HAT cases. In practice, the poor performance of microscopy compromises this opportunity. Training and quality assurance should be reinforced.

The most sensitive trypanosome detection techniques, including mAECT, rely on recognition of live trypanosomes. In particular in the context of elimination, where laboratory staff is decreasingly confronted with HAT, setting up a quality control system for visualisation of live trypanosomes, as well as for serological test reading, emerges as a priority.

7. Future for a HAT vaccine?

So far, no vaccine for trypanosomiasis is available. The immunopathology of HAT remains poorly understood and most of our understanding comes from experimental *T.b. brucei* infections in mice, which also serve as a model for vaccine development. Experimental *T.b. brucei* infection gives rise to general memory B-cell destruction and abrogates vaccine-induced protection [16,115,116]. If these findings are confirmed in HAT, this would imply the need of revaccination of HAT patients after anti-trypanosomal therapy. Moreover, development of a vaccine against the disease would be hard to achieve [117].

7.1 HAT-polyB study

In the HAT-polyB study we wanted to address memory B-cell destruction and vaccine induced protection at the human level. The pool of memory B- and T- cells in peripheral blood can be assessed by taking blood on a stabilizer, which allows shipment of samples for subsequent flow cytometry. The vaccine-induced memory response in HAT is difficult to assess. Firstly, one is limited to vaccines that provide life-long protection and have been administered to the majority of the population and prior to trypanosomiasis infection. Secondly, loss of protection cannot be tested by challenge with the pathogen.

We therefore had to rely on surrogate markers. In the presence of an intact immune system, iso-agglutinins to the missing A or B red blood cell carbohydrate antigens are always found, even if there has been no exposure to red blood cells carrying these antigens. Natural IgM antibodies against A and B carbohydrate antigens are T-cell independent, while a T-cell dependent antibody response results in IgG1 and IgG3 antibodies [118]. These antibodies were therefore used to asses T-cell independent and T-cell dependent humoral immunity. Measles were selected as a second surrogate marker. A high proportion of the population is expected to have antibodies against measles since the measles vaccine is part of the standard vaccination programs [119]. Half-life of measles IgG antibodies is around 3000 years so they should be measurable in all subjects that have been infected or were immunized [120]. Moreover, the absolute level of antibodies needed to fully protect against infection is known, as well as the concentration below which no protection is obtained [121,122].

Based on the above assumptions, the HAT-polyB study [A16] addressed the following questions:

1. does *gambiense* HAT affect peripheral blood memory B- and T-cells
2. does *gambiense* HAT influence iso-agglutinin levels and antibody levels against measles, and;
3. are these effects reversible upon cure from *gambiense* HAT?

Blood was taken from HAT patients before and 6 months after treatment, and from matched controls at the same time points.

In gambiense HAT patients compared to controls, significantly higher percentages of memory B- and memory T-cells were present in peripheral blood (Figure 13). Six months after treatment, the percentage of memory T-cells normalized while the percentage of memory B-cells did not yet fully normalize.

Iso-agglutinin IgM end-titres were slightly lower in gambiense HAT, which might indeed point to a moderate effect on the T-independent antibody response [16]. This effect seemed partially reversible upon treatment. Although anti-measles antibody levels were, and remained, lower in gambiense HAT patients than in controls (Figure 14), no significant difference could be observed in the number of individuals with levels above the cut-off for protection.

Figure 13: Flow cytometry of the CD20+ B-cell population in a HAT patient before and after treatment (T=0 and 7 months), and a control at the same time points. B-cell subsets were based on the CD27 and IgM cell surface markers. Cut-offs for cell surface markers are shown as solid lines.

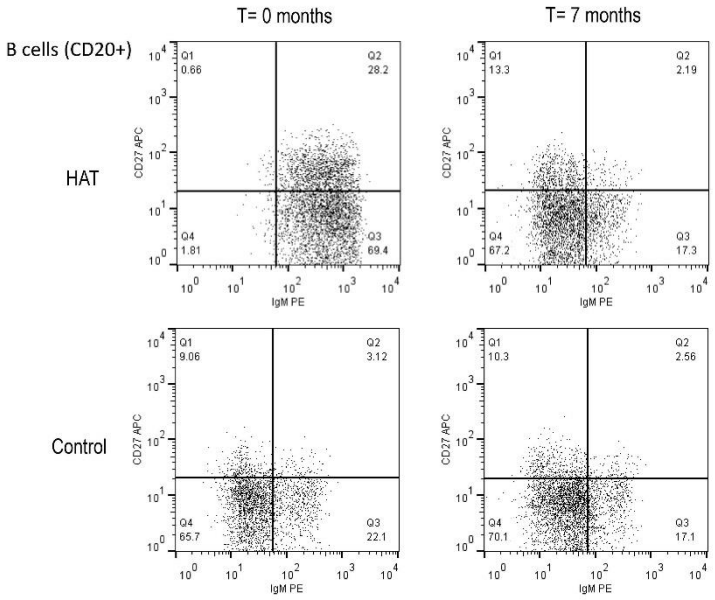
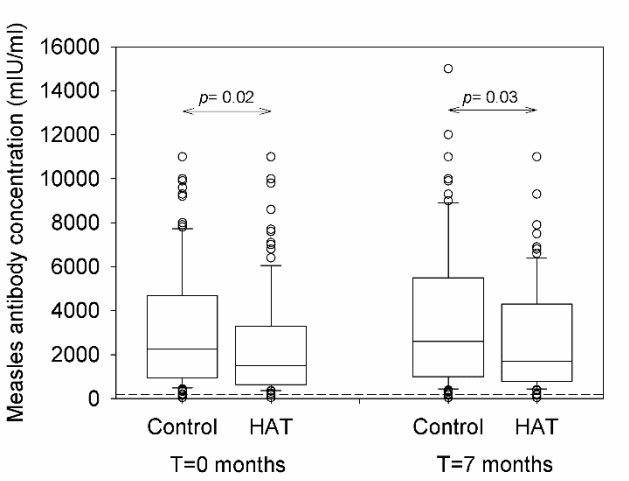


Figure 14: Box plot of measles antibody concentrations in HAT and controls before treatment (T=0 months, n=116), and after treatment or at the same time point (T=7 months, n=99). The cut-off value for protective immunity of ≥200 mIU/ml is indicated by the dashed line.



7.2 Impact in the field and perspectives

Overall, our results in gambiense HAT do not suggest massive memory cell destruction, or loss of antibody levels. If some degree of immunity loss may exist in *T.b. gambiense* HAT patients, it does not seem of clinical relevance. Moreover, evidence for an increased occurrence of vaccine preventable diseases in cured HAT patients is missing, although such relationships may be easily overlooked due to weak surveillance systems in HAT endemic countries. Differences in immune-suppression and B-cell apoptosis observed between gambiense HAT and experimental infections may be linked to the differences in parasitemia [115,123]. It might therefore be worth to perform similar investigations in *T.b. rhodesiense* HAT, which is characterized by higher parasitemias.

8. Ongoing and future research

In between, the HAT epidemic has been controlled. With 2184 HAT cases declared in 2016, elimination is approaching. Elimination by 2020 of *T.b. gambiense* HAT as public health problem is targeted, meaning less than 1 new case per 10000 in at least 90% of foci, and less than 2000 cases worldwide. By 2030, elimination with zero transmission, has been targeted [124,125]. However, important hurdles still have to be taken:

- **A shift from active to passive case detection:** Active case detection by mobile teams has largely reduced the HAT prevalence. At low prevalence, cost-effectiveness of active case detection decreases and integration of case finding into routine activities of peripheral health centres becomes crucial. HAT case detection by the peripheral health system with limited resources requires adapted diagnostic tests and algorithms [126].
- **Elimination is not eradication.** Continued post-elimination monitoring is required to ensure sustainability of zero transmission and to avoid re-emergence caused by remaining *T.b. gambiense* reservoirs [127,128].
- New drugs without safety or efficacy concerns are needed for HAT treatment at peripheral health level. Therapeutic trials are hampered by the 18 months follow-up required to decide on treatment efficacy, slowing down the drug developmental process. Furthermore, due to repeated lumbar punctures, treated patients rarely fully comply with follow-up. **Tests of cure for faster assessment of treatment outcome are needed** [64].

As shown in the previous chapters, significant progresses have been made in HAT diagnostics, but some questions remain. Different screening RDTs are available, but prospective phase 3 field studies on disease suspects combining these tests, as well as data from West-Africa are lacking. LAMP has been introduced as a low-technology platform for molecular testing, but phase 2 and 3 diagnostic accuracy studies are missing. Neopterin seems to have high accuracy for disease outcome assessment, but confirmation is indicated. Detection of SL RNA may also represent a promising alternative for disease outcome assessment, but needs confirmation.

Standardized antibody detection ELISAs allow sensitive screening dried blood spots and are applicable in regional reference centers [A45], in combination or not with trypanolysis or PCR [129]. A real-time PCR platform has been phase I validated for the detection of *Trypanozoon* (18S gene) and identification of *T.b. gambiense* (TgsGP gene) in blood. These PCRs are suited for high-throughput molecular testing [130].
Setting up a quality control system for visualisation of live trypanosomes, as well as for serological test reading, emerges as a priority, and can be realized using tablets that allow recording of pictures and videos of microscopic images.

These challenges and observations form the basis of **the DiTECT-HAT project**, a project supported by the European and Developing Countries Clinical Trial Partnership (EDCTP2, Horizon 2020 program) that I coordinate.

8.1 Diagnostic tools for HAT elimination and clinical trials (DiTECT-HAT)

The general objective of DiTECT-HAT is to deliver new, cost-effective and ready to implement diagnostic tests and test algorithms for *T.b. gambiense* HAT elimination.

Specific objectives are:

1. For passive case detection: to determine the diagnostic performance in peripheral health centres in low prevalence HAT foci of RDTs and to establish diagnostic algorithms combining RDTs at point-of-care and remote serological and/or molecular reference tests on dried blood spots.
2. For post-elimination monitoring: to determine cost-effectiveness of different diagnostic algorithms of serological and molecular high-throughput tests with or without previous RDT blood screening.
3. For test of cure in therapeutic trials: to determine the accuracy of SL-RNA detection in blood and cerebrospinal fluid and of neopterin quantification in cerebrospinal fluid, for assessing treatment outcome.

The DiTECT-HAT project consists of 3 diagnostic trials, each related to one of the above concepts.

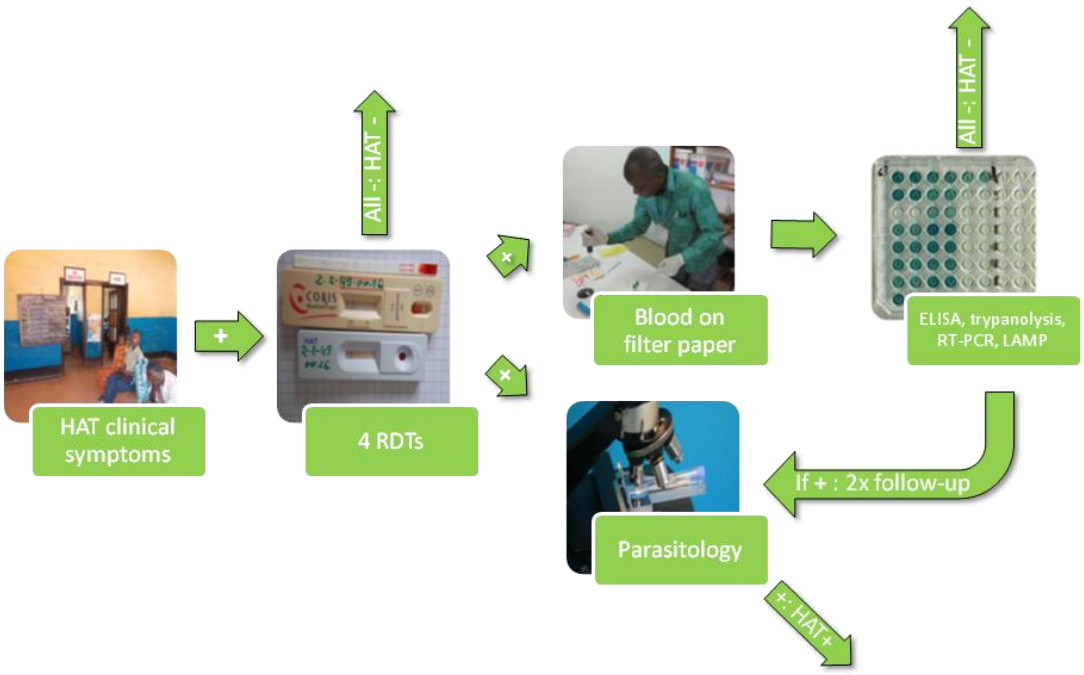
8.1.1 Passive case detection (DiTECT-HAT-WP2)

The objective of the DiTECT-HAT-WP2 trial is to validate the performance of diagnostic tools and algorithms for diagnosis of *T.b. gambiense* HAT under conditions of passive case detection. The specific objectives are to determine the diagnostic performance and costs for passive case detection in peripheral health centres in low prevalence HAT foci of:

1. HAT rapid diagnostic tests performed on fresh blood
2. Combinations of HAT rapid diagnostic tests on fresh blood
3. Diagnostic algorithms of HAT rapid diagnostic tests on fresh blood, with serological and/or molecular reference tests on dried blood spots

The DiTECT-HAT-WP2 study (Figure 15), is conducted in centres for diagnosis and treatment and in sites for serological screening in Guinea (n=12), Côte d'Ivoire (n=10) and DR Congo (n=27).

Figure 15: Summary of DiTECT-HAT-WP2 study design



Clinical suspects are tested with several commercially available RDTs for HAT. Clinical suspects with at least one RDT positive result, undergo 1° parasitological examination and 2° blood collection on filter paper for reference analysis in trypanolysis, LAMP, ELISA and real-time PCR in the regional reference laboratory (INRB for DR Congo and CIRDES for West-Africa). If the reference laboratory tests and parasitological examinations are all negative, the suspect is informed and considered free of HAT. If at least 1 reference test is positive, parasitological examinations are repeated at least twice at three months interval, unless trypanosomes are detected. We expect to test around 20000 clinical suspects including 2000 RDT positives and 120 HAT patients.

In order to assess the sensitivity, specificity, positive predictive values and negative predictive values of each assay in these multiple populations, the data in the 3 countries will be used to estimate the assay performances in the absence of a gold standard. As we will collect full cost information for the different algorithms, we will, in addition be able to estimate the cost of each assay in each setting, and rank this jointly with assay performance.

The results will enable us to propose cost-effective test algorithms to detect HAT, adapted to peripheral health centres. Keeping in mind the development of safe oral (single dose) HAT drugs, effective for both disease stages, we will examine if our algorithms with high positive predictive values might allow test-and-treat scenarios without the need for complicated parasitological confirmations.

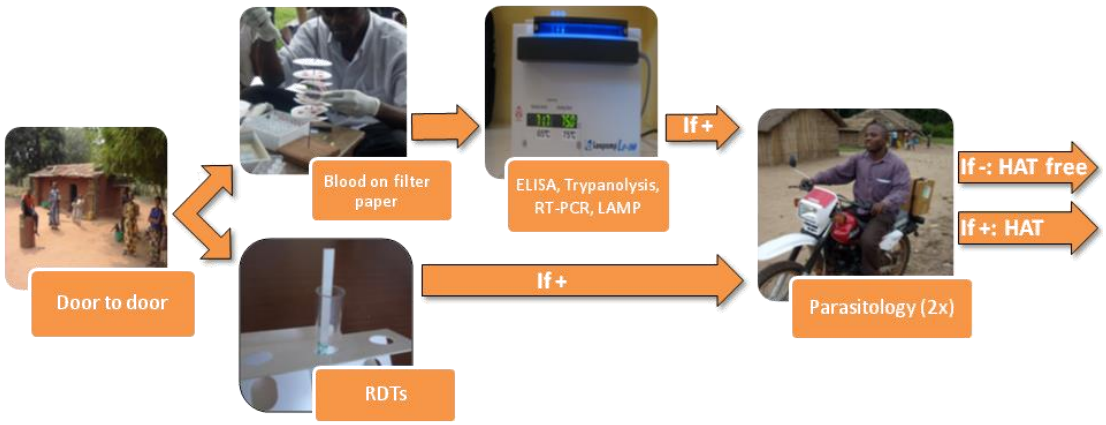
8.1.2 Post elimination monitoring (DiTECT-HAT-WP3)

The general objective of DiTECT-HAT-WP3 is to validate the performance and cost of diagnostic algorithms of serological and molecular high throughput tests with and without previous rapid diagnostic test blood screening for early detection of *T.b. gambiense* HAT re-emergence. The specific objectives are to determine the diagnostic performance, feasibility and cost for monitoring of eliminated *T.b. gambiense* HAT foci based on:

1. Serological high-throughput test on dried blood spots
2. Molecular high-throughput test on dried blood spots
3. Serological and molecular high-throughput test algorithms on dried blood spots
4. Algorithms of a rapid diagnostic test on fresh blood with serological and molecular high throughput tests on dried blood spots

The DiTECT-HAT-WP3 study (Figure 16), is conducted in villages in low to zero prevalence foci in DR Congo, Côte d'Ivoire and Burkina Faso.

Figure 16: Summary of DiTECT-HAT-WP3 study design



In these villages, a health worker goes from house to house to 1) register all consenting inhabitants in a Personal Digital Assistant; 2) spot a blood sample on filter paper 3) perform 2 rapid diagnostic tests. All dried blood spots are sent to the reference laboratory for high-throughput testing (ELISA, trypanolysis, LAMP and real time-PCR). Subjects positive in at least 1 test – the RDTs or high-throughput tests – are revisited twice for parasitological confirmation. In each country, blood specimens of 6000 persons are tested.

The relative effectiveness and overall cost of the different diagnostic algorithms will be investigated. We will quantify the break-even point for an imperfect test algorithm by formulating a decision criterion to assess how many false negatives, but particularly how many false positives can be tolerated while still achieving an intervention with a reasonable cost burden. The results will enable us to propose a test algorithm and a threshold to send out specialised mobile teams for stopping HAT re-emergences, without unnecessarily raising the alarm.

8.1.3 Early test of cure (DiTECT-HAT-WP4)

The objective of the DiTECT-HAT-WP4 study is to validate the diagnostic performance of cerebrospinal fluid neopterin quantification and of blood and cerebrospinal fluid trypanosomal spliced leader RNA detection for assessing treatment outcome.

The DiTECT-HAT-WP4 study is embedded into a therapeutic phase II/III study (DNDi-OXA-02-HAT, short title OXA002) testing a new oral single dose drug against HAT, acoziborole. Approximately 210 confirmed late stage HAT patients and 150 confirmed first and intermediate stage HAT patients will be included in the OXA002 study, in approximately 9 treatment centres in DR Congo and/or Guinea. After treatment with acoziborole, patients have post-treatment examinations, including blood and cerebrospinal fluid examination at day 11, and during follow-up at month 6, month 12 and month 18.

Combination of DiTECT-HAT-WP4 with the OXA002 study in 5 study sites and on 250-300 HAT patients allows evaluation of new treatment outcome assessment markers during follow-up, without the need for additional lumbar or venipunctures. The volumes of venous blood and cerebrospinal fluid taken are increased by 2.5 mls for the DiTECT-HAT-WP4 study. The diagnostic data collected pre- and post-treatment for OXA002, are shared with DiTECT-HAT-WP4.

Reverse transcriptase real time PCR for spliced leader RNA detection in blood and cerebrospinal fluid and neopterin detection will be carried out in the reference laboratory in Kinshasa (index tests). For evaluation of diagnostic performance of the index tests, the reference standard will consist of classification of treatment outcome according to international standards applied for the OXA002 trial but excluding for DiTECT-HAT-WP4 patients lost to follow-up (including those who died for other reasons than HAT). Receiver operator curves, sensitivity and specificity of the different index tests for treatment outcome assessment will be determined at each follow-up time point. If sufficiently accurate, trypanosomal spliced leader RNA detection in blood would allow post-treatment follow-up without the need for lumbar punctures. Improved treatment outcome assessment will not only facilitate follow-up by avoiding the lumbar puncture but also speed up the development and implementation of new drugs. In addition, it may also improve management of patients in routine.

8.2 In the long term …

HAT shares a number of similarities with animal trypanosomosis, the leishmaniasis and Chagas disease, which call for more "inter- trypanosomatid exchange" to fill the diagnostic gaps. The difficult treatment outcome assessment, existence of reservoirs, latent infections, and occurrence of multiple species represent major challenges for disease control and elimination [S2]. The following diagnostic research priorities emerge[131].

For gambiense HAT, besides the topics already dealt with in DiTECT-HAT, development of simple ultrasensitive confirmation tests stays high on the agenda. Markers to detect latent infection, differentiating between self-cure, ongoing latent infection and individuals that will develop HAT, would be an asset [132,A1]. A sensitive test to discriminate subspecies, in particular *T.b. gambiense* from other *Trypanozoon*, would help research on animal reservoirs, but also improve recognition of atypical human infections with animal trypanosomes.

For animal trypanosomosis, rapid diagnostic tests detecting antibodies against *T. congolense* and *T. vivax* merit improvement. There is a need for simple tests for active parasite carriage, applicable in the field. Molecular tests discriminating between *Trypanozoon* species and subspecies, are important to differentiate between mild and severe animal pathogens in a trade and disease management context.

For Chagas' disease, the current tests are considered sufficient to allow detection of acute *T. cruzi* infection and screening of blood and organ donors. A test for early assessment of treatment response and a point of care test for congenital Chagas' disease are considered as the highest diagnostic priorities, as well as reliable diagnostics for Chagas' disease-related complications and a point of care test for chronic phase disease [131,133].

The main gaps in diagnosis of leishmaniasis are differentiation between active disease and asymptomatic carriers -including differentiation of infectious subsets of persons and between self-clearing infection and progression to clinical leishmaniasis [134]. A marker of cure for visceral leishmaniasis remains a priority taking into account development of post kala-azar dermal leishmaniasis and its infectiousness to sand-flies. Reliable diagnostic tests for the dermal forms of leishmaniasis for health clinics remain needed [131].

9. Collaborations

Research is rarely individual. The work described in previous chapters is the result of a multitude of pleasant and fruitful collaborations, amongst others with:

- Belgian Technical Cooperation (CTB), Kinshasa, RD Congo
- Biomedical Proteomics Research Group, Medical University Centre, Geneva, Switzerland
- Centre de Développement Integral, Kinshasa, RD Congo
- Centre International de Recherche-Développement sur l'Elevage en zones Subhumides (CIRDES), Bobo-Dioulasso, Burkina Faso.
- Department of Communicable Disease Surveillance and Response, World Health Organisation, Switzerland
- Drugs for Neglected Diseases initiative (DNDi), Geneva, Switzerland
- EPICENTRE, Paris, France
- Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland
- Geneva University Hospital, Switzerland
- Institut d'Epidémiologie et de Neurologie Tropicale, Limoges, France
- Institut de Recherche pour le Développement (IRD), Montpellier, France.
- Institut National de Recherche Biomédicale (INRB), Kinshasa, RD Congo
- Institut Pierre Richet, Bouaké, Côte d'Ivoire
- Institute for Public Health, Brussels, Belgium
- Institute of Clinical Neuroscience, Department of Neurology; Goteborg, Sweden
- Institute of Tropical Medicine, Antwerp, Belgium (all departments)
- Instituto de Combate y Control de la Tripanosomiase (ICCT), Luanda, Angola
- Instituto de Medicina Tropical Alexander Von Humboldt, Lima, Peru
- International Medical Corps, South Sudan
- International Trypanotolerance Centre, Banjul, The Gambia
- Koirala Institute of Health Sciences, Dharan, Nepal
- Koninklijk Instituut der Tropen (KIT), Amsterdam, The Netherlands
- Laboratoire de Neurochimie, Université Catholique de Louvain, Brussels, Belgium
- Makerere University (Depts. of Veterinary Parasitology, Microbiology & Biochemistry) Kampala, Uganda
- Médecins sans Frontières, France & Spain
- Medische Missie Samenwerking (Memisa), Belgium & DR Congo
- National Reference Centre for Parasitology McGill University, Montreal, Canada.
- Neurochemisches Labor, Universität Göttingen, Göttingen, Germany
- Programme Nationale de Lutte contre la Trypanosomiase, Conakry, Guinea
- Programme Nationale de Lutte contre la Trypanosomiase, Kinshasa, RD Congo
- Projet de Recherches Cliniques sur la Trypanosomiase, Daloa, Côte d'Ivoire
- Swiss Tropical and Public Health Institute, Basel, Switzerland
- University of Geneva, Biomedical Proteomics Group, Switzerland
- University of Liverpool, UK

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11. Résumé en français

Ma recherche a essentiellement porté sur différents aspects du diagnostic de la Trypanosomiase humaine africaine (THA) due à *Trypanosoma brucei* (*T.b.*) *gambiense* : à savoir, **détermination du stade** de la maladie, **suivi post-thérapeutique**, **développement et évaluation des tests diagnostiques** (découverte des peptides mimotopiques, évaluation en phase 1 et 2 de tests de diagnostic rapides, évaluation sur le terrain des tests parasitaires et moléculaires, évaluation externe de qualité).

Dans des modèles expérimentaux chez des souris, les infections à *T. brucei* provoquent une destruction des cellules B de mémoire avec un impact négatif sur la persistance des réponses immunitaires induites par des vaccins. Nous avons alors examiné si l'infection à *T.b. gambiense* chez l'homme détruit des lymphocytes B de mémoire et abroge la protection induite par des vaccins.

À l'heure actuelle, je coordonne le projet européen d'EDCTP, DiTECT-HAT, visant à fournir de nouveaux tests diagnostiques et des algorithmes d'un bon rapport coûts-efficacité pour l'élimination de la THA.

11.1 Détermination du stade

Une détermination précise du stade de la maladie est essentielle pour sélectionner un traitement adapté, avec un risque minimal pour le patient, en particulier lorsque des médicaments toxiques tels que le mélarsoprol sont utilisés pour traiter la THA au deuxième stade. La détermination du stade est effectuée par examen du liquide céphalorachidien (LCR). Afin d'identifier de meilleurs paramètres pour la détermination du stade, j'ai étudié la réponse immunitaire humorale et cellulaire dans le système nerveux central. Ensuite, j'ai développé un test de stade adapté au conditions de terrain, LATEX/IgM. La relation entre les résultats du traitement (guérison ou échec) et certains des marqueurs du stade de la maladie a été évaluée. Depuis 2007, je collabore avec l'Université de Genève pour des analyses protéomiques et pour l'étude de différentes chimokines, molécules d'adhésion, et néoptérine dans le LCR.

11.1.1 Réponse neuro-immunitaire humorale dans la THA

La quantification d'immunoglobulines, d'albumine et d'anticorps spécifiques aux trypanosomes dans le sérum et le LCR permet de quantifier la réponse immunitaire humorale suite à une infection cérébrale et de caractériser le dysfonctionnement de la barrière hémato-encéphalique. Le dysfonctionnement de la barrière hémato-encéphalique chez les patients trypanosomés est rare, tardif et si présent, modéré. Le stade neurologique de la THA est caractérisé par une réponse en immunoglobulines de deux à trois classes (Figure 3), avec une réponse de classe IgM prédominante. La réponse IgM intrathécale prédominante, dépasse la réponse IgG et IgA en fréquence et en force:

- La fréquence de la synthèse intrathécale d'IgM est plus élevée que celle des autres immunoglobulines. Par exemple, pour le groupe du deuxième stade tardif (colonne de droite sur la Figure 3), les fréquences sont respectivement de 87 %, 65 % et 60 % pour la synthèse des IgM, IgA et IgG (98 %, 80 % et 73 % pour les patients avec >20 globules blancs/µl)
- La réponse de classe IgM est prédominante: la synthèse intrathécale d'IgG ou d'IgA ne se produit pas sans synthèse d'IgM et la fraction intrathécale d'IgM est plus élevée que celle d'IgA ou d'IgG.

La réponse immunitaire humorale intrathécale des patients trypanosomés commence tôt et peut déjà se produire au cours du premier stade de la maladie. De tels patients de premier stade « mal classés » peuvent ensuite recevoir un traitement inadéquat à la pentamidine.

11.1.2 LATEX/IgM, un test de terrain pour la détermination de stade

Bien avant le début de mes recherches, on savait déjà que les fortes concentrations d'IgM dans le LCR sont typiques pour la THA au stade méningo-encéphalitique, ce que nous avons confirmé. Cependant, malgré sa pertinence, la détection des IgM dans le LCR pour la détermination du stade ne se faisait pas en pratique. Cela s'explique par l'absence de tests simples et robustes, applicables dans les régions rurales africaines où la maladie sévit.

Sur la base des tests d'agglutination existants pour la détection des anticorps dans la trypanosomiase, un test d'agglutination sur carte pour la détection des IgM, LATEX/IgM, a été développé et évalué. Le test LATEX/IgM était simple et rapide et le réactif lyophilisé restait stable à 45° C. Le titre final du LCR ou la dilution la plus élevée provoquant toujours une réaction d'agglutination dans LATEX/IgM, correspondait bien à la concentration totale d'IgM dans l'échantillon. La limite de détection était d'environ 1 mg/l (Figure 4). À un seuil de 8, les titres finaux de LATEX/IgM avaient une spécificité de 92,7 % et une sensibilité de 89,4 % pour la synthèse intrathécale d'IgM, reflétant la pathologie du système nerveux central. En utilisant ce seuil de 8, le nombre d'échantillons positifs et négatifs sur 937 patients trypanosomés a été déterminé (Tableau 2). Parmi ces derniers, 342 et 27 échantillons de LCR ont été respectivement analysés avec LATEX/IgM au Sud-Soudan et en République centrafricaine, montrant la faisabilité du test. Parmi les échantillons de deuxième stade tardif, 88 % (483/548) étaient positifs à LATEX/IgM (93 % avec plus que 20 globules blancs/µl, et 39 % avec moins que 20 globules blancs/µl mais avec des trypanosomes dans le LCR). Parmi les patients au premier stade, 11.5 % étaient positifs à un seuil ≥ 8 dans LATEX/IgM. Ces patients, probablement traités à la pentamidine, couraient une forte risque d'échec thérapeutique, mais leur résultat de traitement reste inconnu.

11.1.3 Réponse neuro-immunitaire cellulaire dans la THA

La protéine acidique fibrillaire gliale (GFAp) et la sous-unité légère de neurofilament sont des marqueurs d'astrogliose et de dégénération neurologique, et peuvent être détectés dans le LCR par ELISA. Nous avons étudié le GFAp et le neurofilament chez des patients trypanosomés. L'augmentation des taux de GFAP dans le LCR a confirmé l'astrogliose observée dans la THA et les modèles expérimentaux de THA. Les taux anormaux de neurofilament dans le LCR ont montré une dégénérescence neurologique chez un tiers des patients étudiés. Le GFAp et le neurofilament dans le LCR n'ont pas été retenus en tant que marqueurs de stade, puisque les deux protéines étaient principalement anormales chez les patients au nombre de globules blancs dans le LCR déjà très élevé.

Peu de données étaient disponibles sur les cytokines, en particulier dans le LCR, chez les patients trypanosomés en stade méningo-encéphalitique. Nous avons mesuré les concentrations d'IL-6, d'IL-8, d'IL-10, de TNF-α et d'IFN-γ dans les échantillons de sérum et de LCR de 46 patients trypanosomés, avant et immédiatement après le traitement. Le TNF-α et l'IFN-γ n'ont été que sporadiquement augmentés. Dans le sérum, des concentrations élevées d'IL-6, d'IL-8 et d'IL-10 ont été observées chez tous les patients. Par rapport aux patients au premier stade, des concentrations sériques plus élevées d'IL-10 ont été détectées chez les patients au deuxième stade, qui ont diminué après traitement. Dans le LCR, des concentrations élevées d'IL-6, d'IL-8 et en particulier d'IL-10 ont été observées chez les patients au 2ème stade avancé (> 20 globules blancs/µl dans LCR), qui immédiatement après traitement, sont tombés sous la limite de détection (Figure 5). Le IL-10 dans le LCR a été retenu comme un paramètre intéressant pour la détermination du stade, en raison de ses niveaux très élevés dans le LCR chez les patients au deuxième stade avancé par rapport aux patients au premier stade.

11.1.4 Réponse immunitaire intrathécale et résultat du traitement

D'après nos précédentes études, il était clair qu'une réponse immunitaire intrathécale pouvait survenir chez des patients au premier stade. D'autre côté, une grande partie des patients avec 6-20 globules blancs/µl, indépendamment de la présence de trypanosomes, n'avait pas de réponse immunitaire intrathécale. Un traitement réussi à la pentamidine chez des patients au deuxième stade ayant jusqu'à 20 cellules/µl a été rapporté. Nous avons pu étudier la relation entre la réponse immunitaire intrathécale et les échecs thérapeutiques de deux groupes de patients atteints de THA à *T.b. gambiense*. Pour ces deux études, nous avons émis l'hypothèse qu'un traitement infructueux avec la pentamidine ou la suramine, était lié à une infection du système nerveux central non guéri.

Une première étude a été réalisée sur des patients trypanosomés avec un nombre de globules blancs ≤20 /µl, indépendamment de la présence de trypanosomes dans le LCR. Dans cette étude, respectivement 21/49 et 16/49 des patients traités par pentamidine et mélarsoprol ont récidivé. Nous avons pu démontrer que des taux de 11-20 globules blancs/µl dans le LCR, la présence de synthèse intrathécale d'IgM, et les titres finaux du LATEX/IgM ≥4, ≥8 et ≥16 dans le LCR ou du LCR LATEX/*T.b. gambiense* positif, étaient associés à un risque accru d'échec thérapeutique par pentamidine (Tableau 3). Une telle relation n'a pas pu être identifiée dans le groupe de patients traités par mélarsoprol. Ceci confirme que ces paramètres peuvent être utiles pour l'évaluation de l'atteinte du système nerveux central, qui est incurable par pentamidine.

La deuxième étude a été réalisée sur des patients trypanosomés au premier stade (≤5 globules blancs/µl, pas de trypanosomes dans le LCR). En raison d'une pénurie de pentamidine, ceux-ci ont été traités avec de la suramine. Quatorze patients sur 60 (23 %) ont récidivé. De nouveau, la présence d'une synthèse d'IgM intrathécale, d'une concentration élevée d'IgM dans le LCR et de concentrations élevées d'IL-10 dans le LCR ont été associées à un échec du traitement (Tableau 4). Aucune association entre l'échec thérapeutique et les anticorps spécifiques dans le LCR ou la protéinorachie dans le LCR n'a été observée. Pris ensemble, l'intérêt de la synthèse d'IgM intrathécale, et les concentrations d'IgM et d'IL-10 dans le LCR comme marqueurs pour la détermination du stade a été confirmé dans ces études.

11.1.5 Collaboration avec l'Université de Genève

Après les études ci-dessus, je me suis principalement concentrée sur le suivi post-thérapeutique. Pour ma recherche sur la détermination du stade j'ai collaboré avec l'Université de Genève.

En utilisant l'électrophorèse bidimensionnelle et la spectrométrie de masse quantitative, 73 protéines ont été trouvées surexprimées chez des patients atteints de THA au deuxième stade. Deux de ces protéines, l'ostéopontine et la β-2-microglobuline, ont été confirmées comme marqueurs de stade potentiels par Western blot et ELISA.

En parallèle, la protéine H-FABP, la protéine GSTP-1 et la protéine S100β – des marqueurs connus des lésions cérébrales – et treize protéines liées à l'inflammation (IL-1rα, IL-1b, IL-6, IL-9, IL-10, G -CSF, VEGF, IFN-γ, TNF-α, CCL2, CCL4, CXCL8 et CXCL10) ont été quantifiées. Cette étude a identifié CXCL10 comme un biomarqueur pour la détermination de stade des patients atteints de THA à *T.b. gambiense*. Dans une étape ultérieure, des matrix métallo-protéinases et des molécules d'adhésion cellulaire (MMP-2, MMP-9, ICAM-1, VCAM-1 et E-sélectine) ont été testées dans le LCR et comparées à CXCL10, CXCL8 et la protéine H-FABP pour la détermination de stade. Cette étude a identifié ICAM-1 et MMP-9 dans le LCR comme les marqueurs de stade les plus puissants.

Enfin, le potentiel de CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, β-2-microglobuline, IgM et néoptérine a été évalué sur 512 patients de l'Angola, du Tchad et de la RD Congo. Parmi les marqueurs testés, l'IgM et la néoptérine présentaient la meilleure précision diagnostique pour la détermination du stade (Tableau 5).

11.1.6 Impact sur le terrain et perspectives

Nos résultats ont mis en évidence la synthèse intrathécale d'IgM, la concentration d'IgM, la concentration d'IL-10 et la concentration de néoptérine dans le LCR en tant que marqueurs performants pour la détermination du stade de la THA due à *T.b. gambiense*. Ces marqueurs ont été repris dans des recherches ultérieures sur le suivi post-thérapeutique. L'intérêt de la néoptérine dans le LCR pour le stade de la THA a été confirmé récemment par la recherche sur la métabolomique. Pour les IgM, LATEX/IgM, un test de terrain semi-quantitatif, était disponible.

Au cours des années, l'impact de la détermination du stade a progressivement diminué, car le mélarsoprol a été remplacé par des traitements de première ligne moins toxiques. À partir de 2001, l'éflornithine est devenue disponible gratuitement pour le traitement de la THA due à *T.b. gambiense*. L'association médicamenteuse nifurtimox-éflornithine (NECT) étant plus sûre et plus facile à administrer que l'éflornithine seule, a été incluse dans la liste des médicaments essentiels en 2009 et est devenue le traitement de première intention du deuxième stade de la THA à *T.b. gambiense*. Compte tenu de la sécurité relative de NECT, le choix entre la pentamidine et le NECT pour le traitement est devenu moins important, bien que contrairement à la pentamidine, l'administration de NECT nécessite toujours une hospitalisation et reste lourde.

Dans un proche avenir, la détermination du stade de la THA due à *T.b. gambiense* deviendra probablement redondante. Il a été démontré récemment que le fexinidazole, un médicament administré par voie orale, est efficace, sûr et simple pour le traitement des deux stades de la THA. L'acoziborole, un nouveau médicament oral à dose unique, est actuellement testé pour tous les stades de la THA à *T.b. gambiense* dans un essai thérapeutique de phase II/III, et peut encore simplifier le traitement.

11.2 Suivi post-thérapeutique

Comme aucun des médicaments utilisés pour traiter la THA n'est efficace à 100 %, les patients traités doivent être suivis après le traitement. Jusqu'à récemment, pour évaluer le résultat thérapeutique, les patients devaient être suivis pendant 2 ans après traitement : à 3 et 6 mois, et tous les 6 mois par la suite. Un patient trypanosomé était déclaré guéri si aucun trypanosome n'était détecté et si le nombre de globules blancs dans le LCR était redevenu normal. La détection des trypanosomes pendant le suivi est une preuve absolue de l'échec du traitement, mais peut se produire tardivement. Ainsi, le diagnostic d'une rechute est souvent basé sur les symptômes cliniques et le nombre de globules blancs, bien qu'il n'y ait pas de directives précises là-dessus. En raison de l'augmentation des échecs du traitement au mélarsoprol, de la transmissibilité de l'infection, du risque de séquelles neurologiques et de décès, la détection précoce et précise de l'échec du traitement est vitale.

Notre approche pour raccourcir et améliorer le suivi post-thérapeutique a été double. Afin de proposer des seuils et des algorithmes pour son interprétation, nous avons d'une part examiné l'évolution du **nombre de globules blancs dans le LCR,** le paramètre le plus souvent utilisé pour évaluer les résultats du traitement. D'autre part, les **marqueurs pour la détermination du stade** ont été évalués quant à leur utilité dans le suivi post-thérapeutique. Nous avons également examiné certains marqueurs supplémentaires : le **CATT** sur le sérum et la **détection d'ADN et d'ARN**.

Pour ces études, des données et des échantillons biologiques avant traitement et pendant le suivi post-thérapeutique de patients trypanosomés étaient disponibles à partir de 2 groupes. Un premier groupe (groupe Bwamanda) comprenait des échantillons de sérum et de LCR provenant de 278 patients au deuxième stade avancé, ainsi que des échantillons biologiques provenant de 73 patients au premier stade et de 60 patients au deuxième stade précoce. Un deuxième groupe (groupe Mbuji-Mayi) était composé de spécimens biologiques de 360 patients atteints de THA, inclus dans une étude longitudinale sur le suivi post-

thérapeutique, réalisée à Mbuji-Mayi. Le foyer de THA de Mbuji-Mayi avait un taux d'échec thérapeutique de 59 % avec le mélarsoprol.

11.2.1 Nombre de globules blancs dans le suivi post-thérapeutique

Dans une première tentative pour établir des seuils concernant le nombre de globules blancs dans le LCR pour le suivi post-thérapeutique, 10 critères existants ont été évalués sur le groupe de Bwamanda (Tableau 6). Le critère d'échec « Présence de trypanosomes dans le sang, la lymphe ou le LCR OU ; augmentation du nombre de globules blancs dans le LCR jusqu'à ≥ 50/µl » a été retenu. Il est simple et ne fait pas référence aux valeurs de comptage précédentes. Sa sensibilité augmentait en fonction du temps de 56 % à 6 mois, 64 % à 12 mois, 78 % à 18 mois, alors qu'il était toujours > 97 % spécifique. Nous avons également observé que l'interprétation du nombre de globules blancs dans le LCR à 3 mois conduisait à des faux positifs et devait être évitée.

Lors d'une analyse plus poussée du nombre de globules blancs dans le liquide céphalo-rachidien chez les patients au deuxième stade du groupe Bwamanda, le nombre de globules blancs présentait une précision diagnostique acceptable à partir de 6 mois après le traitement. Les seuils entre 8 et 10 globules blancs/µl fournissaient le meilleur compromis entre spécificité et sensibilité. Nous avons également observé que dans le groupe des patients guéris, le nombre médian de globules blancs se normalisait déjà 6 mois après le traitement (Figure 7). Un marqueur diagnostique pour la guérison, permettant une sortie précoce des patients à faible risque de rechute, permettrait un suivi plus intensif du plus petit groupe de patients à haut risque. Un critère de ≤5 globules blancs/µl à six mois semblait prometteur à cet égard, car dans nos données, un sous-groupe de 63 % (92/146) des patients a présenté un « faible risque de rechute », laissant un sous-groupe de 37 % nécessitant un suivi intensif. Ce dernier groupe comprenait 81% (26/32) de toutes les rechutes.

Dans le groupe Mbuji-Mayi, on a constaté que le nombre de globules blancs dans le LCR présentait une précision diagnostique acceptable à partir de 3 mois après le traitement. Dans ce groupe, les seuils d'environ 20 globules blancs/µl ont fourni le meilleur compromis entre spécificité et sensibilité. Sur la base de ces observations, nous avons construit 4 algorithmes avec des durées de suivi variables (Tableau 7) :

- Lors de toute visite de suivi, les patients avec ≤5 globules blancs/µl ont été considérés comme « guéris » (arrêter le suivi). Les patients avec ≥50 globules blancs/µl ou des trypanosomes ont été considérés comme en « échec » (retraitement). Les patients avec 6-49 globules blancs/µl ont été considérés comme en « évolution incertaine » (continuer le suivi).
- Lors du dernier test de guérison (6, 12, 18 et 24 mois post-traitement pour les algorithmes D, C, B et A), les patients avec présence de trypanosomes ou >20 globules blancs/µl ont été classés comme en « échec thérapeutique », ceux avec ≤ 20 globules blancs/µl ont été considérés comme guéris.

La précision de ces algorithmes a été vérifiée par rapport aux données des patients du 2ème stade du groupe Mbuji-Mayi (Figure 8, Tableau 7) et du groupe Bwamanda (Tableau 7). Nous avons retenu l'algorithme C pour le suivi avec une spécificité d'environ 98 %, et des sensibilités de respectivement 94 et 79 % dans les groupes Mbuji-Mayi et Bwamanda :

- 6 mois après le traitement, les patients avec ≤5 globules blancs/µl sont considérés comme « guéris » et arrêtent le suivi. Les patients avec ≥50 globules blancs/µl ou des trypanosomes sont en « échec thérapeutique » et reçoivent de nouveau un traitement. Les patients avec 6-49 globules blancs/µl sont en « évolution incertaine » et continuent le suivi.
- 12 mois après le traitement, les patients présentant des trypanosomes ou >20 globules blancs/µl sont en « échec thérapeutique » et doivent être retraités, les patients avec ≤20 globules blancs/µl sont guéris.

11.2.2 Marqueurs de stade pour le suivi post-thérapeutique

Dans le groupe de Bwamanda, les concentrations d'IL-10, le titre final LATEX/IgM, le LATEX/*T.b. gambiense* et la concentration totale en protéines ont été déterminés dans le LCR. Contrairement au nombre de globules blancs, qui présentait une bonne précision diagnostique à 6 mois après le traitement, les concentrations d'IL-10, le titre final LATEX/IgM et la concentration totale en protéines ne sont parvenus à une précision diagnostique suffisante qu'à partir de 12 mois seulement, LATEX/*T.b. gambiense* à partir de 18 mois. Les résultats du groupe de Bamanda ont été confirmés dans le groupe Mbuji Mayi : la précision de LATEX/IgM pour un diagnostic correct du résultat du traitement a été suffisante 12 mois seulement après traitement (sensibilité 69 %, spécificité 97 % au seuil ≥1: 16). Le manque de précision pendant le suivi post-thérapeutique du LATEX/IgM était principalement causé par la lente diminution d'IgM chez les patients guéris (Figure 9). En collaboration avec l'Université de Genève, la néoptérine, le CXCL10, CXCL13, ICAM-1, VCAM-1, MMP-9, β-2-microglobuline et IgM dans le LCR ont été déterminés sur un sous-ensemble du groupe Mbuji-Mayi. Le nombre de globules blancs, la néoptérine suivie par le CXCL13 ont montré les plus grandes précisions pour le diagnostic du résultat du traitement. Ceux-ci ont été vérifiés sur le reste du groupe Mbuji-Mayi (n = 242). Trois mois après traitement, les précisions diagnostiques de la néoptérine, de la CXCL13 et du nombre de globules blancs étaient similaires. 6 et 12 mois après traitement, la néoptérine était plus précise que le nombre de globules blancs (Tableau 8). La néoptérine a donc été retenue comme un marqueur prometteur pour l'évaluation précoce des résultats du traitement.

11.2.3 CATT, détection d'ADN et d'ARN pour le suivi post-thérapeutique

La présence d'anticorps spécifiques anti-trypanosomes dans le sang, détectables par test CATT, a été démontrée jusqu'à 24 mois après un traitement réussi. Nous avons examiné la valeur d'un CATT normalisant comme marqueur de guérison. Nous avons observé que les titres de CATT diminuaient après le traitement, tant chez les patients qui ont connu un échec thérapeutique que chez les patients guéris. Un résultat CATT négatif post-thérapeutique n'indique donc pas de guérison.

La PCR a été considérée comme prometteuse dans le diagnostic de la THA, la détermination du stade et le suivi post-thérapeutique. Cependant, très peu d'approches de PCR ont été évaluées sur un grand nombre de spécimens et aucune n'a été évaluée pour le suivi post-thérapeutique dans une étude longitudinale prospective. Nous avons donc évalué la PCR pour le diagnostic, la détermination du stade et le suivi post-thérapeutique du groupe Mbuji-Mayi. Nous avons utilisé des amorces ciblant le gène multi-copies de l'ARN ribosomique 18S. La PCR sur le sang a montré une spécificité de 99% (98-100%) pour le diagnostic de la THA, mais seulement 88 % (84-93%) de sensibilité. Pour la détermination du stade, la PCR sur le LCR a montré une sensibilité de 88% (85-92%) et une spécificité de 83% (71-95%). Pour le suivi post-thérapeutique, la PCR sur le sang n'était ni sensible (13-50%) ni spécifique (81-92%). Par ailleurs, la PCR sur le LCR a montré une précision diagnostique limitée avec des sensibilités allant de 40 à 72% et des spécificités de 56 à 84%. nQuelle que soit la source de la positivité de la PCR, le rôle de la PCR ou d'autres techniques de détection de l'ADN pour le suivi post-traitement de la THA reste incertain.

La détection d'ARN messager, en particulier de l'ARN « spliced leader » (SL), est considérée comme l'un des meilleurs marqueurs pour les organismes viables et a été proposée pour le suivi post-thérapeutique dans la THA. Nous avons donc évalué la détection de ARN SL, comme marqueur de guérison, dans le sang de 61 patients traités. La détection d'ARN SL a montré des spécificités de 100 et 98% dans le premier et deuxième essai. La détection d'ARN SL pourrait donc être un marqueur précis pour le suivi post-thérapeutique.

11.2.4 Impact sur le terrain et perspectives

Nos observations ont mis en évidence le rôle du nombre de globules blancs dans le LCR et de la néoptérine comme marqueurs pour le suivi post-thérapeutique dans la THA. Une preuve de principe du potentiel de la détection de l'ARN SL a été obtenue.

L'intérêt de notre algorithme « C » a été confirmé par Epicentre et MSF sur des données provenant de l'Ouganda, du Soudan, de l'Angola, de la République centrafricaine, de la République du Congo et de la RD Congo, correspondant à 2190 patients atteints de THA *gambiense*. Compte-tenu des observations récentes selon lesquelles le taux de rechute est ≤2 % avec NECT et <5 % avec pentamidine, que le taux de suivi en routine est extrêmement bas, et que les patients traités qui deviennent symptomatiques retournent à l'hôpital, l'OMS a adapté ses recommandations pour le suivi. Pour la routine, aucun suivi systématique n'est recommandé, afin que le suivi et les examens du LCR se concentrent sur les patients symptomatiques. Dans les cas où un examen de suivi est effectué, notre algorithme « C » a été adopté. La recommandation de l'OMS ci-dessus ne s'applique pas aux nouveaux schémas thérapeutiques ou aux essais cliniques pour lesquels une évaluation précise et précoce des résultats du traitement reste déterminante.

En raison de la normalisation rapide de la néoptérine dans le LCR immédiatement après le traitement, et son augmentation dans les échecs thérapeutiques, la néoptérine offre une grande précision pour le suivi post-thérapeutique et pourrait être un marqueur plus précoce et plus précis que le nombre de globules blancs dans le LCR. Une confirmation sur d'autres groupes reste indiquée.

Le manque de spécificité de la PCR au cours du suivi nous a surpris, mais a confirmé d'autres observations fragmentaires. La détection d'ARN SL peut représenter une alternative prometteuse, mais nécessite une confirmation supplémentaire. La nécessité de prélever des spécimens sur un tampon de stabilisation d'ARN a entravé la recherche. Les études antérieures n'ont pas recueilli d'échantillons de LCR sur ce type de tampon, ni d'échantillons biologiques provenant de cas d'échecs de traitement.

11.3 Développement et évaluation des tests diagnostiques

Le diagnostic de la THA à T. b. *gambiense* est généralement effectué par étapes (Figure 2). On procède tout d'abord au dépistage d'anticorps dans le sang, puis on recherche la présence de trypanosomes chez les personnes qui présentent des anticorps.

Afin d'améliorer le sérodiagnostic, notre laboratoire a investi dans la découverte d'antigènes améliorés et dans le développement de nouveaux formats de test. J'ai été particulièrement impliquée dans l'utilisation du « phage display » (expression phagique) pour identifier les **mimotopes peptidiques des glycoprotéines de surface variables** d'intérêt diagnostique. Des **tests de diagnostic rapide** (TDR) ont été récemment développés pour la THA, et j'ai contribué à l'évaluation de leur **précision diagnostique**.

Après le sérodiagnostic, les personnes avec des anticorps subissent des examens parasitologiques. Les études sur la performance diagnostique de la parasitologie sur des larges séries de patients sont rares. Dans le cadre de la surveillance de la THA, l'importance des analyses moléculaires à distance pourrait augmenter, mais là encore, les études à grande échelle manquent. Nous avons donc évalué la **précision diagnostique des techniques parasitologiques et moléculaires** pour le diagnostic de la THA.

Enfin, **l'évaluation externe de la qualité** de l'examen microscopique de la goutte épaisse pour le diagnostic du paludisme et de la THA souligne la nécessité 1) d'une formation régulière ; 2) d'une évaluation régulière de la qualité et 3) d'établir des SOP (procédures d'opération standardisées) claires pour améliorer les pratiques diagnostiques.

11.3.1 Mimotopes peptidiques de VSG

Ma recherche sur les antigènes améliorés reposait sur le principe de 1) l'identification d'une alternative synthétique pour les glycoprotéines variables de surface (VSG) natives LiTat 1.3 et 1.5 ; et 2) l'utilisation de « phage display » pour découvrir des mimotopes de VSG.

Nous avons appliqué deux approches différentes pour la recherche de mimotopes. Dans un premier temps, nous avons utilisé des anticorps monoclonaux contre LiTat 1.3 et LiTat 1.5 VSG, pour le « phage display ». En tenant compte du fait que les anticorps monoclonaux pourraient avoir manqué certains mimotopes ayant un potentiel diagnostique important, et que les systèmes immunitaires de la souris et de l'humain pourraient reconnaître différents épitopes, nous avons également utilisé des anticorps humains pour le « phage display ». Pour cette approche, des anticorps spécifiques de LiTat 1.3 et 1.5 VSG ont été purifiés à partir de sérums humains.

Les deux approches ont identifié des mimotopes peptidiques, et les peptides mimotopiques correspondants ont été synthétisés. En outre, un certain nombre de séquences de VSG contre lesquelles nous avons trouvé de nombreuses réactions (LiTat 1.5 VSG amino-acides 81-109 et 268-281, LiTat 1.3 VSG amino-acides 78-110 et 424-439) ont également été synthétisées.

La performance diagnostique de tous les peptides synthétisés et de la VSG native a été testée avec 102 sérums de patients trypanosomés et 102 contrôles négatifs (Tableau 9). Nous avons confirmé le potentiel diagnostique de certains peptides synthétiques, qui pourraient remplacer les VSGs LiTat 1.3 et LiTat 1.5, bien qu'ils soient moins performants.

11.3.2 Précision diagnostique de TDRs

J'ai contribué à l'évaluation de la première génération de TDR HAT Sero *K*-SeT qui a été commercialisé par Coris Bioconcept, Belgique. La preuve de principe (étude de phase 1), réalisée sur 99 plasmas de patients trypanosomés et de 99 témoins endémiques, mélangés à des globules rouges d'un volontaire sain, a montré une sensibilité de 93.9 % (87.9-99.9 %) et une spécificité de 99.0 % (96.5-100 %).

La précision diagnostique de HAT Sero *K*-SeT a été évaluée sur le terrain (RD Congo, étude de phase 2) sur du sang frais de 134 cas de THA et 356 témoins négatifs endémiques recrutés dans des centres de traitement et lors du dépistage actif. HAT Sero *K*-SeT a montré une sensibilité et une spécificité de respectivement 98.5 % (94.7-99.6 %) et de 98.6 % (96.8-99.4 %). La principale limite de cette étude était le fait qu'une partie des patients confirmés avaient été détectés lors d'un précédent dépistage CATT, impliquant un risque de surestimation de la sensibilité du test.

Une autre évaluation de phase 2 a été réalisée sur des TDRs de première génération commercialement disponibles, HAT Sero *K*-SeT et SD Bioline HAT (Standard Diagnostics, Corée). Cette étude rétrospective a utilisé pour la première fois des échantillons provenant d'Afrique de l'Ouest, incluant 231 patients trypanosomés et 257 témoins endémiques. Nous n'avons pas observé de différence significative de sensibilité entre HAT Sero *K*-SeT (99.1 %, 96.9-99.9 %) et SD Bioline HAT (99.6 %, 97.6-100 %). La spécificité des deux tests, respectivement 87.9 % (83.3-91.7 %) et 88.3 % (83.8-92.0 %) s'est révélée faible. La combinaison des 2 TDRs, augmentait la spécificité à 93.4 % (IC 89,6-96,1 %) en maintenant la sensibilité. Pour cette étude, un biais de sélection dû au dépistage précédent avec CATT ne pouvait par ailleurs être exclu. Autre limitation : l'utilisation de plasma conservé au lieu de sang frais. Jusqu'à présent, aucune étude prospective de phase 3 n'a été réalisée sur des suspects cliniques dans des centres de santé en utilisant tous les TDRs en parallèle. De plus, la performance des TDRs en d'Afrique de l'Ouest reste peu documentée.

11.3.3 Précision diagnostique des techniques parasitologiques et moléculaires

L'étude sur la performance des tests parasitologiques et de la PCR a inclus 237 personnes qui étaient séropositives au CATT sur une dilution de ¼ du sang ou présentaient des symptômes cliniques s de la THA. Cent quarante-trois de ces personnes ont été confirmées parasitologiquement (Tableau 10).

Aucun participant n'a été positif à la centrifugation par micro-hématocrite (mHCT) seul, ce qui montre qu'il n'y a aucun avantage à effectuer la mHCT lorsque la minicolonne (mAECT) ou mAECT sur buffy coat (BC) sont réalisées. La mAECT-BC était significativement plus sensible que la mAECT (91 % contre 80 %, p <0,001). Bien que mAECT-BC soit la technique parasitologique la plus sensible disponible à ce jour, elle est uniquement recommandée aux techniciens expérimentés à cause de sa technicité. La détection des trypanosomes dans l'aspiration des ganglions lymphatiques était positive à 39 %, mais elle a détecté 4 % des cas qui autrement seraient passés inaperçus. La combinaison de l'aspiration ganglionnaire et de l'examen mAECT (-BC) est donc recommandée.

Pour la PCR, le sang a été conservé sur du papier filtre (FP) et du tampon guanidine-EDTA (GE). Sur les cas confirmés parasitologiquement, la sensibilité de PCR-GE était similaire à mAECT-BC ou moins. La détection d'ADN n'offre donc pas un diagnostic plus sensible que la parasitologie. La répétabilité et la reproductibilité étaient modestes. Les problèmes de reproductibilité étaient plus graves chez les suspects sérologiques aparasitémiques, ce qui confirme des observations antérieures. Dans une étude distincte réalisée sur les mêmes échantillons, le résultat du test LAMP présentait des sensibilités similaires à la PCR. Cependant, l'extraction d'ADN a été réalisée dans un laboratoire de référence à partir de sang conservé dans un tampon GE. Le test LAMP sur le terrain est réalisé sur du sang conservé sur du papier filtre Whatman n°1, mais il reste à effectuer des études de précision de diagnostic de phase 2 et 3 en utilisant ce protocole.

11.3.4 Evaluation externe de qualité (EEQ)

Bien que la sensibilité des gouttes épaisses pour le diagnostic de THA soit faible (environ 25 %), la technique est adéquate pour les centres de santé non spécialisés qui n'ont pas d'électricité et ne peuvent pas appliquer de techniques de concentration. Bien que la lecture des gouttes épaisses pour le diagnostic de la THA semble simple, les observations de deux EEQ en RD Congo ont été alarmantes. Une première expérience a été réalisée dans le cadre d'une EEQ pour le paludisme incluant une lame avec des trypanosomes. Parmi les laboratoires participants, 50,4 % ont manqué le diagnostic de trypanosomiase. Dans l'EEQ suivante en RD Congo, une lame contenait des trypanosomes et une autre lame contenait des *Plasmodium falciparum* et des trypanosomes. La lame contenant uniquement des trypanosomes a été correctement lue par 45 % des participants. Cependant, 84 % n'ont pas vu les trypanosomes dans la lame de coïnfection paludisme-trypanosomiase. Seulement 13 % des participants ont reconnu les trypanosomes dans les deux lames. Ces résultats montrent que la formation et l'assurance qualité doivent être renforcées. Les techniques les plus sensibles de détection des trypanosomes, y compris le mAECT, reposent sur la reconnaissance des trypanosomes vivants. Dans le contexte d'élimination de la THA, le personnel de laboratoire est de moins en moins confronté à des trypanosomes. La mise en place d'un système de contrôle de qualité pour la mise en évidence des trypanosomes vivants, ainsi que pour la lecture sérologique, est considérée comme une priorité.

11.4 L'étude HAT-polyB

L'immuno-pathologie de la THA reste mal connue à ce jour. Nos connaissances viennent surtout des infections expérimentales à *T.b. brucei* chez les souris. La souris sert également de modèle pour le développement des vaccins. L'infection expérimentale par *T.b. brucei* provoque une destruction générale des lymphocytes B mémoire et efface la protection induite par les vaccins. Si ces résultats expérimentaux

sont confirmés dans la THA, cela impliquerait la nécessité d'une revaccination des patients atteints de THA après leur traitement. En outre, le développement d'un vaccin contre la THA serait difficile à réaliser.

Dans l'étude HAT-polyB, nous voulions étudier au niveau humain 1) la destruction des cellules B mémoire et 2) la persistance de la protection induite par les vaccins. Nous avons examiné si la THA affecte 1) les cellules B et T mémoire dans le sang périphérique; 2) les niveaux d'iso-agglutinines et les taux d'anticorps contre la rougeole; et 3) si ces effets sont réversibles après guérison. Dans le sang périphérique des patients trypanosomés, des pourcentages de lymphocytes B et T mémoire étaient significativement plus élevés par rapport aux témoins (Figure 13). Six mois après le traitement, le pourcentage de cellules T mémoire s'était normalisé alors que le pourcentage de cellules B mémoire ne s'était pas encore complètement normalisé. Les titres des iso-agglutinines de classe IgM étaient légèrement plus faibles dans la THA, ce qui pourrait indiquer un effet modéré sur la réponse des anticorps T-indépendants. Cet effet semblait partiellement réversible après traitement. Bien que les taux d'anticorps anti-rougeoleux étaient et restaient plus faibles chez les patients trypanosomés que chez les témoins (Figure 14), aucune différence significative n'a pu être observée dans le nombre d'individus ayant des concentrations supérieures au seuil de protection. Dans l'ensemble, nos données suggèrent que si un certain degré de perte d'immunité peut exister dans la THA, il ne semble pas cliniquement pertinent.

11.5 Recherches en cours

Entre-temps, l'épidémie de THA a été contrôlée et l'élimination de la THA a été ciblée. Néanmoins, des défis importants demeurent:

- **Le passage du dépistage actif à passif:** Le dépistage actif de la THA par des équipes mobiles spécialisées a fortement réduit la prévalence. À faible prévalence, sa rentabilité diminue. L'intégration du dépistage de la THA dans la routine des centres de santé périphériques devient cruciale. Le diagnostic de la THA par le système de santé périphérique avec ses ressources limitées nécessite des tests de diagnostic et des algorithmes adaptés.
- **Elimination n'est pas éradication:** Après l'élimination de la THA, une surveillance continue est nécessaire pour assurer la pérennité d'une « transmission zéro » et pour éviter des réémergences causées par des réservoirs restants de *T.b. gambiense*.
- De nouveaux médicaments pour le traitement de la THA au niveau du système sanitaire périphérique sont nécessaires. Les essais thérapeutiques sont ralentis par le suivi post-thérapeutique de 18 mois. En outre, en raison de ponctions lombaires répétées, les patients traités respectent rarement ce suivi. Des **tests précoces de guérison** sont nécessaires pour une évaluation plus rapide de l'efficacité du traitement.

Ces défis et observations constituent la base du projet DiTECT-HAT, un projet soutenu par le Partenariat pour les Essais Cliniques des Pays Européens et en Développement (EDCTP2, programme Horizon 2020), que je coordonne. L'objectif général de DiTECT-HAT est de fournir de nouveaux tests diagnostiques et des algorithmes d'un bon rapport coût-efficacité pour l'élimination de la THA due à *T.b. gambiense*. Le projet DiTECT-HAT consiste en 3 essais diagnostiques, chacun lié à l'un des concepts ci-dessus.

11.5.1 Dépistage passif (DiTECT-HAT-WP2)

L'objectif de l'essai diagnostique DiTECT-HAT-WP2 est de valider les performances de différents tests et algorithmes pour le dépistage passif de la THA. Ses objectifs spécifiques sont de déterminer les performances diagnostiques et les coûts de la détection passive des cas de THA dans les centres de santé périphériques dans les foyers de faible prévalence de THA en utilisant:

1. Des TDRs de la THA effectués sur du sang frais

2. Des combinaisons de TDRs de la THA sur du sang frais
3. Des algorithmes diagnostiques avec des TDRs sur sang frais et des tests de référence sérologiques et / ou moléculaires sur du sang séché sur papier filtre

L'étude DiTECT-HAT-WP2 (Figure 15) est menée dans des centres de santé et des hôpitaux en Guinée (n = 12), en Côte d'Ivoire (n = 10) et en RD Congo (n = 27). Des suspects cliniques sont testés avec plusieurs TDRs pour la THA. Ceux ayant au moins un résultat positif au TDRs, subissent un examen parasitologique et un prélèvement sanguin sur papier filtre pour des tests ultérieurs dans un laboratoire régional de référence (trypanolyse, LAMP, ELISA et PCR en temps réel). Si les tests du laboratoire de référence et les examens parasitologiques sont tous négatifs, le suspect est considéré comme indemne de THA. Si au moins un test de référence est positif, les examens parasitologiques sont répétés à deux reprises. Nous prévoyons de tester environ 20000 suspects cliniques, dont 2000 positifs en TDR et 120 patients atteints de THA. Comme nous allons collecter des informations complètes sur les coûts, nous serons en mesure d'estimer le coût de chaque test ou algorithme dans chaque circonstance. Les résultats nous permettront de proposer des algorithmes de tests pour détecter la THA, adaptés aux centres de santé périphériques et d'un bon rapport coût-efficacité.

11.5.2 Monitorage post-élimination (DiTECT-HAT-WP3)

L'objectif général de DiTECT-HAT-WP3 est de valider des algorithmes de tests sérologiques et moléculaires à haut débit pour une détection précoce d'une réémergence de la THA. Les objectifs spécifiques sont de déterminer la performance diagnostique, la faisabilité et le coût du monitorage dans les foyers où la THA a été éliminée, sur la base de:

1. Tests sérologiques à haut débit sur sang séché sur papier filtre
2. Tests moléculaires à haut débit sur sang séché sur papier filtre
3. Tests sérologiques et moléculaires à haut débit sur sang séché sur papier filtre
4. Algorithmes de TDRs sur sang frais avec des tests sérologiques et moléculaires à haut débit sur sang séché sur papier filtre

L'étude DiTECT-HAT-WP3 (Figure 16) est menée dans des foyers de faible à zéro prévalence en RD Congo, en Côte d'Ivoire et au Burkina Faso. Un agent de santé fait des visites de porte-à-porte pour prélever un échantillon de sang sur papier filtre et effectuer deux TDRs. Les papiers filtres sont envoyés au laboratoire de référence pour des tests d'ELISA, de trypanolyse, de LAMP et de PCR en temps réel. Les sujets positifs dans au moins un test - les TDR ou les tests à haut débit - sont revus deux fois pour une confirmation parasitologique. L'efficacité et le coût global des différents algorithmes de diagnostic seront étudiés. Les résultats de DiTECT-HAT-WP3 nous permettront de proposer un algorithme de tests et d'établir un seuil de réémergence, qui déclenchera l'envoi d'équipes mobiles spécialisées aptes à stopper la réémergence.

11.5.3 Test précoce de guérison (DiTECT-HAT-WP4)

L'objectif de l'étude DiTECT-HAT-WP4 est de valider la précision diagnostique de la concentration de néoptérine dans le LCR et la détection d'ARN SL dans le sang et le LCR pour le suivi post-thérapeutique. L'étude DiTECT-HAT-WP4 est intégrée dans l'étude thérapeutique phase II/III d'acoziborole. La combinaison des deux études permet d'évaluer de nouveaux marqueurs pour le suivi post-thérapeutique, sans nécessiter de ponctions lombaires ou veineuses supplémentaires. Si elle est suffisamment précise, la détection d'ARN SL dans le sang permettrait un suivi post-thérapeutique sans ponctions lombaires.

11.6 Perspectives de recherche sur le long terme

La THA, la trypanosomose animale, la leishmaniose et la maladie de Chagas, toutes dues à des parasites kinétoplastides de la famille des Trypanosomatidés, partagent des lacunes diagnostiques qui continuent de compliquer leur contrôle et leur élimination : l'évaluation difficile des résultats thérapeutique, l'existence de réservoirs et d'infections latentes, et la présence de multiples espèces.
Pour la THA à *T.b. gambiense*, outre les sujets abordés dans DiTECT-HAT, le développement de tests de confirmation simples et ultrasensibles reste une priorité. Des marqueurs d'infection latente, pouvant reconnaitre une auto-guérison, une infection latente et un individu qui développera la THA, seraient un atout. Un test sensible pour discriminer les sous-espèces, en particulier *T.b. gambiense* d'autres *Trypanozoon*, aiderait la recherche sur les réservoirs animaux.

Pour la trypanosomose animale, les TDRs qui détectent des anticorps contre *T. congolense* et *T. vivax* méritent d'être améliorés. Des tests simples pour la mise en évidence du parasite, applicables sur terrain, restent nécessaires. Les tests moléculaires capables de reconnaitre les espèces de *Trypanozoon* et les sous-espèces, sont importants pour différencier les pathogènes « légers » et « sévères » dans un contexte de commerce international et pour la prise en charge.

Pour la maladie de Chagas, les tests actuels sont suffisants pour la détection d'une infection aiguë à *T. cruzi* et pour le dépistage des donneurs de sang et d'organes. Un test précoce pour évaluer la réponse thérapeutique et des tests « point of care » pour les infections congénitales et chroniques sont considérés comme des priorités diagnostiques, ainsi qu'un test de pronostic des complications liées à la maladie.

Les principales lacunes dans le diagnostic de la leishmaniose sont la différenciation entre la maladie active et les porteurs asymptomatiques. Ceci comprend la différenciation des personnes infectieuses, de l'auto-guérison et de la progression vers une leishmaniose clinique. Un marqueur de guérison de la leishmaniose viscérale reste une priorité, compte tenu du développement de la leishmaniose dermique post-kala-azar et de son infectiosité pour les phlébotomes. Des tests diagnostiques fiables pour les formes dermiques de la leishmaniose, applicables dans les centres de santé, restent indispensables.

12. Selection of publications

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IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test

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Summary An increased IgM concentration in cerebrospinal fluid (CSF), occurring as a consequence of massive intrathecal IgM synthesis, is a marker of interest for diagnosis of the meningo-encephalitic stage in human African trypanosomiasis. However, in current practice, IgM in CSF is not determined because of the lack of a simple and robust test that is applicable in African rural regions where the disease prevails. We describe the development of a sensitive semiquantitative card agglutination test, LATEX/IgM, for IgM quantification in CSF. The test is simple and fast and the lyophilized reagent remains stable even at 45 °C. CSF end-titres obtained with LATEX/IgM parallel the IgM concentrations determined by nephelometry and enzyme-linked immunosorbent assay. Detection of intrathecal IgM synthesis is the most sensitive marker for CNS involvement in sleeping sickness. At a cut-off value of ≥ 8, the sensitivity and specificity of LATEX/IgM for intrathecal IgM synthesis are 89.4 and 92.7%. As a consequence, patients with LATEX/IgM end-titres ≥ 8 are likely to have intrathecal IgM synthesis, thus central nervous system involvement and therefore should be treated accordingly. Further studies should concentrate on the relationship between the LATEX/IgM end-titres, presence of intrathecal IgM synthesis and occurrence of treatment failures in patients treated with pentamidine.

keywords cerebrospinal fluid, IgM, stage determination, latex microspheres, card agglutination, *Trypanosoma brucei gambiense*, sleeping sickness, intrathecal IgM synthesis

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Introduction

Human African trypanosomiasis or sleeping sickness is caused by infection with the protozoan parasites *Trypanosoma brucei (T.b.) gambiense* or *T.b. rhodesiense*. Gambiense sleeping sickness occurs in West to Central sub-Saharan Africa whereas rhodesiense sleeping sickness is found east of the African rift. The disease is transmitted through the bites of infected tsetse flies. After the infective bite, trypanosomes spread and proliferate in blood and lymph, corresponding to the first or haemo-lymphatic disease stage, followed by central nervous system invasion corresponding to the second or meningo-encephalitic disease stage. As treatment is stage-dependent and treatment of the second stage is not without risks and requires hospitalization (Van Nieuwenhove 1999), differentiation between the disease stages is crucial. Although for stage determination and follow-up the examination of

cerebrospinal fluid (CSF) on cell count, total protein concentration and presence of trypanosomes is prescribed (WHO 1998), these criteria are not 100% accurate and in reality seldom combined. As a consequence, stage determination may be incorrect and diagnosis of relapse may be problematic.

The CSF of *T.b. gambiense* sleeping sickness patients in the meningo-encephalitic stage often contains high concentrations of IgM (Greenwood & Whittle 1973) originating partially from serum whose IgM concentration is about five times as high in *T.b. gambiense* patients as in controls (Bisser *et al.* 1997), but also, and mainly, from intrathecal IgM synthesis (Bisser *et al.* 1997, 2002; Lejon *et al.* 1998a). Such high CSF IgM concentrations are pathognomic for sleeping sickness in the meningo-encephalitic stage (Mattern 1968). Furthermore, successful treatment would be followed by a fall in CSF IgM levels (Greenwood & Whittle 1973), and relapses are characterized by high CSF IgM (Mattern 1967; Whittle *et al.* 1977). Even today IgM in CSF of sleeping sickness patients is rarely determined because of the lack of a test which is feasible in rural health settings in endemic regions.

Hence, our aim was to develop a simple, robust IgM quantification test for CSF which would not require sophisticated and expensive equipment, such as a nephelometer or enzyme-linked immunosorbent assay (ELISA) equipment, often not available in health centres where the disease is diagnosed. The reagent should be sufficiently stable to allow transport at ambient temperature and long-term storage. We developed a latex card agglutination test for semiquantitative detection of IgM in the CSF (Lejon *et al.* 1998b), which is easy to perform with only pipettes and a rotator – generally already in use for serological screening by card-agglutination test with stained trypanosomes (CATT) (Magnus *et al.* 1978). However, batch to batch reactivity variations were observed with this test, inherent to the use of different batches of polyclonal anti-IgM antibodies (personal observation).

Here we describe how coating of monoclonal antibodies on the latex particles by another coupling procedure eliminated batch to batch variation and reduced the reaction time to 5 min. Semi-quantitative IgM detection with LATEX/IgM was compared with quantitative IgM detection by nephelometry. The feasibility of the improved LATEX/IgM test in the field was studied in Sudan and in the Central African Republic. Sensitivity and specificity of LATEX/IgM for intrathecal IgM synthesis, which is the most sensitive marker for neuro-inflammation in sleeping sickness, are investigated and a cut-off for CSF LATEX/IgM end-titre representing intrathecal synthesis is established.

Based on LATEX/IgM results of 937 CSF samples from *T.b. gambiense* patients from several countries, the relationship of LATEX/IgM end-titres to the cell count and presence of trypanosomes in CSF, which are the conventional WHO criteria for stage determination in sleeping sickness, is shown.

Materials and methods

Preparation of LATEX/IgM reagent

One gram of carboxylated polystyrene latex (diameter 0.857 μm, 9 μeq. COOH, K1-080 red, Estapor France) was washed twice in 40 ml phosphate buffered sodium dodecyl sulphate (SDS) (0.01 M, pH 6, 0.01% SDS) by centrifugation (3400 *g*, 45 min, 4 °C). The latex was activated by resuspension of the sediment in 100 ml freshly prepared phosphate buffered SDS containing 10 mg/ml carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl, Pierce] and 6 mg/ml *N*-hydroxysuccinimide (Sigma). After 15 min of gentle mixing on an end-to-end mixer, the latex was centrifuged and the sediment was washed by resuspension and centrifugation in 40 ml HCl–SDS solution (2 mM HCl, 0.01% SDS) followed by resuspension and centrifugation in 40 ml of 2 mM HCl. The activated sediment was resuspended in 40 ml phosphate buffer (0.02 M, pH 7.4). For coupling, 10 mg of mouse anti-human IgM (clone MH15-1, provided as 2 mg/ml, purified from ascites, in 0.02 M Tris and 0.15 M NaCl, CLB, the Netherlands), diluted in phosphate buffer to a total volume of 10 ml, were added. The suspension was stirred overnight at ambient temperature and centrifuged. The sediment was washed three times by centrifugation in 60 ml of phosphate buffer. After the last centrifugation, the latex was resuspended in stabilization buffer (0.02 M Tris, pH 7.4, 0.8% NaCl, 1% BSA, 10% sucrose and 0.1% NaN_3) to a 1% w/v latex suspension. The suspension was sonicated on ice (Vibra-cell, 6 mm probe, amplitude 80, 1 min, pulse 3 s, 9 W output) and monodispersity was checked microscopically (400× magnification). Aliquots containing 10 mg of sensitized latex were dispensed in penicillin vials, snap-frozen in liquid nitrogen and lyophilized with the following temperature settings: 24 h at − 30 °C, 7 h at 0 °C and 24 h at 25 °C. Afterwards the vials were flushed with nitrogen gas and stoppered.

LATEX/IgM test protocol

Lyophilized latex reagent was resuspended with 1 ml phosphate buffered saline (PBS) (0.01 M, pH 7.4) supplemented with 5 mg/l phenol red for colour contrast. Twofold serial dilutions of CSF were prepared in the same buffer. On the reaction zone of a test card (white reaction zones with a diameter of 1.5 cm), 20 μl of latex reagent

were mixed with 20 µl of diluted or undiluted CSF and spread over the reaction zone. The card was rocked on a horizontal rotator (eccentric deviation 12 mm) at 70 rpm. After 5 min, the degree of agglutination was scored. Invisible or hardly visible agglutination was considered negative, visible up to maximal agglutination was considered positive. The end-titre of a CSF sample, defined as the highest dilution still yielding a positive result, was determined. When the CSF reacted undiluted, but no agglutination was observed in the 1:2 dilution, an end-titre of 1 was given. If no agglutination was obtained even with undiluted CSF, an end-titre of 0 was assigned.

Total IgM and albumin concentration in serum and CSF by nephelometry and ELISA

The IgM and albumin concentration in serum and CSF was determined by nephelometry (BN100, Dade Behring). In samples with an IgM concentration below the detection limit of nephelometry (4 mg/l), IgM was measured by a sandwich ELISA. Briefly, microplates were coated overnight with mouse antihuman IgM (CLB, the Netherlands), control wells received PBS. After blocking with a skimmed milk solution, the plates were incubated with CSF (twofold dilutions of 1:4–1:32 in PBS-Blotto), a standard (CSF containing 20 mg/l IgM, diluted 1:40–1:5120) and a control (CSF containing 24 mg/l IgM, diluted 1:40–1:320). Then the wells were filled with rabbit antihuman IgM peroxidase (DAKO, Denmark). Plates were developed using a commercial ABTS solution (Boehringer). The optical density was read at 415 nm (Multiskan RC Version 6.0, Labsystems) and the IgM concentrations in the CSF samples and the control were interpolated from the standard curve using the four-parameter algorithm provided with the software (Genesis Lite) of the reader.

Intra-assay variability, batch to batch variation and stability of the LATEX/IgM reagent

In order to test intra-assay variability of the reagent, the end-titres of two CSF samples were determined 20 consecutive times using the same vial of LATEX/IgM reagent. Batch to batch variation and stability of the reagent were checked with a set of five reference human serum samples, originating from sleeping sickness patients (R1–3), and normal controls (R4 and 5). These sera were conserved in capillaries of 20 µl at − 80 °C, and for each experiment, a new aliquot was used. Batch to batch variation was tested with LATEX/IgM reagent produced on seven different dates, using two different batches of mouse antihuman IgM for coupling onto the particles. Stability of the reagent was assessed after storage of LATEX/IgM reagent (batch 16/06/99) at 4 and 37–45 °C for up to 31 months. After 0 (immediately after lyophilization), 35, 92, 183, 289, 406, 497, 727 and 938 days, the reagent was tested.

For these experiments, a maximal variation in end-titre of 1 dilution factor, which can occur as a consequence of different interpretation of doubtful reactions, was considered acceptable.

Serum and CSF samples

All serum and CSF samples from sleeping sickness patients used for this study were collected during routine diagnostic activities. On blood, these include serological (antibody detection in blood or serum by CATT; Magnus *et al.* 1978) and parasitological examination (wet blood film, thick blood film, microhematocrite centrifugation or mini-anion exchange centrifugation technique). CSF was examined for stage determination of the disease (cell count and detection of trypanosomes) (Van Meirvenne 1999). No systematic screening for other infections or other neuro-inflammatory diseases in these patients was performed. The origin of the samples is specified for each experiment. No control CSF samples of nonsleeping sickness patients from endemic regions were available.

Based on CSF cell count and presence of trypanosomes, four patient groups were defined: a group with 0–5 cells/µl and no trypanosomes in the CSF (first stage patients), a group with 6–20 cells/µl and no trypanosomes in the CSF (early second stage patients), a group with ≤ 20 cells/µl and trypanosomes in CSF (another early second stage group) and a group with > 20 cells/µl or trypanosomes in the CSF (second stage patients).

Comparison between CSF end-titre in LATEX/IgM and the IgM concentration

The IgM concentrations of CSF samples from 435 *T.b. gambiense* patients before treatment, originating from Central-West Côte d'Ivoire (102 samples) and from Arua district, Northern Uganda (333 samples), were determined. Samples were grouped according to their LATEX/IgM end-titres and the mean IgM concentration for each group was calculated. Differences between the groups, mean IgM concentrations were assessed by ANOVA statistics (Pagano & Gauvreau 2000).

Intrathecal IgM synthesis and determination of a LATEX/IgM cut-off end-titre

We calculated the intrathecal fraction of 93 patients from Côte d'Ivoire and 205 patients from Northern Uganda,

whose complete serum and CSF albumin and IgM data were available. Twenty-eight patients had 0–5 cells/μl and no trypanosomes in CSF, 54 patients had 6–20 cells/μl and no trypanosomes in CSF, 43 patients had ≤ 20 cells/μl and trypanosomes in CSF and 173 patients had > 20 cells/μl. Blood–CSF barrier function was evaluated using the CSF/serum albumin quotient, Q_{Alb}. The age related upper reference limits for Q_{Alb} are 5×10^{-3} (up to 15 years), 6.5×10^{-3} (up to 40 years) and 8×10^{-3} (up to 60 years) (Reiber & Felgenhauer 1987). For each CSF/serum pair the maximum IgM quotient [Q_{Lim}(IgM)] in absence of intrathecal immunoglobulin synthesis was calculated using the formula

$$Q_{Lim}(IgM) = (a/b) \times (Q_{alb}^2 + b^2)^{1/2} - c$$

(with $a/b = 0.67$, $b^2 = 120 \times 10^{-6}$, $c = 7.1 \times 10^{-3}$). The intrathecally synthesized fraction of IgM, in percentage of total CSF IgM, was calculated as

$$IF_{IgM} = [1 - Q_{Lim}(IgM)/Q_{IgM}] \times 100$$

(with Q_{IgM} = measured CSF/serum IgM quotient) (Reiber & Peter 2001). An $IF_{IgM} > 0\%$ indicates intrathecal synthesis and was considered positive. Alternatively, the IgM quotient can be graphically represented in function of Q_{Alb} (Reiber & Peter 2001). The reference range of the blood derived IgM fractions in CSF is under the hyperbolic reference curve Q_{Lim}(IgM). Values above Q_{Lim}(IgM) represent the intrathecal IgM fractions as percentage of total CSF concentrations, independent of the blood-IgM concentration and blood–CSF barrier function, and can be directly read from the Reiber quotient diagrams, with the Q_{Lim} as 0% synthesis.

A modified receiver–operator characteristic (ROC) curve was constructed for estimation of sensitivity and specificity of the LATEX/IgM for intrathecal IgM synthesis (Jacobson 1998).

LATEX/IgM end-titre, cell count and presence of trypanosomes in CSF

The LATEX/IgM test was performed on CSF samples of 937 patients before treatment originating from Equator Province in R.D. Congo (259 samples), Central-West Côte d'Ivoire (104 samples), Arua district, Northern Uganda (205 samples), Southern Sudan (342 samples) and from Central African Republic (27 samples). LATEX/IgM testing on CSF samples from R.D. Congo, Côte d'Ivoire and Uganda was performed at the Institute of Tropical Medicine, samples from Sudan and Central African Republic were tested on the spot. A total of 191 patients had 0–5 cells/μl and no trypanosomes in CSF, 198 patients had 6–20 cells/μl and no trypanosomes in CSF, 51 patients had ≤ 20 cells/μl and trypanosomes in CSF and 497 patients had > 20 cells/μl.

Results

Intra-assay variability, batch to batch variation and stability of the LATEX/IgM reagent

The end-titres of two CSF samples, tested 20 consecutive times with the same batch of reagent, showed no variation, indicating minimal intra-assay variability. With one sample an end-titre of 8 was obtained 20 times, with the other CSF sample an end-titre of 128 was observed 20 times. The reactivity of seven batches of LATEX/IgM reagent (produced with two different batches of mouse antihuman IgM) with the references showed a maximum difference of one titre step in one reference sample, observed on one occasion, indicating minimal batch to batch variation.

All stored reagents remained monodisperse and the reaction with PBS was always negative when the stability of the reagent was checked. The reagent stored at 4 °C maintained its original reactivity throughout the experiment as reflected by the end-titres which differed maximally one titre step (Table 1). With the reagent stored at 45 °C, in all samples (R1–R5), differences of at most one titre step were observed, with one exception (R3), which differed two titre steps on one occasion. At day 406, an unexplained drop in end-titre from 3200 to 800 occurred in this sample.

Concordance between CSF end-titre in LATEX/IgM and the IgM concentration

The CSF end-titres obtained with LATEX/IgM in function of the corresponding IgM concentration in the samples are shown in Figure 1. A parallel increase of end-titre and CSF IgM concentration is observed. Although within each of the 11 end-titre groups, large variations of IgM concentrations can occur, the mean IgM concentrations of these groups differed significantly ($P < 10^{-3}$).

Intrathecal IgM synthesis and determination of a LATEX/IgM cut-off end-titre

Using the presence of intrathecal IgM synthesis ($IF_{IgM} > 0\%$) as the reference, the sensitivity and specificity of LATEX/IgM for intrathecal IgM synthesis were calculated on 289 patients for each cut-off and compared using a modified ROC curve (Figure 2). The best combination of sensitivity and specificity for intrathecal IgM synthesis were obtained at LATEX/IgM cut-off ≥ 8 (specificity 92.7%, sensitivity 89.4%), although at

Table 1 End-titres of five reference sera with LATEX/IgM reagent which was stored for up to 938 days at 4 and 37–45 °C

LATEX/IgM storage time	Storage temperature	R1	R2	R3	R4	R5	PBS
0 days		12800	6400	3200	800	400	Negative
35 days	4 °C	6400	6400	3200	800	400	Negative
	45 °C	6400	3200	3200	800	400	Negative
92 days	4 °C	6400	6400	1600	800	400	Negative
	45 °C	6400	3200	1600	400	400	Negative
183 days	4 °C	12800	6400	1600	800	200	Negative
	45 °C	12800	6400	1600	800	200	Negative
289 days	4 °C	6400	3200	3200	800	400	Negative
	45 °C	6400	3200	3200	800	400	Negative
406 days	4 °C	6400	3200	1600	800	400	Negative
	45 °C	6400	3200	800	800	400	Negative
497 days	4 °C	12800	6400	3200	800	400	Negative
	45 °C	12800	6400	3200	800	400	Negative
727 days	4 °C	12800	6400	3200	800	400	Negative
	45 °C	12800	6400	3200	800	400	Negative
938 days	4 °C	6400	3200	1600	800	400	Negative
	45 °C	12800	3200	1600	800	400	Negative

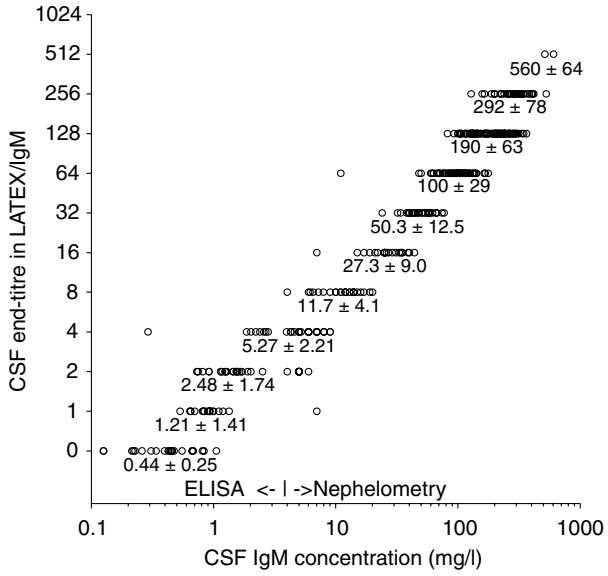

Figure 1 End-titre in LATEX/IgM of 435 CSF samples in function of the CSF IgM concentration measured by nephelometry (concentrations > 4 mg/l) and ELISA (concentrations < 4 mg/l). For each end-titre, the mean value ± standard deviation is indicated.

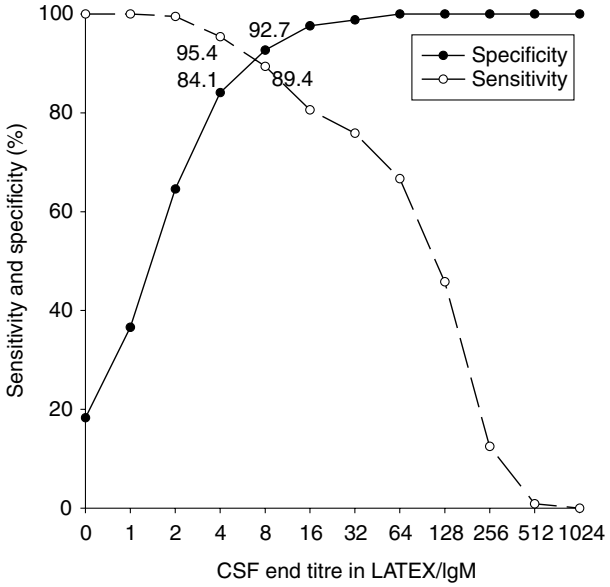

Figure 2 Modified receiver–operator characteristics curve of sensitivity and specificity for intrathecal IgM synthesis as a function of different cut-off LATEX/IgM end-titres.

cut-off ≥ 4 specificity and sensitivity were still high, 84.1 and 95.4%, respectively. The relationship between Q_{IgM}, Q_{Alb} and the LATEX/IgM cut-off end-titre of ≥ 8 is visualized in a Reiber quotient diagram (Figure 3).

Of the patients with 0–5 cells/μl and no trypanosomes in CSF, 7/28 had end-titres ≥ 8, including all four patients with intrathecal IgM synthesis. Among the patients with 6–20 cells/μl and no trypanosomes in CSF, 15/54 had end-titres ≥ 8, including 13/25 of the patients with intrathecal IgM synthesis. A total of 16/43 of the patients with ≤ 20 cells/μl and trypanosomes in CSF had end-titres ≥ 8, including 14/23 with intrathecal IgM synthesis. Of the 173 patients with > 20 cells/μl, 168 had an end-titre ≥ 8, including 162/164 of the patients with intrathecal IgM synthesis.

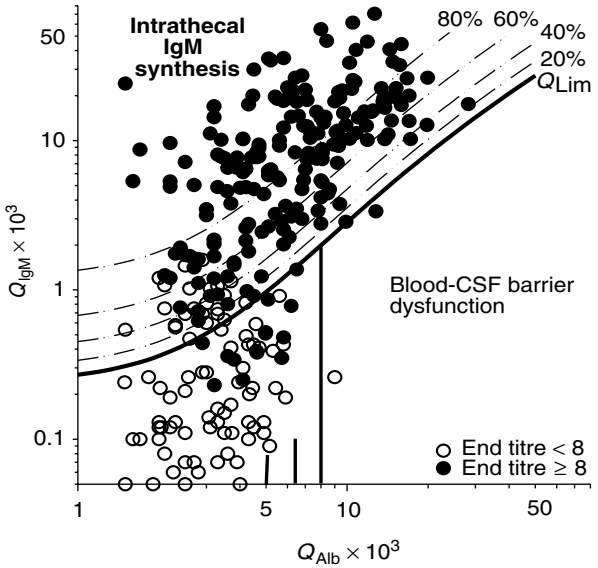

Figure 3 CSF/serum quotient diagram for IgM with hyperbolic graphs (Reiber & Peter 2001). The normal range of blood-derived IgM fraction in CSF is under the bold hyperbolic line Q_{Lim}. Values above the Q_{Lim} line represent intrathecal IgM fractions (IF_{IgM}), as percentage of total CSF concentration. The bold vertical lines indicate the age-dependent upper reference range for normal blood–CSF barrier function. Patients with LATEX/IgM end-titres < 8 are indicated by open circles, patients with LATEX/IgM end-titres ≥ 8 by black dots.

LATEX/IgM end-titre compared with CSF cell count and presence of trypanosomes

Using a cut-off value of ≥ 8, the number of positive and negative samples in a total of 937 *T.b. gambiense* patients, including the ones discussed above, were determined (Table 2). About 93.2% of the patients with > 20 cells/μl had CSF LATEX/IgM end-titres ≥ 8. Of the conventional first-stage patients (0–5 cells/μl, trypanosome negative), 11.5% were positive at this cut-off. Among the second-stage patients with ≤ 20 cells/μl (6–20 cells/μl and trypanosome negative, 0–20 cells/μl but trypanosome positive) between 32.8 and 39.2% were positive at cut-off ≥ 8.

Discussion

In view of the limitations of the currently used tests, and the importance of accurate diagnosis, first of the disease stage and secondly of possible relapses after treatment, alternative parameters for central nervous system involvement and follow-up in *T.b. gambiense* sleeping sickness patients are necessary. The massive intrathecal IgM synthesis in sleeping sickness leads to highly increased CSF IgM levels (Bisser *et al.* 2002). Detection of intrathecal IgM could therefore be replaced by the simple detection of IgM in CSF, which is, however, less accurate. IgM in CSF has been proven to be a marker of interest, but has never been used because of the technical limitations encountered at rural health centres in endemic regions.

Our latex agglutination assay for IgM quantification of sleeping sickness patients can be applied in the field, as was performed for this study in Sudan and in Central African Republic. The result, the end-titre or highest dilution still causing an agglutination reaction, corresponds well with the total IgM concentration in the sample. The detection limit of LATEX/IgM is around 1 mg/l, which is close to the upper limit for the normal CSF IgM concentration of 0.05–0.8 mg/l in Caucasian individuals (Felgenhauer 1998). Of the 407 CSF samples containing IgM concentrations higher than 0.8 mg/l, 99.3% had a LATEX/IgM end-titre higher or equal to 1. Application of LATEX/IgM for other neurological disorders accompanied by smaller increases in CSF IgM concentrations could therefore be studied. Application of the test for stage determination in *T.b. rhodesiense* patients remains to be evaluated, although IgM estimations in CSF for determination of meningo-encephalitic stage proved to be a valid indicator in *T.b. rhodesiense* sleeping sickness as in *T.b. gambiense* disease (Itazi 1981).

Intrathecal IgM synthesis indicates neurological involvement of *T.b. gambiense* infection (Greenwood & Whittle 1973; Lejon *et al.* 1998a; Bisser *et al.* 2002). Detection of intrathecal IgM synthesis (Reiber 1998) or of oligoclonal IgM (Sindic *et al.* 1994) in CSF are, however, not possible in most health centres or hospitals where the disease is endemic, while LATEX/IgM is.

Table 2 Number of positive and negative samples in four groups of *T.b. gambiense* patients using a cut-off LATEX/IgM CSF end-titre of ≥ 8

LATEX/IgM end-titre	0–5 cells/μl, no trypanosomes ($n = 191$)	6–20 cells/μl, no trypanosomes ($n = 198$)	≤ 20 cells/μl, trypanosome positive ($n = 51$)	> 20 cells/μl ($n = 497$)
LATEX/IgM < 8	169 (88.5%)	133 (67.2%)	31 (60.8%)	34 (6.8%)
LATEX/IgM ≥ 8	22 (11.5%)	65 (32.8%)	20 (39.2%)	463 (93.2%)

LATEX/IgM end-titres at a cut-off ≥ 8 show 92.7% specificity and 89.4% sensitivity for intrathecal IgM synthesis, reflecting CNS pathology. At this cut-off, the increased serum IgM concentration in sleeping sickness seems to influence the CSF concentration only to a limited extent. But we emphasize that this high cut-off end-titre is only valid for *T.b. gambiense* sleeping sickness with its high serum IgM concentrations, and that if LATEX/IgM were applied for other neurological diseases, a new, disease-specific cut-off should be determined based on occurrence of intrathecal IgM synthesis.

Of the classical first-stage patients, based on CSF cell count and presence of trypanosomes in the CSF, 11.5% show an increased (≥ 8) CSF end-titre in LATEX/IgM. More than half of these patients show intrathecal IgM synthesis thus central nervous system involvement and therefore might have relapsed after pentamidine treatment. Unfortunately, details on the outcome of the tested patients after treatment are not available. As a consequence, patients with a normal CSF cell count but with CSF end-titres of ≥ 8 in LATEX/IgM should be considered at risk for relapse when treated with pentamidine, and followed with special attention.

Among the *T.b. gambiense* patients with cell counts ≥ 20 cells/μl, intrathecal synthesis is observed in 95% of the patients, confirming that most, if not all of these patients, have CNS involvement and should be treated as such. This was confirmed by the end-titres, which were ≥ 8 in about 95% of the patients. Based on these results, application of LATEX/IgM on patients with > 20 cells/μl for decision on second-stage treatment seems redundant.

In the early second-stage groups (6–20 cells/μl and trypanosome negative, ≤ 20 cells with trypanosomes in CSF) about 34% of the patients have a CSF LATEX/IgM end-titre of ≥ 8, of which 87 and 94% also had intrathecal IgM synthesis. Current practice in Angola and Côte d'Ivoire is to treat patients with cell counts up to 20 cells/μl and in whom no trypanosome can be detected in CSF, with pentamidine (Doua *et al.* 1996; Stanghellini & Josenando 2001). The success of this approach is poorly documented. A recent clinical trial showed that 43% of such patients relapse after pentamidine treatment (D. Legros, personal communication). As discussed above, patients with end-titres ≥ 8 are to be considered at high risk of relapse if treated with Pentamidine.

Based on the results obtained with first and early second-stage patients, further studies should concentrate on the relationship between the LATEX/IgM end-titres, presence of intrathecal IgM synthesis and occurrence of treatment failure.

In addition to its use for stage determination, the use of LATEX/IgM for follow-up after treatment should be investigated based on evidence that relapses are characterized by a strong rise in CSF IgM (Whittle *et al.* 1977). The minimal batch to batch variation and the high stability of the LATEX/IgM reagent open perspectives for its use for follow-up of sleeping sickness patients. But at present there are not enough data on the evolution of CSF end-titres in LATEX/IgM during follow-up.

Acknowledgements

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MAJOR ARTICLE

How to Shorten Patient Follow-Up after Treatment for *Trypanosoma brucei gambiense* Sleeping Sickness

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Background. Clinical management of human African trypanosomiasis requires patient follow-up of 2 years' duration. At each follow-up visit, cerebrospinal fluid (CSF) is examined for trypanosomes and white blood cells (WBCs). Shortening follow-up would improve patient comfort and facilitate control of human African trypanosomiasis.

Methods. A prospective study of 360 patients was performed in the Democratic Republic of the Congo. The primary outcomes of the study were cure, relapse, and death. The WBC count, immunoglobulin M level, and specific antibody levels in CSF samples were evaluated to detect treatment failure. The sensitivity and specificity of shortened follow-up algorithms were calculated.

Results. The treatment failure rate was 37%. Trypanosomes, a WBC count of $\geqslant$100 cells/μL, and a LATEX/immunoglobulin M titer of $\geqslant$1:16 in CSF before treatment were risk factors for treatment failure, whereas human immunodeficiency virus infection status was not a risk factor. The following algorithm, which had 97.8% specificity and 94.4% sensitivity, is proposed for shortening the duration of follow-up: at 6 months, patients with trypanosomes or a WBC count of $\geqslant$50 cells/μL in CSF are considered to have treatment failure, whereas patients with a CSF WBC count of $\leqslant$5 cells/μL are considered to be cured and can discontinue follow-up. At 12 months, the remaining patients (those with a WBC count of 6–49 cells/μL) need a test of cure, based on trypanosome presence and WBC count, applying a cutoff value of 20 cells/μL.

Conclusion. Combining criteria for failure and cure allows follow-up of patients with second-stage human African trypanosomiasis to be shortened to a maximum duration of 12 months.

Human African trypanosomiasis (HAT) is endemic in sub-Saharan Africa. *Trypanosoma brucei gambiense* causes chronic HAT in West and Central Africa. In 2006, a total of 11,382 cases were reported [1]. There are 2 disease stages: first stage (characterized by the presence of trypanosomes in peripheral tissues and organs) and second stage (characterized by the presence of trypanosomes in the brain). Treatment is stage dependent, and untreated HAT is fatal.

Clinical management of HAT requires patient follow-up of 2 years' duration, with visits occurring at 3, 6, 12, 18, and 24 months after treatment [2]. At each follow-up visit, lymph, blood, and cerebrospinal fluid (CSF) samples are examined for trypanosomes, and CSF white blood cell (WBC) counts are determined. Compliance of patients with HAT with scheduled follow-up decreases with time, from 65%–85% at 12 months to 25%–70% at 24 months, whereas 40%–90% of relapses occur within 12 months and 70%–90% occur within 18 months [3]. For clinical research, 18 months of follow-up were recommended [3].

Patients with second-stage disease who had a CSF WBC count of $\leqslant$5 cells/μL at 6 months are at low risk of relapse [4], and defining relapse as "trypanosomes present and/or $\geqslant$50 WBC/μL CSF" allowed timely and

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accurate detection of relapse at any time point during follow-up [5]. Using detection of immunoglobulin M (IgM) and trypanosome-specific antibodies, in combination with the CSF WBC count and presence of trypanosomes, might also allow for a shorter follow-up [4] and, thus, improve patient comfort and facilitate HAT control.

This study investigates whether and how follow-up of patients with HAT can be shortened by applying novel biological criteria under field conditions. We report the treatment outcomes of a prospectively recruited cohort of patients, evaluate risk factors and biomarkers for treatment failure, and validate criteria for shorter follow-up periods.

METHODS

Study design. A longitudinal study was performed to evaluate biomarkers for monitoring the clinical outcome of patients treated for HAT. Primary outcomes of the study were relapse, cure, and death. The secondary outcome was the time of treatment failure. Ethics committees of The Ministry of Health, Democratic Republic of the Congo, and the University of Antwerp, Belgium, provided ethics clearance.

Study site. The study was conducted at Dipumba Hospital, Mbuji Mayi, East Kasai Province, Democratic Republic of the Congo. In 2005, the national control program (Programme National de Lutte contre la Trypansomiase Humaine Africaine [PNLTHA]) reported that the prevalence of HAT in this province was 0.84% among 327,498 persons screened. Treatment failure rates for patients treated with melarsoprol in Dipumba Hospital were close to 50% in 2003–2004 [6].

Patients. The study was performed between May 2005 and May 2008, with patient recruitment continuing until February 2006. Inclusion criteria were the presence of trypanosomes in lymph, blood, or CSF, regardless of disease stage; age $\geqslant$12 years; and residence within a 100-km perimeter around Mbuji-Mayi. Exclusion criteria were pregnancy, no guarantee of follow-up, moribund status, hemorrhagic CSF, and concurrent serious illness (tuberculosis or bacterial or cryptococcal meningitis). Patients who had never been treated previously for HAT (ie, treatment-naive patients) were classified as "primary cases," and those who presented with relapse at enrollment were classified as "retreatment cases." Informed consent was received from the patients or their guardian before enrollment.

Diagnosis. Each patient underwent a clinical examination. Whole blood was tested for specific antibodies, by use of the card agglutination test for trypanosomiasis (CATT) [7]. CATT-positive individuals and those showing suggestive clinical signs were examined for the presence of trypanosomes in lymph or in blood via capillary tube centrifugation or a mini-anion exchange centrifugation technique [8, 9]. A CSF WBC count was determined using disposable counting chambers (Uriglass; Menarini), and a search for trypanosomes was conducted using modified single centrifugation [10]. Retreatment cases were enrolled on the basis of a trypanosome-positive CSF sample. Disease staging was as follows: first stage was defined by a WBC count of 0–5 cells/μL and no trypanosomes present in CSF, and second stage was defined by a WBC count of >5 cells/μL and/or presence of trypanosomes in CSF. Trypanosome-specific antibodies and the total IgM level in CSF were measured using LATEX/*T. b. gambiense* and LATEX/IgM [11, 12]. All tests were repeated 24 h after the last drug administration and at each follow-up assessment. Demographic and clinical characteristics were recorded on a case report form and were entered into an Epi-Info database (version 3.4.3; Centers for Disease Control and Prevention and World Health Organization [WHO]). Human immunodeficiency virus (HIV) infection status was determined with the Vironostika HIV Uni-Form II Ag/Ab (bioMérieux), followed by the INNO-LIA HIV I/II Score (Innogenetics), if the finding was reactive. The INNOTEST HIV Antigen mAb enzyme-linked immunosorbent assay (Innogenetics) was used to detect early seroconversions. HIV polymerase chain reaction analysis of peripheral blood mononuclear cells was performed to confirm HIV infection [13, 14]. HIV-seropositive patients who consented to be informed about their status were referred to the HIV counseling service at Dipumba Hospital.

Treatment. Patients received free treatment and food during hospitalization. Pretreatment consisted of mebendazole (100 mg twice daily for 3 days) and a single dose of combination treatment with 500 mg of sulfadoxine and 25 mg of pyrimethamine (1 tablet per 20 kg of body weight). HAT treatment (Table 1) was given in accordance with PNLTHA guidelines.

Patient monitoring after treatment. Treatment outcome was assessed 1 day (ie, at the end of treatment) and at 3, 6, 12, 18, and 24 months after treatment. During follow-up, patients were classified as having the following outcomes, in accordance with WHO recommendations [3]: death, relapse, probable relapse, favorable evolution, or uncertain evolution (with the following adaptations). Probable relapse was defined by no trypanosomes detected and either a CSF WBC count that increased by >30 cells/μL, compared with the lowest previous WBC count, or neurologic signs not resulting from a condition other than HAT and requiring rescue treatment in the opinion of the physician in charge. Favorable evolution was defined as follows: for stage 1, a WBC count of $\leqslant$5 cells/μL and no trypanosomes detected; for stage 2, either no trypanosomes detected and a WBC count of $\leqslant$20 cells/μL or no trypanosomes detected and a WBC count that had not increased by >10 cells/μL, compared with the lowest previous value, and without clinical deterioration. Uncertain evolution was defined by no trypanosomes detected and failure to be classified as belonging to any other category. For test-of-cure

Table 1. Treatment Regimens Administered to Patients with Human African Trypanosomiasis (HAT) Who Were Included in a Study Performed at Dipumba Hospital, Democratic Republic of the Congo

Drug(s) and dosage(s)	Case status
Pentamidine, 4 mg/kg/day IM for 8 days	Primary case, first stage
Melarsoprol,[a] 2.2 mg/kg/day IV for 10 days	Primary case, second stage
	Retreatment case after treatment of first-stage disease with pentamidine
Melarsoprol, 1.8 mg/kg/day IV for 10 days, PLUS nifurtimox; 15 mg/kg/day PO for 14 days	Retreatment case with no intolerance to melarsoprol noted during previous treatment
Eflornithine, 4 × 100 mg/kg/day IV every 6 h for 14 days	Primary case, second stage
	Primary case, second stage, with intolerance to melarsoprol developing within 8 days of treatment
	Retreatment case with intolerance to melarsoprol having developed during previous treatment
	Retreatment case treated with melarsoprol-nifurtimox combination therapy that was prematurely terminated when nifurtimox was no longer available
	Retreatment case previously treated with melarsoprol-nifurtimox combination therapy
Eflornithine, 4 × 100 mg/kg/day IV every 6 h for 14 days, PLUS melarsoprol, 3.6 mg/kg/day IV; 3 series of 3 daily injections were administered at 7-day intervals	Retreatment case, with no drug intolerance noted during previous treatments with melarsoprol, eflornithine, or a combination of both
Eflornithine, 4 × 100 mg/kg/day IV every 6 h for 14 days, PLUS nifurtimox, 15 mg/kg/day PO for 14 days	Retreatment case; patients had previously received treatment with eflornithine or had developed intolerance to previous treatment with regimens containing melarsoprol

NOTE. IM, intramuscular; IV, intravenous; PO, by mouth.

[a] At the first sign of reaction, treatment was interrupted; treatment received until day 8 was considered to be complete.

(ToC) assessment at 24 months or later, WHO recommendations were followed [3].

Data analysis. Tolerance windows of 2–4, 5–9, 10–16, 17–21, and ⩾22 months were defined around the scheduled follow-up at 3, 6, 12, 18, and 24 months [3]. Patients attaining a final treatment outcome (nonresponder status, relapse, probable relapse, or death) before the ToC assessment at 24 months were excluded from data analysis at subsequent time points. EpiInfo software (version 3.4.3) and Stata software (version 10; Stata) were used for data analysis, with statistical significance denoted by $P = .05$. Comparisons between treatment regimens or algorithms were done with Kruskal-Wallis, Pearson's $\chi 2$, or Fisher's exact tests.

Patients classified as experiencing relapse, probable relapse, failure to respond, or death possibly related to HAT during follow-up were considered to have treatment failure. To calculate the risk factors for treatment failure, to determine marker accuracy, and to evaluate algorithms for follow-up, only data for patients with second-stage disease were analyzed, excluding patients whose deaths were not associated with HAT and those with unknown treatment outcomes. To assess the risk of treatment failure, adjusted odds ratios were estimated based on logistic regression models.

The accuracy of continuous markers (the CSF WBC count and the LATEX/IgM titer) in the determination of treatment failure was assessed by constructing the receiver operator characteristic (ROC) curve and calculating the area under the curve (AUC). When the AUC value was >0.80, the Youden index was determined for the whole range of cutoff values, and the cutoff value with the maximal Youden index was retained. The sensitivity and specificity of some additional cutoff values for the WBC count (⩽5 cells/μL, ⩽20 cells/μL, and ⩾50 cells/μL) were calculated [3–5]. For the binary marker (LATEX/*T. b. gambiense*), accuracy was estimated by calculating sensitivity, specificity, and Youden index.

Several clinical algorithms for detecting failure in association with a shorter follow-up were constructed and evaluated using the cohort data. The best algorithms were validated using an independent data set obtained in a clinical trial conducted between 1998 and 2001 in Bwamanda, Democratic Republic of the Congo [4, 5, 15].

RESULTS

Study population. A total of 360 patients with HAT were enrolled (Figure 1). Their characteristics at baseline are shown in Table 2. More male patients than female patients were enrolled in the groups for the treatment of second-stage disease ($P < .001$), but the sex ratio was not different from 1 in the group with first-stage disease. Two hundred forty-two patients were primary cases, and 118 were retreatment cases; 41 had first-stage disease, and 319 had second-stage disease. Trypan-

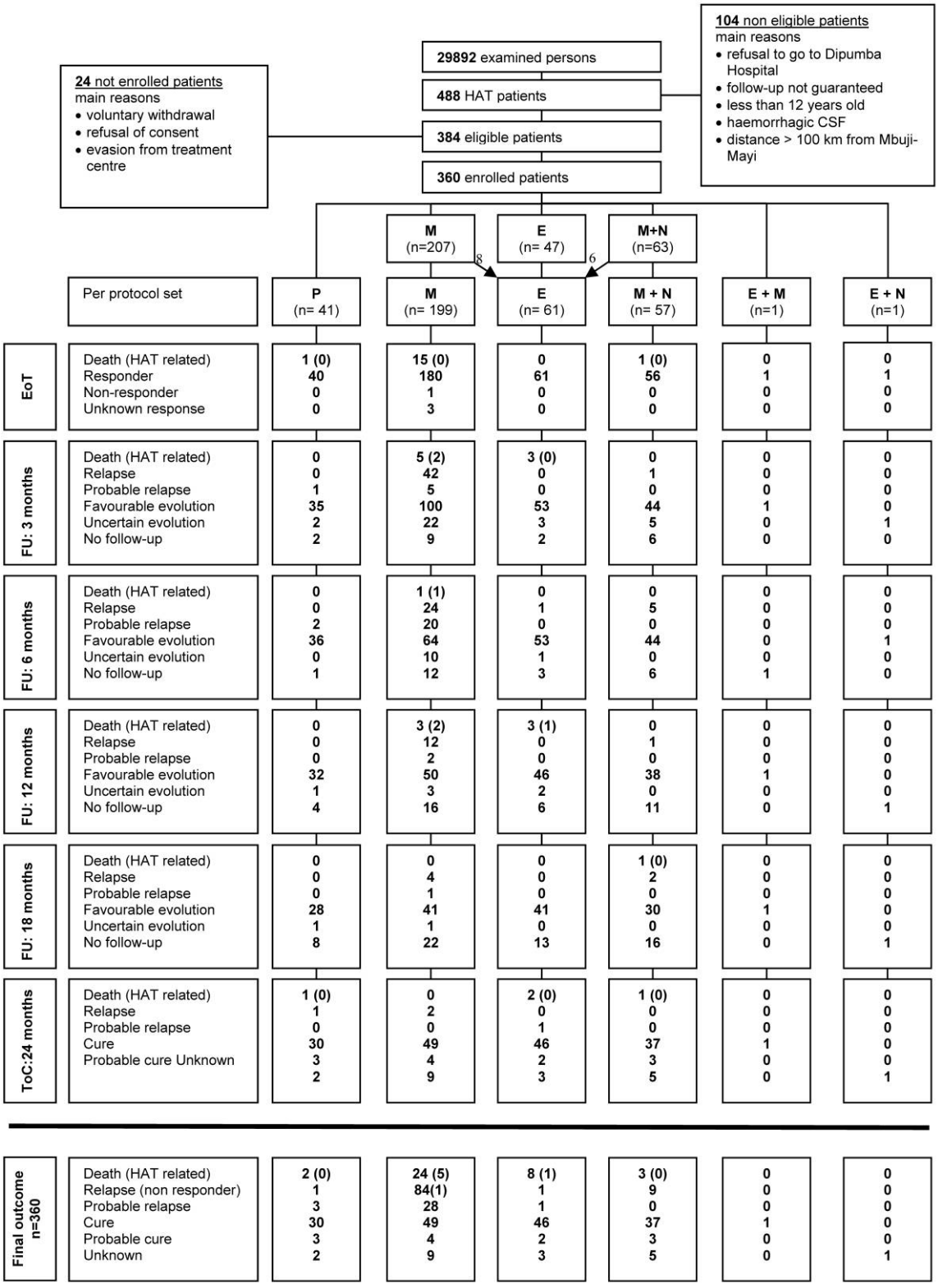

Figure 1. Overview of study profile. CSF, cerebrospinal fluid; E, eflornithine; E-M, eflornithine-melarsoprol combination therapy; E-N, eflornithine-nifurtimox combination therapy; EoT, end-of-treatment assessment; interim FU, follow-up assessments performed at 3, 6, 12 and 18 months; HAT, human African trypanosomiasis; M, melarsoprol; M-N, melarsoprol-nifurtimox combination therapy; N, nifurtimox; P, pentamidine; ToC, test-of-cure assessment performed 24 months after treatment.

Table 2. Baseline Characteristics of 360 Patients Enrolled in the Study, by Treatment Received

Characteristic	All patients (n = 360)	P (n = 41)	M (n = 199)	E (n = 61)	M-N (n = 57)	M-E (n = 1)	E-N (n = 1)
Demographic							
Ratio of male patients to female patients, no.	250:110	18:23	143:56	44:17	44:13	1:0	0:1
Age, mean ± SD, years	34 ± 12	35 ± 13	34 ± 13	34 ± 11	34 ± 12	32	28
Weight, mean ± SD, kg	56 ± 10	53 ± 9	55 ± 10	58 ± 9	59 ± 11	60	63
Height, mean ± SD, cm	168 ± 10	163 ± 9	167 ± 10	170 ± 9	169 ± 10	167	170
Active:passive case detection ratio[a]	61:299	…	…	…	…	…	…
Primary:retreatment case ratio[b]	242:118	41:0	192:7	9:52	0:57	0:1	0:1
Trypanosomes detected[c]							
In lymph	166/227	30/39	133/182	3/6	0/0	0/0	0/0
In blood							
By CTC	24/86	6/11	18/65	0/8	0/0	0/0	0/0
By mAECT	17/59	5/5	8/45	4/8	0/0	0/0	0/0
In CSF							
In cell-counting chamber	152/360	0/41	96/199	30/61	25/57	1/1	0/1
By modified single centrifugation	142/208	0/41	78/103	31/31	32/32	0/0	1/1
CSF biological							
WBC count, mean ± SD, cells/μL	267 ± 295	2 ± 1	329 ± 341	276 ± 216	233 ± 179	214	208
LATEX/IgM titer, mean ± SD	70 ± 100	1 ± 3	75 ± 112	84 ± 84	92 ± 90	16	64
LATEX/*T. b. gambiense* result, n/N[d]	259/357	3/41	150/197	56/61	48/56	1/1	1/1
HIV infection status, n/N[d]	11/360	0/41	9/199	1/61	1/57	0/1	0/1

NOTE. CSF, cerebrospinal fluid; CTC, capillary tube centrifugation; E, eflornithine; E-N, eflornithine-nifurtimox combination therapy; HIV, human immunodeficiency virus; IgM, immunoglobulin M; M, melarsoprol; mAECT, mini-anion exchange centrifugation technique; M-E, melarsoprol-eflornithine combination therapy; M-N, melarsoprol-nifurtimox combination therapy; N, nifurtimox; P, pentamidine; SD, standard deviation; *T. b. gambiense, Trypanosoma brucei gambiense;* WBC, white blood cell.

[a] Data are the no. of patients with active case detection/no. of patients with passive case detection.

[b] Data are the ratio of patients considered to be "primary cases" (ie, patients who had never been treated previously for human African trypanosomiasis [treatment-naive patients]) to patients considered to be "retreatment cases" (ie, patients who presented with relapse at enrollment).

[c] Data are the no. of patients with trypanosomes detected/no. of patients assessed.

[d] Data are the no. of patients with a positive result/no. of patients assessed.

osomes were detected in the CSF of 294 patients. Most patients with second-stage disease had high CSF WBC counts (mean ± SD, 301 ± 297 cells/μL) and positive results of LATEX/*T. b. gambiense* performed on CSF samples. HIV prevalence was 3.1% (11 of 360 patients; 95% confidence interval [CI], 1.6%–5.6%).

Treatment outcomes. The patients (Figure 1) were treated as follows: 41 received pentamidine; 207 started receiving melarsoprol (M) monotherapy, but 8 developed intolerance to M before day 8 and had treatment switched to eflornithine (E); 63 received combination therapy with M and nifurtimox (M-N), but 6 had treatment switched to E when nifurtimox (N) was no longer available; 1 received combination therapy with M and E; and 1 received combination therapy with N and E. A total of 61 patients received E monotherapy. A total of 250 patients completed prescribed treatment without interruptions, and 66 patients (28 who were receiving E, 15 who were receiving M, and 23 who were receiving M-N) experienced minor temporary treatment interruptions. Forty-four patients (1 who was receiving E, 36 who were receiving M, and 7 who were receiving M-N) had treatment terminated before the total prescribed dosage was administered; however, the required minimum dosage [3] was reached for all.

Adherence to follow-up examinations (including lumbar puncture) was, respectively, 94.4% (323 of 342 patients) at 3 months, 91.9% (262 of 285 patients) at 6 months, 83.6% (194 of 232 patients) at 12 months, 71.6% (151 of 211 patients) at 18 months, and 84.2% (171 of 203 patients) at 24 months. It was not significantly different between treatment groups.

The overall cure rate was 48.6% (175 of 360 patients), and the case-fatality rate was 10.3% (37 of 360 patients). Seventeen patients died during treatment: 5 (4 receiving M and 1 receiving M-N) died of advanced HAT, 10 died of M toxicity, 1 (who was receiving M) died of septicemia; and 1 (who was receiving P) died of an unknown cause. During follow-up, 20 deaths occurred, 6 of which were due to HAT (5 patients who died were receiving M, and 1 patient was receiving E); 1, to E toxicity; 1, to hepatorenal failure (the patient was receiving M); 1, to status epilepticus (the patient was receiving E); and 11, to unknown causes (5 patients were receiving E, 3 were receiving M,

2 were receiving M+N, and 1 was receiving P). At the end of treatment, 1 patient with trypanosomes in CSF was classified as a nonresponder. The overall treatment failure rate was 36.9% (133 of 360 patients): 1 patient had no response, 94 experienced relapse, 32 had a probable relapse, and 6 died of HAT-related causes during follow-up. In the remainder of this article, those events will be jointly classified as denoting "failure." The highest failure rate, 58.8% (117 of 199 patients), was observed in the M group, and the lowest failure rate (0%–5%) was observed for patients receiving regimens containing E. One relapse and 3 probable relapses occurred in the P group (9.8% [4 of 41 patients]). Of all failures, 38.3% (51 of 133 patients) were detected within 3 months, 78.2% (104 of 133 patients) within 6 months, 91.7% (122 of 133 patients) within 12 months, and 97.0% (129 of 133 patients) within 18 months.

Characteristics at baseline as risk factors for treatment failure in patients with second-stage disease. A WBC count of $\geqslant$100 cells/μL at baseline ($P = .008$), presence of trypanosomes in CSF ($P = .005$) at baseline, and a LATEX/IgM titer of $\geqslant$1:16 at baseline ($P < .001$) were significantly associated with higher failure rates after adjustment for drug regimen in patients with second-stage disease. Sex, age, trypanosome-specific antibodies (LATEX/*T. b. gambiense*), early treatment termination, and HIV infection status were not (Table 3). In the final logistic regression model, only the drug regimen and the LATEX/IgM titer at baseline were retained as independent predictors of failure.

Posttreatment evolution of CSF parameters in patients with second-stage disease. In patients considered to be cured, the median CSF WBC count decreased from 213 cells/μL to 36 cells/μL at the end of treatment and steadily decreased during follow-up (Figure 2), whereas among patients considered to have experienced treatment failure, it decreased from 259 cells/μL to 59 cells/μL at the end of treatment, increased to 101 cells/μL at 6 months, and subsequently decreased to 37 cells/μL at 24 months. In cured patients, median LATEX/IgM titers were 1:64 before treatment and decreased to 1:2 at 18 and 24 months, whereas in patients with treatment failure, they decreased until 3 months, stabilized at 1:16 between 3 and 12 months, and decreased further thereafter. The proportion of patients with a positive LATEX/*T. b. gambiense* result decreased from 82% before treatment to 14% at 24 months in patients considered to be cured, whereas in patients with treatment failure, it decreased from 80% before treatment to 52% at 3 months and increased to 56%–68% between 6 and 18 months.

Accuracy of criteria for detection of treatment failure in patients with second-stage disease. For the CSF WBC count, the AUC was >0.80 from months 3 to 24 (table 4). The Youden index was highest at 12 and 18 months, with 87%–89% sensitivity and 100% specificity at cutoff values of 23 and 29 cells/μL, respectively (table 4), although a cutoff value of >20 cells/μL had similar high sensitivities and specificities. A cutoff WBC count of >5 cells/μL was 89%–96% sensitive, and a cutoff WBC count of $\geqslant$50 cells/μL was 95%–100% specific.

For the CSF LATEX/IgM titer, the AUC was >0.80 at 12 months only (0.84; 95% CI, 0.72–0.96), with a cutoff value of $\geqslant$1:16. Sensitivity was 69%, and specificity was 97%.

For trypanosome-specific CSF antibodies, the maximum Youden index was 0.43 at 12 months. Sensitivity was 65%, and specificity was 78%.

Evaluation of algorithms for shortening the duration of follow-up. On the basis of the information presented above, we constructed several algorithms with variable durations of follow-up and used a composite definition to discriminate between cure and treatment failure at each follow-up visit. According to this definition, patients with a WBC count of $\leqslant$5 cells/μL at any follow-up visit are considered to be "cured" and do not require further follow-up, whereas those with a count of $\geqslant$50 cells/μL are considered to have "failure" and should receive rescue treatment. Trypanosome-negative patients with a WBC count of 6–49 cells/μL are considered to have "uncertain evolution" and should continue follow-up. At the final ToC assessment, patients with trypanosomes present or >20 WBCs/μL are considered to have "treatment failure." Combining these follow-up and ToC criteria, 4 algorithms were formulated, and their accuracy was checked against our study data (Figure 3).

In algorithm A, follow-up lasted for 24 months. Of patients with second-stage disease with known treatment outcome, 85 were considered to be cured at 6 months, 40 at 12 months, 7 at 18 months, and 10 at 24 months (for a total of 142 patients, including 4 whose outcomes were wrongly classified); 56 patients are considered to have treatment failure at 6 months, 10 at 12 months, and 6 at 18 months (for a total of 72 patients, including 2 whose outcomes were wrongly classified). Over 24 months, specificity is 98.6% (138 of 140 patients; 95% CI, 95%–100%) and sensitivity is 94.6% (70 of 74 patients; 95% CI, 87%–99%).

In algorithm B, follow-up lasted for 18 months. Eighty-five patients were considered to be cured at 6 months, 40 at 12 months, and 9 at 18 months (for a total of 134 patients, including 4 whose outcomes were wrongly classified); 56 patients were considered to have treatment failure at 6 months, 10 at 12 months, and 6 at 18 months (for a total of 72 patients, including 2 whose outcomes were wrongly classified). Specificity was 98.5% (130 of 132 patients; 95% CI, 95%–100%), and sensitivity was 94.6% (70 of 74 patients; 95% CI, 87%–99%).

In algorithm C, follow-up lasted for 12 months; 85 patients were considered to be cured at 6 months and 50 at 12 months (for a total of 135 patients, including 4 whose outcomes were wrongly classified); 56 patients were considered to have treatment failure at 6 months and 15 patients at 12 months (for a

Table 3. Risk Factors at Baseline for Treatment Failure in 272 Patients with Second-Stage Gambiense Sleeping Sickness

	Patients with treatment failure		Unadjusted		Adjusted for treatment		Multivariate model[a]	
Risk factor	*n/N*	%	OR	*P*	OR	*P*	OR	*P*
Treatment				<.001[b]				<.001[b]
E	3/51	6	1				1	
M	117/171	69	35.62				46.1	
M-E	0/1	0	–				–	
M-N	9/49	18	3.60				1.56	
Sex				.392		.655		
Female	39/75	52	1		1			
Male	91/197	46	0.79		0.86			
Age, years				.894		.726		
<25	34/69	49	1		1			
25–39	54/117	46	0.88		1.18			
⩾40	42/86	49	0.98		0.90			
WBC count, cells/μL				.091		.008[b]		
<100	22/63	35	1		1			
100–199	32/58	55	2.29		4.22			
200–399	38/79	48	1.73		3.21			
⩾400	38/71	54	2.15		1.95			
Trypanosomes in CSF				.467		.005[b]		
No	8/20	40	1		1			
Yes	122/252	48	1.41		1.52			
LATEX/IgM titer				.019[b]		<.001[b]		<.001[b]
<1:16	7/26	27	1		1		1	
⩾1:16	122/241	51	2.78		6.97		6.97	
LATEX/*T. b. gambiense* result				.611		.203		
Negative	26/51	51	1		1			
Positive	103/219	47	0.85		0.83			
Early treatment termination				.108		.648		
No	112/243	46	1		1			
Yes	18/29	62	1.91		1.22			
HIV infection status				.140		.334		
Negative	124/264	47	1		1			
Positive	6/8	75	3.39		2.42			

NOTE. Forty-seven patients were excluded because of missing data. CSF, cerebrospinal fluid; E, eflornithine; E-N, eflornithine-nifurtimox combination therapy; HIV, human immunodeficiency virus; IgM, immunoglobulin M; M, melarsoprol; M-E, melarsoprol-eflornithine combination therapy; M-N, melarsoprol-nifurtimox combination therapy; N, nifurtimox; OR, odds ratio; *T. b. gambiense; Trypanosoma brucei gambiense;* WBC, white blood cell.

[a] Multivariate model obtained by stepwise model selection using all factors significant in treatment-adjusted analysis.

[b] Statistically significant difference ($P<.05$).

total of 71 patients, including 3 whose outcomes were wrongly classified). Over 12 months, specificity was 97.8% (131 of 134 patients; 95% CI, 94%–100%), and sensitivity was 94.4% (68 of 72 patients; 95% CI, 86%–98%).

In algorithm D, the ToC criterion was applied at 6 months; 136 patients were considered to be cured (including 13 patients whose outcomes were wrongly classified); 77 patients were considered to have treatment failure (including 13 patients whose outcomes were wrongly classified). Specificity was 90.4% (123 of 136 patients; 95% CI, 84%–95%), and sensitivity was 83.1% (64 of 77 patients; 95% CI, 73%–91%).

To validate our findings, we applied these algorithms to a set of data for patients with second-stage and intermediate HAT who were treated with M, N, and a combination of both drugs (M-N) in another clinical trial [4, 5, 15]. The specificity and sensitivity for algorithm B were 98.9% (173 of 175 patients; 95% CI, 96%–100%) and 84.2% (32 of 38 patients; 95% CI, 69%–94%), respectively, and those for algorithm C were 98.8%

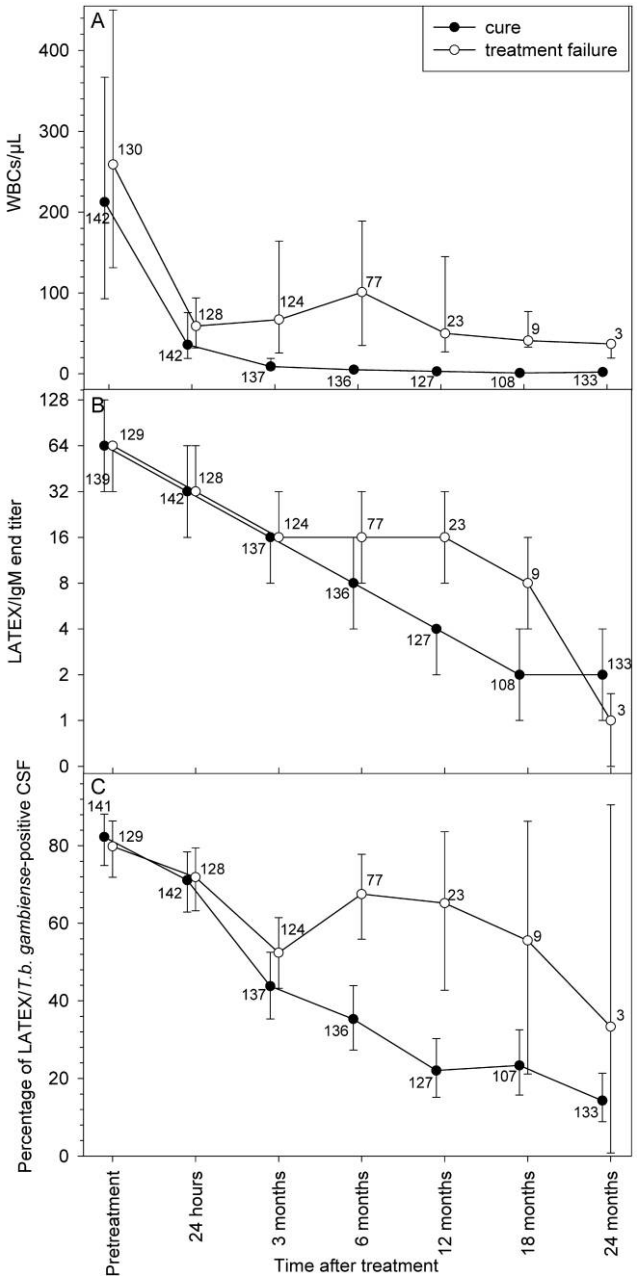

Figure 2. Evolution of the cerebrospinal fluid (CSF) white blood cell (WBC) count (expressed as the median and interquartile range) *(top),* LATEX/immunoglobulin M titer (expressed as the median and interquartile range) *(middle),* and LATEX/*Trypanosoma brucei gambiense* positivity (expressed as the percentage of CSF-positive patients and 95% confidence interval) *(bottom)* after treatment of human African trypanosomiasis in patients with second-stage disease. The no. of patients is indicated next to the data point.

(167 of 169 patients; 95% CI, 96%–100%) and 79.4% (27 of 34 patients; 95% CI, 62%–91%), respectively. For algorithm A, specificity was 99.5% (207 of 208 patients; 95% CI, 97%–100%), and sensitivity was 85.4% (35 of 41 patients; 95% CI, 71%–94%).

DISCUSSION

The present study was conducted to assess how the recommended 24 months of posttreatment follow-up for patients with HAT could be shortened using field-applicable criteria. The overall relapse rate in the cohort was 37%, but it was 59% after treatment with M for 10 days, a rate that is much higher than that reported elsewhere [16]. High rates of treatment failure after M monotherapy have also been observed in the Equateur province, Democratic Republic of the Congo [17]; in Uganda [18]; and in Angola [19]. It remains unclear whether these high rates are associated with the parasite or with patient characteristics. In 2006, PNLTHA replaced the 10-day M regimen with 14 days of E or M-N combination therapy, which subsequently reduced the treatment failure rate.

The >71% rate of adherence to follow-up in this cohort study was excellent and was achieved thanks to active tracing of patients who defaulted from follow-up. A lack of means of transportation was the main obstacle to completion of follow-up. Financial constraints constituted not only a barrier for early diagnosis and treatment [20] but also for follow-up compliance.

Within 6 months after treatment, 78% of all relapses were detected, a rate that is considerably higher than that noted in other studies with active follow-up: 17%, (in the 2004 study by Priotto detailed in [3]), 33% [16], and 50% [21]. The early relapse rate might be associated with the low efficacy of M, but it may also result from active tracing of patients defaulting from follow-up combined with the use of a very sensitive trypanosome detection technique, the modified single centrifugation of CSF. Also, the choice of criteria for relapse and probable relapse influences the sensitivity for and delay in detection of treatment failure [5].

HIV prevalence among the patients was 3.1%. This prevalence corresponded to the rates reported for the adult population in the study region [22].

After adjustment for the drug regimen received, a baseline CSF WBC count of $\geqslant$100 cells/μL, presence of trypanosomes in CSF, and a LATEX/IgM titer of $\geqslant$1:16 were found to be risk factors for treatment failure, a finding that corresponds to observations noted elsewhere [4, 16, 18, 23]. Although treatment failure was more common among HIV-positive patients, the difference was not significant.

To prevent serious sequelae resulting from late detection of trypanosomes during follow-up, it has been suggested to administer rescue treatment to patients for whom treatment failure (ie, those who are trypanosome negative) is suspected on the basis of indirect markers. The WBC count, LATEX/IgM titer, and trypanosome-specific antibody titer in CSF have been identified as markers for treatment failure [4, 24, 25], and we investigated their potential for earlier assessment of treatment outcome. At 3 months of follow-up, diagnosis of treatment failure should exclusively be based on trypanosome detection

Table 4. Sensitivity and Specificity of the Cerebrospinal Fluid White Blood Cell (WBC) Count for Detection of Treatment Failure in Patients with Second-Stage Gambiense Sleeping Sickness, by Different Follow-Up Time Points and Different Cutoff Values

Time after treatment	Patients with relapse, no.	Patients with a cure, no.	AUC (95% CI)	Cutoff WBC count, cells/μL	Sensitivity	Specificity	Youden Index
3 months	124	137	0.86 (0.82–0.91)	>30	0.73	0.88	0.61
				>5	0.96	0.32	0.28
				>20	0.80	0.77	0.57
				⩾50	0.57	0.95	0.52
6 months	77	136	0.93 (0.89–0.97)	>25	0.83	0.96	0.79
				>5	0.95	0.60	0.55
				>20	0.83	0.90	0.73
				⩾50	0.70	0.99	0.69
12 months	23	127	0.92 (0.82–1.00)	>23	0.87	1.00	0.87
				>5	0.91	0.87	0.79
				>20	0.87	0.99	0.86
				⩾50	0.52	1.00	0.52
18 months	9	108	0.92 (0.77–1.00)	>29	0.89	1.00	0.89
				>5	0.89	0.94	0.83
				>20	0.89	1.00	0.89
				⩾50	0.33	1.00	0.33
24 months	3	133	0.85 (0.55–1.00)	>36	0.67	1.00	0.67
				>5	0.67	0.99	0.66
				>20	0.67	1.00	0.67
				⩾50	0.00	1.00	0.00

NOTE. AUC, area under the curve; CI, confidence interval.

(ie, relapse), because we previously observed that relying on the CSF WBC count may result in many false-positive results [5], even if, in the present study, the WBC count had good discriminatory power at that time point. For clinical trials, the WHO recommends choosing between a follow-up assessment at 3 or 6 months, depending on the expected efficacy of the study drug [3]. At 6 months of follow-up, the WBC count is definitely more reliable than that noted at 3 months, with an AUC of 0.93. At 12 months of follow-up, both the WBC count and the LATEX/IgM titer could be used, but the AUC value of the latter is inferior. For CSF trypanosome-specific antibodies, the maximal Youden index was observed at 12 months but was only 0.43. Consequently, the CSF WBC count was the marker that distinguished cures from treatment failures soonest and most accurately.

A marker for cure could allow shortening of the duration of follow-up of patients with a low risk of treatment failure [4, 24], thus leading to a considerable workload reduction. We investigated, for the first time, the usefulness to follow-up of a combination of a criterion for treatment failure and a criterion for cure based on 2 markers, presence of trypanosomes, and the CSF WBC count, distinguishing 3 patient groups. Group 1 patients with a WBC count of ⩽5 cells/μL from 6 months onward and no trypanosomes detected are at low risk for treatment failure and are not to return for additional follow-up [4]. Group 2 patients with a WBC count of ⩾50 cells/μL and/or detection of trypanosomes at any follow-up assessment are considered to have treatment failure [5]. Group 3 patients with a WBC count of 6–49 cells/μL and with no trypanosomes detected are considered to have "uncertain evolution status" and should be told to attend the next scheduled follow-up assessment. For ToC assessment, a cutoff value of >20 cells/μL is used, as in other studies [3].

For patients with first-stage disease, the algorithm analysis could not go beyond 6 months, when follow-up would have been terminated in 34 of 36 patients and continued in 2 patients, with a sensitivity of 33.3% and a specificity of 100%. Because these 2 patients were, in reality, considered to have probable relapses, rescue treatment was administered, and no follow-up data were available after 6 months. Therefore, algorithm analysis was restricted to patients with second-stage disease. Application of algorithm B or C would result in a considerable reduction in the number of patients needing further follow-up (beyond 18 and 12 months, respectively) and shorter duration of follow-up. Because their sensitivity was identical, and because the difference in specificity was not statistically significant ($P = 1$), algorithm C, with a ToC assessment at 12 months, seems to be most appropriate for HAT

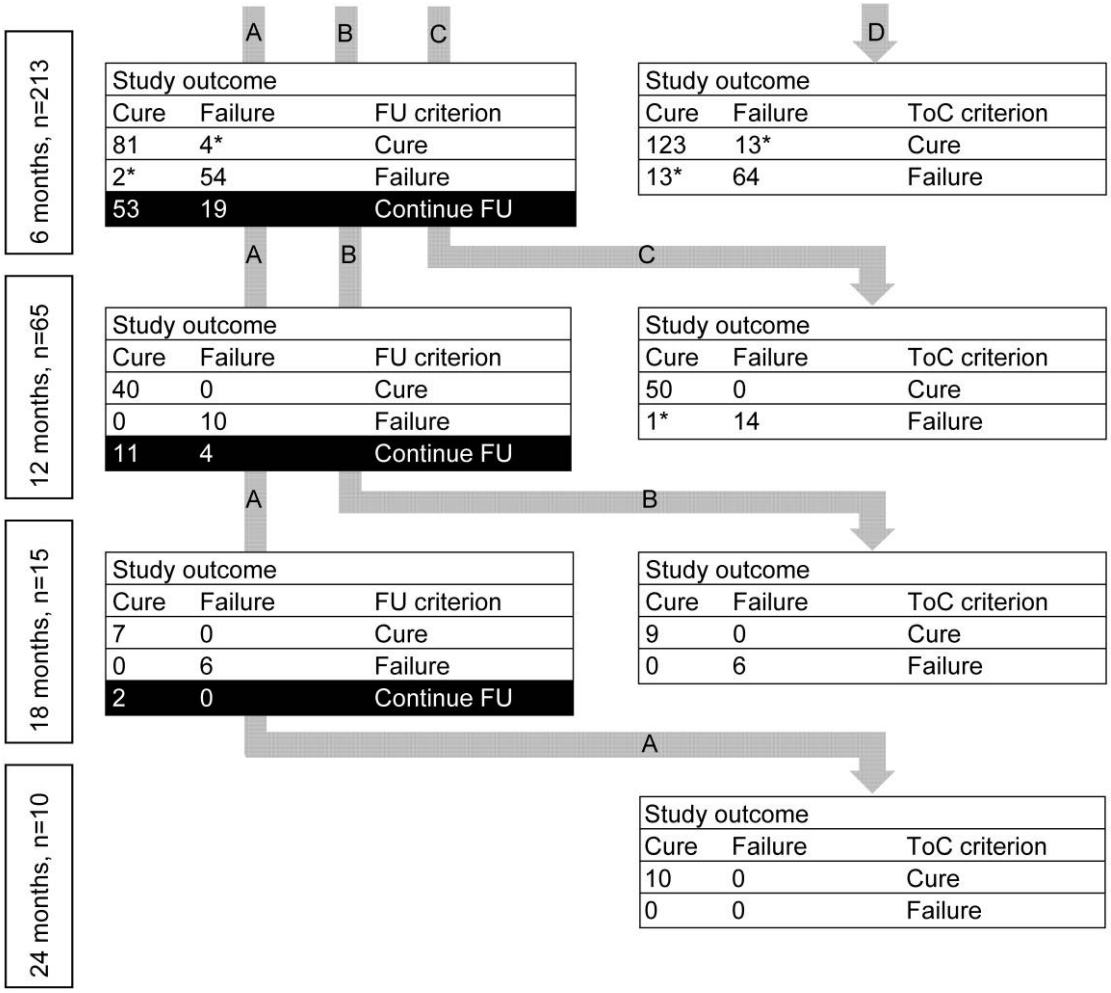

Figure 3. Effect of 4 algorithms *(A–D)* on the duration of follow-up (FU) of enrolled patients with second-stage human African trypanosomiasis (HAT), on the basis of a combination of 2 different criteria for treatment failure and cure. The FU criteria were as follows: for cure, ⩽5 white blood cells (WBCs)/μL cerebrospinal fluid (CSF) and no trypanosomes detected; for failure, ⩾50 WBCs/μL or presence of trypanosomes; for continued FU, 6–49 WBCs/μL and no trypanosomes detected. The criteria for test-of-cure (ToC) assessment were as follows: for cure, ⩽20 WBCs/μL and no trypanosomes detected; for failure, >20 WBCs/μL or presence of trypanosomes. *Wrongly classified outcome (not corresponding with real treatment outcome).

control and clinical trials. This algorithm showed a high specificity on both data sets on which it was tested. With sensitivities of 79% and 94%, a risk of missing treatment failures remains; therefore, patients classified as cured should be instructed to return to follow-up if they feel sick.

Our study had the following limitations. Although several investigated markers had cutoffs based on studies of other foci, and although many of our observations were similar to those of other investigators, our conclusions are based on a cohort in Mbuji Mayi, where the treatment failure rate noted after M monotherapy was unusually high. When we validated our algorithms in a second cohort with lower treatment failure rates, a lower sensitivity was obtained, although the difference was not significant. We were constrained to restrict our algorithm analysis to patients with second-stage disease, because the number of patients with first-stage disease was low. Our analysis comprised only field-applicable tests, whereas more-sophisticated techniques (like polymerase chain reaction, inflammation-related proteins, and/or brain damage markers [26]) might allow reducing the follow-up duration even further. Patients experiencing relapse after previously receiving HAT treatment were included in the cohort. The majority of our patients were treated with M, which increasingly is being replaced by E or N-E combination therapy. Because the timing of treatment failure might be drug regimen dependent, it may be useful to assess the efficacy of the proposed follow-up strategy retrospectively and/or prospectively in other patient cohorts before implementing it on a large scale.

In conclusion, by using simple trypanosome detection and the CSF WBC count as follow-up criteria at 6 months and by

introducing "no trypanosomes and ⩽20 WBCs/μL CSF" as criteria for cure at 12 months, posttreatment follow-up of patients with second-stage *gambiense* HAT may be considerably reduced, from the current 2-year duration to a maximum duration of 12 months. With fewer lumbar punctures (a 74% reduction), overall patient comfort improves considerably.

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Identification of Mimotopes with Diagnostic Potential for *Trypanosoma brucei gambiense* Variant Surface Glycoproteins Using Human Antibody Fractions

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Abstract

Background: At present, screening of the population at risk for *gambiense* human African trypanosomiasis (HAT) is based on detection of antibodies against native variant surface glycoproteins (VSGs) of *Trypanosoma brucei* (*T.b.*) *gambiense*. Drawbacks of these native VSGs include culture of infective *T.b. gambiense* trypanosomes in laboratory rodents, necessary for production, and the exposure of non-specific epitopes that may cause cross-reactions. We therefore aimed at identifying peptides that mimic epitopes, hence called ''mimotopes,'' specific to *T.b. gambiense* VSGs and that may replace the native proteins in antibody detection tests.

Methodology/Principal Findings: A Ph.D.-12 peptide phage display library was screened with polyclonal antibodies from patient sera, previously affinity purified on VSG LiTat 1.3 or LiTat 1.5. The peptide sequences were derived from the DNA sequence of the selected phages and synthesised as biotinylated peptides. Respectively, eighteen and twenty different mimotopes were identified for VSG LiTat 1.3 and LiTat 1.5, of which six and five were retained for assessment of their diagnostic performance. Based on alignment of the peptide sequences on the original protein sequence of VSG LiTat 1.3 and 1.5, three additional peptides were synthesised. We evaluated the diagnostic performance of the synthetic peptides in indirect ELISA with 102 sera from HAT patients and 102 endemic negative controls. All mimotopes had areas under the curve (AUCs) of $\geq$0.85, indicating their diagnostic potential. One peptide corresponding to the VSG LiTat 1.3 protein sequence also had an AUC of $\geq$0.85, while the peptide based on the sequence of VSG LiTat 1.5 had an AUC of only 0.79.

Conclusions/Significance: We delivered the proof of principle that mimotopes for *T.b. gambiense* VSGs, with diagnostic potential, can be selected by phage display using polyclonal human antibodies.

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Introduction

The chronic form of sleeping sickness or human African trypanosomiasis (HAT) in West and Central Africa is caused by the protozoan parasite *Trypanosoma brucei (T.b.) gambiense* while *T.b. rhodesiense* causes a more fulminant, acute form in East and Southern Africa. Both subspecies of *T. brucei* are cyclically transmitted by tsetse flies of the genus *Glossina* and mainly affect poor, rural populations. The true burden of this disease is unknown as many cases remain undiagnosed or unreported [1,2].

Since untreated HAT is almost always fatal and no inexpensive, safe and easily administered drugs are available, accurate case detection is crucial. Parasite detection is laborious and insensitive, and remains therefore limited to disease suspects. In the absence of reliable clinical symptoms or antigen detection tests, HAT suspects are identified through screening of the population at risk for presence of trypanosome specific antibodies. The commonly used antibody detection tests, card agglutination test for trypanosomiasis (CATT) [3], LATEX/*T.b. gambiense* and ELISA/*T.b. gambiense* [4,5] detect antibodies against the highly immunogenic variant surface glycoproteins (VSGs) of *T.b. gambiense*. Even though the genome of *T. brucei* contains >1000 VSG genes, only one variable antigen type (VAT) is expressed at a time. Stochastic switching of VSG allows the trypanosome to evade the specific antibody responses that were raised against earlier VATs [6–10]. Some VATs, such as LiTat 1.3 and 1.5, are recognised by almost all *gambiense* HAT patients and therefore called predominant. The dense VSG monolayer on the living trypanosome shields all non-specific epitopes. The hypervariable N-terminal VSG domain (300–400 residues) is exposed to the immune system and comprises the VAT-specific epitopes, while the relatively conserved C-terminal domain (40–80 residues) is hidden by the intact VSG coat [6,9,11,12].

Author Summary

Control of the chronic form of sleeping sickness or *gambiense* human African trypanosomiasis (HAT) consists of accurate diagnosis followed by treatment. We aim to replace the native variant surface glycoprotein (VSG) parasite antigens that are presently used in most antibody detection tests with peptides that can be synthesised *in vitro*. Antibodies recognising VSG were purified from HAT patient sera and were used to select phage-expressed peptides that mimic VSG epitopes from a Ph.D.-12 phage display library. The diagnostic potential of the corresponding synthetic peptides was demonstrated in indirect ELISA with sera from HAT patients and endemic negative controls. We proved that diagnostic mimotopes for *T.b. gambiense* VSGs can be selected by phage display technology, using polyclonal human antibodies.

Disadvantages of the present antibody detection tests include the occurrence of non-specific reactions. This might be explained by exposure of non-HAT-specific epitopes that are normally shielded on the living trypanosome [12,13]. In addition, diagnostic test production actually requires culture of infective *T.b. gambiense* in large numbers of laboratory rodents and poses an important risk of infection to the manufacturing staff [14].

These drawbacks can be circumvented through the use of synthetic peptides that mimic HAT-specific VSG epitopes (mimotopes) and can be produced in a standardised way [15]. One way to identify such mimotopes is by peptide phage display. This technique is based on DNA recombination resulting in foreign peptides with random sequences that are displayed fused to the pIII surface protein of the M13 phage. After an *in vitro* selection process based on binding affinity and several rounds of enrichment (panning), the encoded peptide insert sequence of the selected phage is deduced from the phage DNA. We previously reported successful identification of mimotopes for VSG LiTat 1.3 and LiTat 1.5 by performing phage display with three monoclonal antibodies [16]. However, by the use of only three monoclonal antibodies, representing only a fraction of the VSG-specific antibody response, some mimotopes with diagnostic potential might have been missed. Additionally, the mouse and human immune system may recognise different B cell epitopes. The use of polyclonal human antibodies might therefore increase chances of selecting diagnostic mimotopes [17]. Polyclonal antibodies from human sera have been previously used for selection of mimotopes with diagnostic potential for e.g. hepatitis C [15], typhoid fever [18] and Epstein Barr virus [17]. Some mimotopes have been patented for incorporation in commercially available tests, e.g. for neurocysticercosis [19].

In this manuscript we describe the identification of mimotopes for VSG LiTat 1.3 and LiTat 1.5 through phage display, using sera from HAT patients and endemic negative persons.

Materials and Methods

Ethics statement

Sera from HAT patients and endemic controls were collected within different diagnostic studies [5,20]. All individuals gave their written informed consent before providing blood. Permission for these studies was obtained from the national ethical committee of the Democratic Republic of the Congo (DR Congo) and from the Institute of Tropical Medicine Antwerp (ITMA) ethical committee, reference number 03 07 1 413 and 04 44 1 472. Forty additional endemic negative control specimens were obtained from the archived specimen bank of the Parasite Diagnostics Unit at ITMA. All specimens were anonymised.

Coating of magnetic particles with VSG LiTat 1.3 or LiTat 1.5

Variant surface glycoproteins were purified from cloned populations of *T.b. gambiense* Variable Antigen Type (VAT) LiTat 1.3 and 1.5 [4]. VSG LiTat 1.3 or LiTat 1.5 were coated onto magnetic particles (MP, Estapor, 10% suspension, 1.04 µm, 9 µeq/g COOH). A volume of 250 µL of MP suspension was washed twice with 1 mL of buffer A (10 mmol/L NaH_2PO_4, pH 6.0). The MP were activated with 2.5 ml of buffer A containing 25 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) and 15 mg of N-hydroxysuccinimide (Sigma). The MP were rotated for 15 minutes at room temperature (rT) and washed with 1 mL of buffer B (2 mmol/L HCl) where after 350 µg of VSG (LiTat 1.3 or LiTat 1.5) in 1.5 mL of buffer C (20 mmol/L NaH_2PO_4/Na_2HPO_4, pH 7.5) was added to the pellet of MP. After rotating for 2 h at rT, the MP were washed three times with buffer C and resuspended to a final concentration of 8% in buffer C containing 100 mmol/L glycin, 1% bovine serum albumin (BSA) and 0.1% NaN_3. Successful coating of the MP was evaluated by agglutination with a HAT positive serum diluted 1/4 in phosphate buffered saline (PBS, 0.01 mol/L phosphate, 0.14 mol/L NaCl, pH 7.4) containing 0.02% w/v NaN_3.

Affinity purification of VSG LiTat 1.3 or LiTat 1.5 specific antibodies from HAT serum

Antibodies specific to VSG LiTat 1.3 or LiTat 1.5 were purified from nine HAT positive sera originating from the DR Congo [20]. One mL of LiTat 1.3 or LiTat 1.5 coated MP was rotated for 2 h at rT with 125 µL of HAT positive serum. After five washes with 800 µL of PBS, the specific antibodies were eluted from the MP by adding 700 µL of 0.2 mol/L glycine/HCl (pH 2.2) followed by magnetic separation after five minutes. The eluates, corresponding to the affinity purified antibody fractions, were neutralised with 100 µL of 1 mol/L Tris/HCl pH 9.1.

Indirect ELISA on VSG

Indirect ELISA was used to screen the affinity purified antibody fractions and all human serum samples on reactivity with VSG LiTat 1.3 and LiTat 1.5. ELISA plates (Nunc MaxiSorp^{TM}) were coated overnight (ON) at 4°C with 100 µL/well of 2 µg/mL of each VSG separately in phosphate buffer (PB, 0.01 mol/L phosphate, pH 6.5) or with 1.7 10^{11} particle/mL of wild type phage (WTP) in PBS. One plate was left empty as antigen negative control (Ag0). The plates were tapped dry, saturated with 350 µL/well of PBS-Blotto (0.01 mol/L phosphate, 0.2 mol/L NaCl, 1% w/v skimmed milk powder, 0.05% w/v NaN_3) during 1 h at rT and washed three times with 0.05% v/v Tween-20 in PBS (PBST) (ELx50, Bio-Tek ELISA washer). The purified antibody fractions were diluted 1/25 and human serum samples 1/150 in PBS-Blotto. One hundred µL/well of each dilution was applied in duplicate and incubated for 1 h at rT. After three washes with PBST 100 µL/well of horse radish peroxidase (PO)-conjugated goat anti-human IgG (H+L) (Jackson), 1/40000 diluted in PBST, was added. An hour and five washes later, wells were incubated for 1 h at rT with 100 µL/well of 2.2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) chromogen/substrate solution (50 mg tablet/100 mL of ABTS buffer, Roche). The plate was shaken for ten seconds and the optical density (OD) was read at 414 nm (Labsystems Multiskan RC 351). The measured OD was corrected (OD_c) with the corresponding OD in the Ag0 wells.

Coating of magnetic particles with human IgG antibodies

Three LiTat 1.3 positive pools, each consisting of three different VSG LiTat 1.3-specific antibody fractions, three LiTat 1.5 positive pools, each consisting of three different VSG LiTat 1.5-specific antibody fractions and one negative pool of four endemic negative sera were prepared. For each pool the antibodies were coated onto anti-human IgG (H+L) functionalised magnetic particles (MP) (1% w/v, 1.05 µm, Estapor/Merck) according to the guidelines of the manufacturer.

Selection of mimotopes for VSG LiTat 1.3 and LiTat 1.5 by panning of phage-displayed peptides

The panning was performed with the Ph.D.-12 (12-mer) phage display library (New England Biolabs, NEB) [21] through two rounds consisting of 1) a positive selection with anti-VSG (LiTat 1.3 or 1.5, respectively) antibodies coated on MP, 2) a negative selection with endemic negative serum antibodies coated on MP and 3) phage amplification [22]. Each positive selection was followed by phage titration and sandwich ELISA. After these two rounds a third positive selection was performed.

Positive selection

Positive selection was performed as previously described [16]. Bound phages were eluted for ten minutes with 600 µL of 0.2 mol/L glycine-HCl containing 1 mg/mL BSA (pH 2.2) and neutralised with 90 µL of Tris-HCl (1 mol/L, pH 9.1).

Negative selection

Six hundred µL of the elution from the positive selection was rotated ON at 4°C with 1 mg of MP coated with endemic negative serum antibodies, in a total volume of 1 mL of PBSG.

Amplification and purification of phages

The unbound phages in 900 µL of the supernatant of the negative selection were amplified, in a culture of *Escherichia (E.) coli* (strain ER2738, NEB) at early log (0.01–0.05 A_{600}), and purified with PEG-NaCl as previously described [16,21].

Titering of phages

Phages from the first, second and third positive selection were diluted in PBS 10^1 to 10^4, 10^2 to 10^5, 10^4 to 10^7, respectively. Ten µL of these dilutions were incubated for five minutes with 200 µL of an *E. coli* culture in mid-log (0.4–0.5 A_{600}). The mixture was then pipetted into 4 mL of Top-Agar (50°C) and poured onto agar plates containing 1 mL/L IPTG/X-gal (1.25 g isopropyl β-D-thiogalactoside, 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 25 mL dimethylformamide); ninety-four blue clones were picked and each clone was inoculated in 200 µL of lysogeny broth (LB) in a sterile culture plate (BD Falcon™ Clear 96-well Microtest™ Plate) [21]. This plate was shaken overnight at 30°C, and then the bacteria were pelleted by 5 min centrifugation at 1312 g. The supernatant was tested in a sandwich ELISA.

Sandwich ELISA with phage particles

ELISA plates were coated ON at 4°C with 100 µL/well of VSG LiTat 1.3- or LiTat 1.5-specific positive antibody pools (5 µg/mL in PBS) or a 1/10000 dilution in PBS of the negative serum pool. The ELISA was performed as previously described [16]. Briefly, the wells were incubated for 1 h at rT with 100 µL of phage dilution in PBS-Blotto (1/3 for culture plate supernatant or 1/20 for PEG-NaCl purified phage). PO-anti-M13 pVIII mAb (GE Healthcare), diluted 1/2000 in PBST was added to the wells for 1 h at rT. The wells were then incubated for 1 h at rT with ABTS and read at 414 nm.

Phage clones were withheld after the first two positive selections if 1) the OD with the corresponding positive pool (OD_{pos})>average OD_{pos}+2*standard deviation (sd_{pos}) and 2) the OD with the negative pool (OD_{neg})<average OD_{neg}.

After the third positive selection, phages were sequenced if 1) OD_{pos}>average OD_{pos}+1* sd_{pos} with at least one of the positive pools, 2) OD_{pos} with the 3rd positive pool >0.150 or 0.200 for phages selected for VSG LiTat 1.3 or LiTat 1.5 respectively and 3) OD_{neg}<average OD_{neg}. Withheld phage clones were sequenced and tested in a similar sandwich ELISA with as capture antibody the nine individual affinity purified antibody fractions, diluted 1/70 in PBS.

Single-stranded DNA extraction, sequencing and sequence analysis

Purification of phage DNA was performed according to the NEB manual [21]. Sequence determination was performed as described before [16]. The obtained sequence chromatograms were read with Chromas 2.33 (Technelysium Pty Ltd). Sequence alignment was performed manually and with RELIC software [23]. A protein data base (pdb) model of the N-terminal domain of VSG LiTat 1.5, was created using SWISS-MODEL [24,25]. Modelling was based on the known structure of VSG MITat 1.2 (pdb 1vsgA), previously derived by X-ray crystallography [26]. For VSG LiTat 1.3 however the server could not find a template with sufficient sequence homology, hence the pdb was created by Thomas Juetteman from the PyMol helpdesk (PyMOL Molecular Graphics System, Schrödinger, LLC). In order to identify possible conformational epitopes, the 3D-Epitope-Explorer (3DEX) [27] was used to find structural homology between the mimotope sequences and the respective VSG protein sequence.

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081) (http://www.cgl.ucsf.edu/chimera).

Peptide synthesis

The peptides were synthesised at >85% purity (Peptide 2.0, Chantilly, VA, U.S.). The GGGS-spacer, separating the library insert and the pIII phage protein, was added to the C-terminus of the peptides that were selected by phage display [16,21]. All peptides were C-terminally elongated with an additional lysine-biotin and amidated (-$CONH_2$), mimicking the uncharged peptide bond in a protein. All synthetic peptides were reconstituted in sterile deionised H_2O to a concentration of 2 mg/mL.

Indirect ELISA on biotinylated synthetic peptides and human sera

First, the reactivity of all biotinylated synthetic peptides was evaluated with the nine sera used for affinity antibody purification and nine endemic negative controls. Second, the diagnostic performance of the synthetic peptides was evaluated with human serum samples that were previously screened (indirect ELISA on VSG, serum dilution 1/100) on reactivity with VSG LiTat 1.3 and 1.5. All 102 serum samples from *gambiense* HAT patients originated from DR Congo [20]. Of the 102 endemic *gambiense* HAT negative serum samples, 71 originated from the DR Congo and 31 from Benin. The indirect ELISA on biotinylated peptides was performed as the indirect ELISA on VSG but 150 µL/well was applied in all but the saturation and washing steps. ELISA plates were coated with 10 µg/mL streptavidin (NEB) in carbonate buffer (0.1 mol/L, pH 9.2) or with 2 µg/mL VSG LiTat 1.3 and LiTat 1.5 in PB, or

wells were left empty (Ag0). After saturation with PBS-Blotto, the peptides were added at a concentration of 2 µg/mL in PBS to the wells containing streptavidin. The peptide-free wells received only PBS. To the VSG-containing and Ag0 wells PBS-5% w/v sucrose was added. After incubation of 1 h at rT the plates were tapped dry, sealed and frozen at −80°C. The serum samples were centrifuged for 5 min at 15700 g and diluted 1/100 in PBS-Blotto. After thawing of the plates and three washes with PBST, the serum dilutions were applied in duplicate. After one hour, we added PO-conjugated goat anti-human IgG (H+L), 1/40000 diluted in PBST. ABTS was used as chromogen/substrate solution and the OD was read as described above. The measured OD was corrected by subtracting the corresponding OD in the peptide-free or Ag0 wells and the average of the duplicate corrected ODs was taken (OD_c).

The accuracy of the synthetic peptides to detect VSG-specific antibodies for diagnosis of sleeping sickness was assessed by the area under the receiver operator characteristics (ROC) curve (AUC) [28]. Confidence intervals were calculated according to DeLong [29]. For the whole range of cut-offs the Youden index was determined (Youden index = sensitivity+specificity−1) [30] and the cut-off with maximal Youden index was retained.

Results

Affinity purification of VSG-specific antibodies

In indirect ELISA, all affinity purified antibody fractions reacted specifically with their corresponding VSG and not with WTP. The antibody fractions that were purified with VSG LiTat 1.3, had an average OD_c of 0.533±0.319 with VSG LiTat 1.3, and average OD_cs of only 0.032±0.032 with VSG LiTat 1.5 and −0.008±0.015 with WTP. The antibody fractions purified with VSG LiTat 1.5 had an average OD_c of 1.406±0.487 with VSG LiTat 1.5, and average OD_cs of only 0.037±0.064 with VSG LiTat 1.3 and −0.017±0.018 with WTP.

The negative serum samples did not react with VSG LiTat 1.3 (OD_c 0.034±0.078), nor with VSG LiTat 1.5 (OD_c 0.028±0.069), nor with WTP (OD_c 0.013±0.029).

Selection of mimotopes for VSG LiTat 1.3

During the selection process, none of 94 phage clones of the first positive selection, eight of 188 phage clones of the second positive selection and 11 of 188 phage clones of the third positive selection reacted in the sandwich ELISA and were sequenced, resulting in 18 sequences (table 1).

The alignment results of VSG LiTat 1.3 [GenBank AJ304413] and the eighteen peptide sequences displayed by the phage clones are presented in figure 1. All peptides could be aligned within amino acid stretch (AA) 72 to 116 of the N-terminal domain of VSG LiTat 1.3 (alignment 1). The common motive (F/W)ExDxK(A/V/L)x(A/V/L) was repeated twice in this VSG AA stretch. Therefore twelve sequences could be aligned twice within this region. The peptide displayed by phage 3-3-F6, ETDNMKPLHLRQ, could even be aligned three times within this region of VSG LiTat 1.3, having ETD, DNxKP and ExD identical within amino acids 78 to 80, 87 to 91 and 102 to 104 of the protein sequence. The peptide sequence displayed by phage 3-2-

Table 1. Peptide sequences of phage clones selected with human anti-VSG LiTat 1.3 antibodies.

Pos selection	Phage clone	Peptide sequence	OD_c (average ± SD)	Synthetic peptide
2	3-2-E2	WDSDCKRSCRVH	0.755±0.924	3-2-E2
2	3-2-G5	LTWVSDSKSGNT	0.530±0.528	3-2-G5
2	3-2-G10	TIAPSWATDSKP	0.449±0.417	3-2-G10
2	3-2-C5	TPNNAQKQPQLP	0.444±0.533	3-2-C5
2	3-2-B12	SWMPDSKVFASH	0.383±0.394	*
2	3-2-D10	WETDQKFKQRVA	0.342±0.347	3-2-D10
2	3-2-D11	WETDQKFKQRVA	na	/
2	3-2-F6	SAYDDVKRFYTN	0.307±0.683	/
3	3-3-F6	ETDNMKPLHLRQ	0.648±1.001	3-3-F6
3	3-3-E3	VNDASKLFYPRS	0.386±0.510	3-3-E3
3	3-3-H3(1)	WPTSWHMWLANR	0.161±0.146	/
3	3-3-A7	GVPDNHKPARTQ	0.131±0.121	/
3	3-3-A2	ALPTHMNWVMPV	0.113±0.074	/
3	3-3-A4	TWPQWWWTNSKG	0.111±0.071	/
3	3-3-B8	NPPIWGTATKGI	0.091±0.058	/
3	3-3-E8	FWKPHRTHFWWG	0.080±0.046	/
3	3-3-H3(2)	YNWETDKPMPVP	0.051±0.034	/
3	3-3-A3	TWWWHSLAKTPH	0.036±0.020	/
3	3-3-C12	TTWNFKHWWPYR	0.021±0.018	/

3-3-H3(1) and 3-3-H3(2) are two different phage clones, na: not applicable, SD: standard deviation,
*not withheld: similar to 3-2-G5 & 3-2-G10.
The phage clones were selected after two or three positive (pos) selections. The peptide sequences that were expressed by these phage clones are given in column three. The average OD_c in sandwich ELISA, using nine purified antibody fractions as capture antibody, is shown in column four. If the peptide sequence was synthesised, the name of the biotinylated peptide is given in column five.
doi:10.1371/journal.pntd.0001682.t001

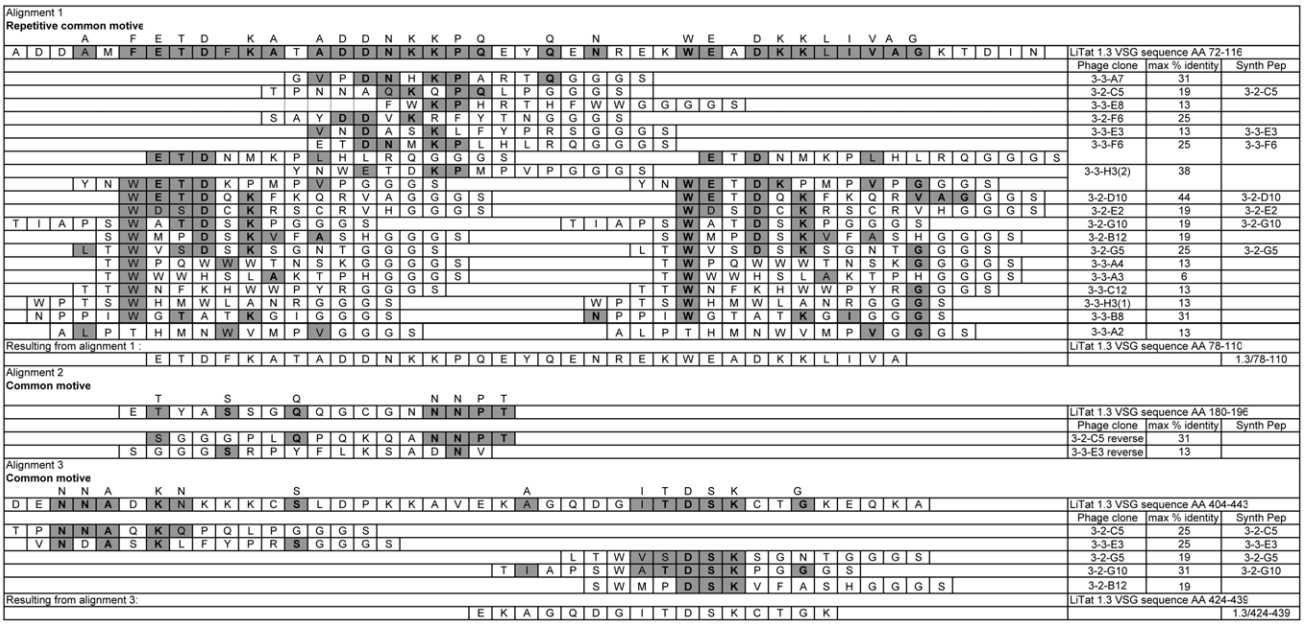

Figure 1. Alignment on VSG LiTat 1.3 of peptides selected with anti-VSG LiTat 1.3 antibody fractions. Homologous sequences between phage displayed peptides and/or the protein sequence of VSG LiTat 1.3 are indicated in grey. Amino acids that are identical to those of the VSG protein sequence are in bold and grey. All peptide sequences include the GGGS-spacer at the C-terminus. Maximum % identity: percentage identity of the peptide sequence with a corresponding stretch of sixteen AA within the protein sequence of VSG LiTat 1.3. Synth peptide: name of the synthesised peptide.
doi:10.1371/journal.pntd.0001682.g001

D10 had the highest identity within AA 72 to 116 of the VSG LiTat 1.3 sequence (7/16 AA, 44%). The reverse sequence of the peptides displayed by phage clones 3-2-C5 and 3-3-E3 showed respectively 31 and 13% identity within AA 180 to 196 (alignment 2). Within the C-terminal domain (alignment 3), the peptide expressed by phage clone 3-2-C5 and 3-3-E3 had 25% identity (4/16 AA) within AA 404 to 443 of VSG LiTat 1.3. The peptide expressed by phage clones 3-2-G10, 3-2-G5 and 3-2-B12 were respectively 31%, 19% and 19% identical within AA 404 to 443 of VSG LiTat 1.3.

All selected phage clones were tested in a sandwich ELISA with the individual purified antibody fractions. The peptides displayed by the seven phage clones with the highest average OD_c were withheld for synthesis as biotinylated peptides (table 1). The peptide displayed by phage clone 3-2-B12 was not withheld, since it was a homologue of 3-2-G10 and 3-2-G5 but had a lower average OD_c. Based on the alignment results, AA stretch 78 to 110 and AA stretch 424 to 439 of the protein sequence of VSG LiTat 1.3 were also synthesised as biotinylated peptides (respectively peptide 1.3/78-110 and peptide 1.3/424-439).

The reactivity of all nine biotinylated synthetic peptides was evaluated in indirect ELISA with the nine HAT positive sera used for affinity antibody purification, and with nine endemic negative controls. Peptide 1.3/78-110 was the best performing peptide with OD_c 1.469. Peptide 1.3/424-439 gave a lower average OD_c (0.246) than peptides 3-2-G10 and 3-2-G5 (0.564 and 0.920), sharing the same common motive, and was not withheld for further testing. Peptide 3-2-E2, a homologue of peptide 3-2-D10, also gave a lower average OD_c (0.541 versus 0.763) and was also not withheld for testing on diagnostic performance.

Selection of mimotopes for VSG LiTat 1.5

During the selection process, one of 94 phage clones of the first positive selection, two of 94 phage clones of the second positive selection and 20 of 188 phage clones of the third positive selection reacted in the sandwich ELISA and were sequenced, resulting in 20 sequences (table 2).

The alignment results of VSG LiTat 1.5 [GenBank HQ662603] and the 20 peptide sequences displayed by the phage clones are presented in figure 2.

The peptide expressed by phage clone 5-1-F9 (19% identity) could be aligned within AA 33 to 47 (alignment 1).

Within the N-terminal domain, 18 phage peptides could be aligned with minimum 6% identity within AA 81 to 119 (alignment 2), by analogy with alignment 1 for VSG LiTat 1.3. The peptides expressed by clones 5-3-C7, 5-3-A8 and 5-3-B5 respectively had 0, 0 and 6% identity within this AA stretch, but shared the common motive (W/F)Y with the peptide expressed by phage 5-3-B8 (19% identity). The peptide of phage clone 5-3-G6 had only 1/16 AA (6%) identity with VSG LiTat 1.5 but two more AA were homologous within this region. The reverse sequences of phage clone peptides 5-3-F7 (13% identity), 5-1-F9 (19% identity) and 5-3-D5 (13% identity) could also be aligned within this VSG LiTat 1.5 region. The peptide expressed by clone 5-3-B9 had the highest identity within the AA 81 to 119 stretch (5/16 AA, 31% identity, if a gap of 1 AA was allowed).

Peptides expressed by phage clones 5-3-C1, 5-3-A4 and 5-3-A6, with common motive "KLANP", could also be aligned between AA 145 to 166 of the VSG LiTat 1.5 protein sequence (alignment 3) with respectively 25, 13 and 13% identity. Within the VSG LiTat 1.5 AA stretch 245 to 281, the peptide expressed by phage clone 5-3-A6, showed 19% identity and the reverse peptide sequence of phages 5-3-B9, 5-3-A3 and 5-3-A4 showed respectively 19, 19 and 13% identity (alignment 4). Within the boundary with the C-terminal domain of VSG LiTat 1.5, showed the peptides expressed by phage clones 5-3-A8, 5-3-C1 and 5-3-B6 respectively 19, 31 and 31% identity within AA stretch 341 to 368, if a gap of three AA was allowed for peptide 5-3-C1 (alignment 5). Within the C-terminal domain of VSG LiTat 1.5, phage clone peptide 5-3-B9 and 5-3-A4 had respectively 31 and 13% identity between AA 468 to 489 (alignment 6).

Table 2. Peptide sequences of phage clones selected with human anti VSG LiTat 1.5 antibodies.

Positive selection	Phage clone	Peptide sequence	OD_c (average ± SD)	Synthetic peptide
1	5-1-F9	AAIMHQEQESNT	0.403±0.693	5-1-F9
2	5-2-D3	SAGFENDGTKLA	0.246±0.120	5-2-D3
2	5-2-H2	TGLPTTNKQTSS	0.234±0.196	5-2-H2
3	5-3-C1	AYSKPTIKLANP	0.496±0.758	5-3-C1
3	5-3-E8	AYSKPTIKLANP	na	/
3	5-3-C5	AYSKPTIKLANP	na	/
3	5-3-B9	LPLATADKNGRT	0.436±0.624	5-3-B9
3	5-3-A4	DKLDNPGGPTVG	0.291±0.404	5-3-A4
3	5-3-E5	DKLDNPGGPTVG	na	/
3	5-3-A6	LQMPHNSKTANP	0.261±0.423	*
3	5-3-G6	INGQFSLKYRNP	0.253±0.188	5-3-G6
3	5-3-D4	LMPNKISNFASA	0.209±0.144	/
3	5-3-A1	DQTCNSPPCPPL	0.186±0.156	/
3	5-3-F7	STLPPPQGKIIH	0.185±0.243	/
3	5-3-B8	WYPLHSGLRSYY	0.156±0.121	/
3	5-3-B6	NKSTTNDFLRSP	0.141±0.122	/
3	5-3-D6	NGDYLQYKAPNP	0.139±0.141	/
3	5-3-B5	NTVRPPTLFYHW	0.126±0.109	/
3	5-3-D5	WHSEYQEPYPLS	0.120±0.103	/
3	5-3-A3	LDKNVLSPPMPL	0.107±0.082	/
3	5-3-C7	HHMSWYSRWLPV	0.091±0.071	/
3	5-3-A8	WWKPWSNFYGST	0.085±0.060	/
3	5-3-B3	LNTQNHAPLPSI	0.080±0.057	/

na: not applicable, SD: standard deviation,
*not withheld: similar to 5-3-C1.
The phage clones were selected after 1, 2 or 3 positive selections. The peptide sequences that were expressed by these phage clones are given in column three. The average OD_c in sandwich ELISA, using nine purified antibody fractions as capture antibody, is shown in column four. If the peptide sequence was synthesised, the name of the biotinylated peptide is given in column five.
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All selected phage clones were tested in a sandwich ELISA with the individual purified antibody fractions (table 2). The peptides displayed by the seven phage clones with the highest average OD_c, were chosen for synthesis as biotinylated peptides, except for the peptide displayed by phage clone 5-3-A6, which was similar to 5-3-C1 but had a lower average OD_c. Based on the alignment results and by analogy with VSG LiTat 1.3, the AA stretch 81 to 109 of the protein sequence of VSG LiTat 1.5 was synthesised as biotinylated peptide (peptide 1.5/81-109).

The reactivity of all eight biotinylated synthetic peptides was evaluated in indirect ELISA with the nine sera used for affinity antibody purification and with nine endemic negative controls. Peptide 5-3-A4 and 5-3-G6 had the lowest average OD_cs (0.145 and 0.109) and shared a common motive with peptide 5-3-C1 with a higher average OD_c (0.289) and were therefore not withheld for testing on diagnostic performance. Peptide 1.5/81-109 had an OD_c of 0.382 and was withheld.

Assessment of the performance of the biotinylated peptides for diagnosis of gambiense HAT

The accuracy of the biotinylated peptides to detect VSG-specific antibodies was assessed with sera from 102 *gambiense* HAT patients and 102 endemic negative controls (table 3). Among the mimotopes for VSG LiTat 1.3, the highest AUC was obtained with peptide 3-2-G5 (0.93) and peptide 3-2-G10 (0.95). Sensitivities and specificities at the cut-off with the highest Youden index were respectively 0.85 and 0.94 for peptide 3-2-G5, and 0.90 and 0.93 for peptide 3-2-G10. Of the mimotopes for VSG LiTat 1.5 the highest AUC was obtained with peptide 5-1-F9 (0.95) and 5-2-D3 (0.94) with respective sensitivities and specificities of 0.94 and 0.95 for peptide 5-1-F9 and 0.92 and 0.89 for peptide 5-2-D3. With peptide 1.3/78-110, an AUC of 0.95 was observed, with a sensitivity of 0.96 and a specificity of 0.85. With peptide 1.5/81-109, an AUC of 0.79, a sensitivity of 0.81 and a specificity of 0.75 were obtained.

VSG LiTat 1.3 and 1.5 obtained an area under the curve of respectively 1.000 and 0.997. The sensitivity and specificity were both 1.000 at cut-off 1.318 for VSG LiTat 1.3 and 1.000 and 0.990 at cut-off 1.182 for VSG LiTat 1.5.

Three dimensional epitope mapping of the peptides with diagnostic potential

By using 3DEX software and setting the number of hits at a minimum of 5 AA, none of the VSG LiTat 1.3 mimotopes with AUC>0.90 could be mapped as a conformational epitope on the protein model of the VSG LiTat 1.3. In contrast, among the VSG

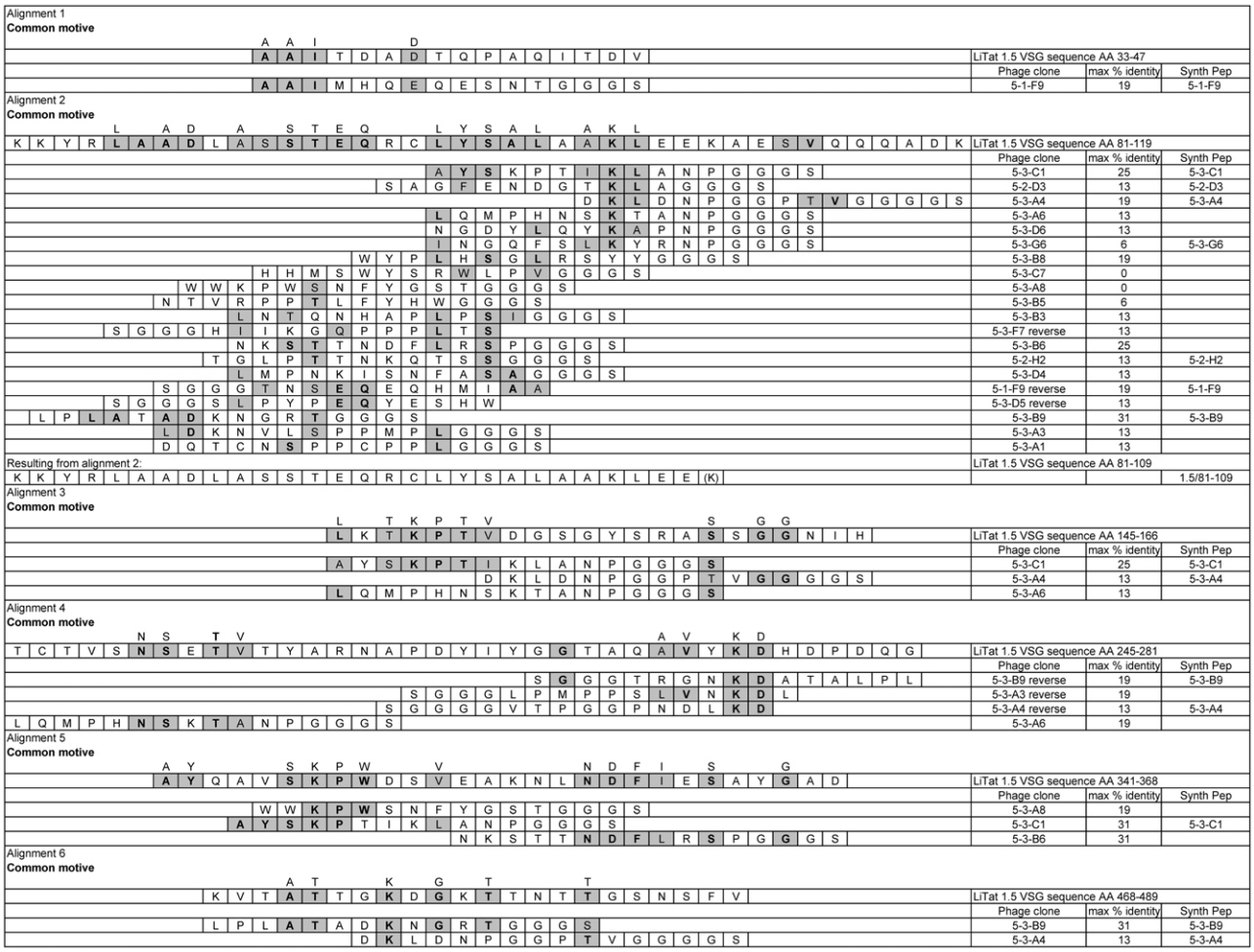

Figure 2. Alignment on VSG LiTat 1.5 of peptides selected with anti-VSG LiTat 1.5 antibody fractions. Homologous sequences between phage displayed peptides and/or the protein sequence of VSG LiTat 1.5 are indicated in grey. Amino acids that are identical to those of the VSG protein sequence are in bold and grey. All peptide sequences include the GGGS-spacer at the C-terminus. Maximum % identity: percentage identity of the peptide sequence with a corresponding stretch of sixteen AA within the protein sequence of VSG LiTat 1.5. Synth peptide: name of the synthesised peptide.
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LiTat 1.5 mimotopes with AUC>0.90, peptide 5-2-D3 could be mapped with 8/12 AA (E 168|N 164|D 152|G 153|T 150|K 146|L 144|A 141) on the three-dimensional VSG LiTat 1.5 protein model (figure 3).

Discussion

In this manuscript we describe how mimotopes and regions that take part in epitope formation for VSGs LiTat 1.3 and LiTat 1.5 of *T.b. gambiense* were identified by screening of a Ph.D.-12 phage display library with polyclonal antibodies that were purified from sera of sleeping sickness patients.

As sera from sleeping sickness patients contain an important fraction of trypanosome unrelated antibodies [31], the risk of selecting mimotopes that are unrelated to sleeping sickness by using human sera for the screening was considerable.

We identified a linear region between amino acid 72 and 114 of the protein sequence of both VSG LiTat 1.3 and LiTat 1.5 wherein most of the peptide sequences could be aligned with the VSG protein sequence. This region is localised in the hypervariable N-terminal domain of the VSG and was for both VSGs synthesised as a linear biotinylated peptide and tested in indirect ELISA with a panel of 102 HAT positive and 102 endemic negative sera.

Peptide 1.3/78-110, corresponding to AA stretch 78 to 110 of VSG LiTat 1.3, had an AUC of 0.95, indicating diagnostic potential. The epitope of VSG LiTat 1.3, recognised by the human serum antibodies used for screening of the peptide library, therefore seems to be linear and located within AA stretch 78 to 110. The peptide sequences that were selected for VSG LiTat 1.3 had in average 3/16 amino acids in common within AA 72 to 114 with a maximum of 7/16 (44%) identical amino acids. Interestingly, a common motive of the peptide sequences was repeated twice within AA 72 to 114 of VSG LiTat 1.3: (F/W)ExDxK(A/L/V)x(A/L/V), from AA 77 to 85 and 101 to 109.

The two mimotopes of VSG LiTat 1.3 with the highest AUC, peptide 3-2-G5 and 3-2-G10, seemed to share a common epitope (correlation coefficient of OD_es with human sera in ELISA 0.71, data not shown), their motive WxxDxK reoccurred twice within AA 72 to 114. Their motive, (I/V/A)(T/S)DSK, could also be aligned within the C-terminal domain (AA 424 to 439). This AA stretch was synthesised as a biotinylated peptide as well, but had a

Table 3. Evaluation of the potential of the biotinylated peptides for diagnosis of *T.b. gambiense* HAT.

Antigen type	Name	AUC (95% CI)	sensitivity (95% CI)	specificity (95% CI)
LiTat 1.3	1.3/78-110	0.95 (0.91–0.98)	0.96 (0.90–0.99)	0.85 (0.77–0.92)
LiTat 1.3	3-2-G10	0.95 (0.91–0.97)	0.90 (0.83–0.95)	0.93 (0.86–0.97)
LiTat 1.3	3-2-G5	0.93 (0.89–0.96)	0.85 (0.77–0.92)	0.94 (0.88–0.98)
LiTat 1.3	3-3-E3	0.89 (0.84–0.93)	0.96 (0.90–0.99)	0.76 (0.67–0.84)
LiTat 1.3	3-3-F6	0.89 (0.84–0.93)	0.82 (0.74–0.89)	0.86 (0.78–0.92)
LiTat 1.3	3-2-C5	0.89 (0.84–0.93)	0.90 (0.83–0.95)	0.82 (0.74–0.89)
LiTat 1.3	3-2-D10	0.86 (0.81–0.91)	0.86 (0.78–0.92)	0.81 (0.72–0.88)
LiTat 1.5	5-1-F9	0.95 (0.91–0.97)	0.94 (0.88–0.98)	0.95 (0.89–0.98)
LiTat 1.5	5-2-D3	0.94 (0.90–0.97)	0.92 (0.85–0.97)	0.89 (0.82–0.94)
LiTat 1.5	5-2-H2	0.88 (0.82–0.92)	0.82 (0.74–0.89)	0.81 (0.72–0.88)
LiTat 1.5	5-3-C1	0.87 (0.82–0.92)	0.86 (0.78–0.92)	0.79 (0.70–0.87)
LiTat 1.5	5-3-B9	0.85 (0.79–0.89)	0.79 (0.70–0.87)	0.83 (0.75–0.90)
LiTat 1.5	1.5/81-109	0.79 (0.73–0.85)	0.81 (0.72–0.88)	0.75 (0.65–0.83)
Native VSG	LiTat 1.3	1.000 (0.982–1.000)	1.000 (0.964–1.000)	1.000 (0.964–1.000)
Native VSG	LiTat 1.5	0.997 (0.973–1.000)	1.000 (0.964–1.000)	0.990 (0.947–1.000)

The ability of biotinylated synthetic peptides to bind human serum antibodies in 102 HAT positive and 102 endemic negative control sera was assessed by indirect ELISA. The area under the receiver operator characteristics curve (AUC) and the sensitivity and specificity at maximum Youden index are shown with 95% confidence intervals (CI).

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low average OD_c upon a first screening with nine HAT sera and was discarded for further evaluation of diagnostic performance. We think it unlikely that peptide 3-2-G5 and 3-2-G10 are mimotopes for a linear epitope localised in the C-terminal domain of VSG LiTat 1.3. Additionally, epitopes localised in the relatively conserved C-terminal domain are more likely to react with non-VSG-specific antibodies.

As for VSG LiTat 1.3, a repetitive motive was present within AA 81 to 114 of VSG LiTat 1.5: (Y/F/W)(x or xx)(A/L/I/V)A(A/I/L)(D or K) (A/L)xxxxE, from AA 83 to 94 and AA 99 to 111. The peptide sequences selected for VSG LiTat 1.5 had in average 2/16 AA in common within AA 82 to 114 of the protein sequence, with a maximum of 5/16 (31%) identical AA. Contrary to the results for peptide 1.3/78-110, peptide 1.5/81-109, corresponding to AA stretch 81 to 109 of VSG LiTat 1.5, had an AUC of only 0.79, while the AUC of all of the individual peptides aligned in this region was >0.85. Motive AYSxxxIKL of peptide 5-3-C1 (AUC 0.87), corresponded to LYSxxxAKL (AA 99 to 106) of the VSG LiTat 1.5 protein sequence. Peptide 5-3-C1 seems therefore to mimic an epitope that is, albeit partly, localised in this region. The similar peptide 5-2-D3, with motive F(x)xxxxKL, performed better in ELISA (AUC 0.94). It is possible that peptide 5-2-D3 and 5-3-C1 are mimotopes for a discontinuous epitope as they share the common motive (A/I/L) (Y/F) xxxxxKLANPG with four other peptides, while ANPG was not found in the VSG protein sequence. We therefore suspect the epitope of VSG LiTat 1.5, recognised by the human serum antibodies used for screening, to be discontinuous and to be at least partly localised within this region. This might explain the weaker performance of the linear peptide 1.5/81-109 compared to the mimotope peptides. This finding was supported by the results of the 3DEX analysis of the mimotopes that had an AUC>90, locating peptide 5-2-D3 with 8/12 AA on the three-dimensional VSG LiTat 1.5 protein model (E 168|N 164|D 152|G 153|T 150|K 146|L 144|A 141).

In a previous study [16] we were able to identify mimotope peptides for the native trypanosomal variant surface glycoproteins by screening of peptide phage display libraries with monoclonal antibodies. Through phage display with polyclonal human antibodies we now identified different mimotopes and regions taking part in epitope formation. Because the three monoclonal antibodies used in the first study represent only a fraction of the VSG-specific antibody response, some mimotopes with diagnostic potential might have been missed. Additionally, the mouse and human immune system may recognise different B cell epitopes. Other factors may have contributed to finding different motives using the two approaches. As a result of a short infection period of two weeks, the mouse monoclonals do not recognise all VSG-epitopes. They were selected for strict VAT-specificity with purified VSGs and identified mimotopes, not necessarily dominant, that were located near the surface of the VSG N-terminal domain. The polyclonal human antibodies result from a long infection and recognise also less exposed VSG-epitopes. It may be that by affinity purification on purified VSG an antibody fraction that recognises non-surface epitopes was mainly retained, as the mimotopes of VSG LiTat 1.3 seem to be located in this region. Another explanation may lie in the presence of self-reactive VSG-specific antibodies in sera from uninfected individuals, as has been demonstrated by Müller et al. [32]. Thus the negative selection with human antibodies from control sera may have eliminated the phages expressing the mimotopes for the VSG-specific epitopes also recognised by the mAbs. Both panning strategies thus seem complementary, in contrast to what has been described by Tang *et al.* [18], who selected a greater number of different 12-mer sequences with polyclonal serum for *Salmonella enterica*, but some of the common motives were also selected by panning of a monoclonal antibody. In the manuscript of Casey *et al.* [17] the mimotopes for Epstein-Barr (EBV) virus, selected with polyclonal EBV immune rabbit and patient sera were also not recognised by the monoclonal antibodies used for mimotope selection in a previous study.

Diagnostic evaluation of individual mimotopes and combinations [patent application GB1202460.0] indicates that screening of

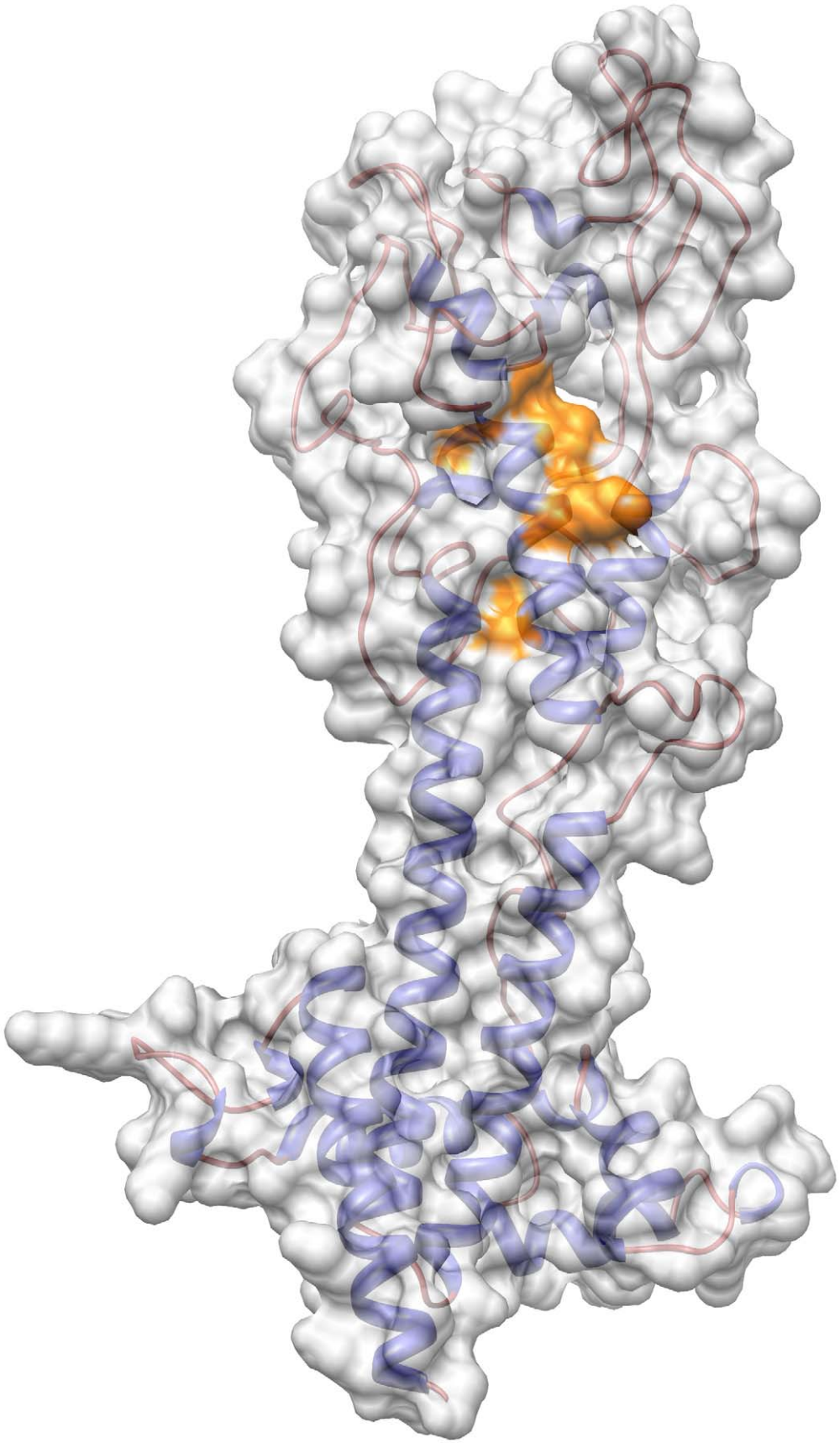

Figure 3. Mapping of peptide 5-2-D3. Peptide 5-2-D3 could be mapped (orange) with AA E 168|N 164|D 152|G 153|T 150|K 146|L 144|A 141 on the three-dimensional model of a VSG LiTat 1.5 N-terminal domain monomer by means of 3DEX and Chimera.
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peptide phage display libraries with patient's antibodies resulted in a more efficient selection of diagnostic peptides than with monoclonal antibodies. We therefore prefer screening with patient's antibodies. As an alternative approach to phage display linear epitopes may be replaced by synthetic peptides identified by scanning of overlapping peptides spanning the native protein sequence [33]. Furthermore, there are alternative *in vitro* methods for phage display such as yeast cell or bacterial display or, non-cellular, ribosome or mRNA display [34].

Our study has nevertheless some limitations. First, considering the broad antibody spectrum in HAT sera due to polyclonal B cell activation [35], we opted to use antibody fractions that were affinity purified for VSG LiTat 1.3 and 1.5. Thus, mimotopes for other predominant VSGs or other trypanosome antigens with diagnostic potential have not been selected. An alternative approach to identify additional diagnostic mimotopes may consist of screening peptide phage libraries with patient antibodies against other candidate diagnostic proteins [36]. Examples are the *T.b. gambiense*-specific glycoprotein TgsGP [37], the *T.b. rhodesiense*-specific serum resistance associated (SRA) protein [38] and *Trypanozoon*-specific trypanosome antigens such as invariant surface glycoprotein (ISG) 65 and ISG 75 [39], microtubule associated repetitive protein 1 (MARP1) and GM6 [40]. Some of them have already been tested for their diagnostic potential in the form of recombinant fusion proteins expressed in *E. coli* but none are yet used in diagnostic tests for HAT. Second, even with affinity purified antibodies there is a risk that non-specific mimotopes are selected with antibodies against VSG epitopes that are normally hidden in the intact VSG coat. Usually, most of the phage particles display a consensus binding sequence after two or three rounds of enrichment [21]. We therefore performed three rounds of positive selection and two selections with negative sera. Remarkably, the mimotopes with the highest AUC for VSG LiTat 1.3 and 1.5 were selected after only two or even one round of panning. Third, no affinity measurements *e.g.* via surface plasmon resonance, have been performed. Considering the polyclonal character of antibodies in patients' sera and the inherent differences in antibody response between individual patients, we opted to assess only the diagnostic potential of the selected peptides by means of ELISA.

Before the native *T.b. gambiense* VSGs LiTat 1.3 and LiTat 1.5 in the currently existing diagnostic formats can be replaced by synthetic peptides, further improvements should be considered. It is possible to define critical residues, essential for binding with the antibody, by e.g. alanine scanning mutagenesis [41]. Thus the epitope of the human serum antibodies might be recreated as has recently been done for a linear epitope on the VP1 protein of foot-and mouth disease virus [42]. Other, non-essential, parts of the peptides can then be eliminated in order to increase specificity. Phage clones that express peptides with a higher binding affinity might be selected by increasing the number of selection rounds and/or the stringency of the washing steps.

In conclusion, with this study we demonstrate that mimotopes of *T.b. gambiense* VSG LiTat 1.3 and 1.5 can be selected from a phage display library and that these mimotopes and corresponding amino acid stretches within the VSGs have diagnostic potential.

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Author Contributions

Conceived and designed the experiments: LVN FB MH PB VL. Performed the experiments: LVN FB. Analyzed the data: LVN FB PB VL. Contributed reagents/materials/analysis tools: LVN TD YG. Wrote the paper: LVN MH TD YG PB VL.

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Gambiense Human African Trypanosomiasis and Immunological Memory: Effect on Phenotypic Lymphocyte Profiles and Humoral Immunity

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Abstract

In mice, experimental infection with *Trypanosoma brucei* causes decreased bone marrow B-cell development, abolished splenic B-cell maturation and loss of antibody mediated protection including vaccine induced memory responses. Nothing is known about this phenomenon in human African trypanosomiasis (HAT), but if occurring, it would imply the need of revaccination of HAT patients after therapy and abolish hope for a HAT vaccine. The effect of *gambiense* HAT on peripheral blood memory T- and B-cells and on innate and vaccine induced antibody levels was examined. The percentage of memory B- and T-cells was quantified in peripheral blood, prospectively collected in DR Congo from 117 *Trypanosoma brucei gambiense* infected HAT patients before and six months after treatment and 117 controls at the same time points. Antibodies against carbohydrate antigens on red blood cells and against measles were quantified. Before treatment, significantly higher percentages of memory B-cells, mainly T-independent memory B-cells, were observed in HAT patients compared to controls (CD20+CD27+IgM+, 13.0% versus 2.0%, $p<0.001$). The percentage of memory T-cells, mainly early effector/memory T-cells, was higher in HAT (CD3+CD45RO+CD27+, 19.4% versus 16.7%, $p=0.003$). After treatment, the percentage of memory T-cells normalized, the percentage of memory B-cells did not. The median anti-red blood cell carbohydrate IgM level was one titer lower in HAT patients than in controls ($p<0.004$), and partially normalized after treatment. Anti-measles antibody concentrations were lower in HAT patients than in controls (medians of 1500 versus 2250 mIU/ml, $p=0.02$), and remained so after treatment, but were above the cut-off level assumed to provide protection in 94.8% of HAT patients, before and after treatment (versus 98.3% of controls, $p=0.3$). Although functionality of the B-cells was not verified, the results suggest that immunity was conserved in *T.b. gambiense* infected HAT patients and that B-cell dysfunction might not be that severe as in mouse models.

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Introduction

Human African Trypanosomiasis (HAT) or sleeping sickness, is a vector-borne parasitic disease occurring in sub-Saharan Africa. About 70 million persons are at risk for infection and 30 000 persons are estimated to be infected [1]. The parasites concerned belong to the *Trypanosoma* genus and are transmitted through the bites of tsetse flies (*Glossina* genus). Two subspecies of *Trypanosoma brucei* (*T.b.*), *T.b. gambiense* and *T.b. rhodesiense*, are responsible for human infection, which is usually fatal if left untreated. Infection with *T.b. gambiense* is responsible for chronic HAT in West- and Central-Africa, and characterized by low parasite numbers. In East-Africa, infection with *T.b. rhodesiense* leads to acute disease with relatively high parasite loads. Control of HAT relies on a combination of accurate diagnosis of cases, treatment of detected cases, and on control of the tsetse fly vector. No vaccine is available yet.

The immunopathology of HAT remains poorly understood and most of our understanding comes from experimental *T.b. brucei* infections in mice, which also serve as a model for vaccine development. In *T.b. brucei* infected mice, host control over disease mainly relies on the T-cell independent IgM antibody response [2–4]. However, mice *T.b. brucei* infection results in decreased B-cell development in the bone marrow [5]. Lymphopoiesis, which is taken over by the spleen, is in turn abrogated by apoptosis of transitional B-cells, permanent loss of splenic marginal zone B-cells (which are important for the early antibody response against T-cell independent antigens) and depletion of follicular B-cells (which normally develop into antibody producing plasma cells and memory B-cells). As a result of B-cell dysfunction, mice become

Author Summary

African trypanosomes are parasites that cause sleeping sickness in humans. In mice models, trypanosomiasis causes loss of the spleen memory B-cell precursors, of the host memory response and of protection against certain pathogens, built up by vaccination. The phenomenon has never been studied in human sleeping sickness, but if occurring, revaccination after treatment would be required. We show that *gambiense* human sleeping sickness is associated with a relevant increase in memory T- and B- cells in peripheral blood, in particular T-independent memory B-cells. As measles vaccination is included in standard vaccination programs, we measured measles antibody concentrations, which, although slightly lower in sleeping sickness patients than in controls, exceeded in 95% of patients the minimum level considered protective. Anti-red blood cell IgM titres, measured to assess the T-cell independent antibody response, were one titre lower in patients than in controls, but normalized after treatment. Overall, our results in *gambiense* HAT patients do not suggest trypanosomiasis associated massive memory cell destruction, or loss of antibody levels, although the antibody's protective capacity remains to be confirmed.

susceptible to repetitive infections by previously encountered *T.b. brucei* variant antigenic types [6]. Furthermore, *T.b. brucei* infection equally affects the protective immune response towards unrelated pathogens, as observed in two experiments. First, in mice immunized against *Trichinella spiralis*, it was observed that upon subsequent infection with *T.b. brucei* and *Trichinella spiralis*, the effect of vaccination was lost in *T.b. brucei* infected mice only [7]. Similarly, in mice vaccinated against diphtheria, tetanus and *Bordetella pertussis*, the vaccine mediated protective effect was abrogated in mice that were infected with *T.b. brucei* prior to a *Bordetella pertussis* challenge, while vaccinated mice that had not been infected with *T.b. brucei*, remained protected upon challenge with *Bordetella* [6]. *In vivo* and *in vitro* correlates of cell-mediated immunity were observed to be depressed as well in rabbits infected with the African trypanosome *T. congolense* [8].

These results indicate that *T.b. brucei* infections can give rise to general memory B-cell destruction in animals, and point to the possibility that *T. brucei* infection may destruct memory B-cell and abrogate vaccine induced protection in humans as well. If confirmed, this would imply the need of revaccination of HAT patients after anti-trypanosomal therapy and development of a vaccine against the disease might be hard to achieve [9]. However, the relevance of the experimental models for humans remained unknown. Data about leukocyte phenotypes in HAT have remained limited to one study showing increased percentages of CD19+ B-cells and activated B-cells in blood of *gambiense* HAT patients, as well as a relative decrease in memory and effector CD8 T-cells [10]. Evidence for an increased occurrence of vaccine preventable diseases in cured HAT patients is missing, although such relationships may be easily overlooked due to weak surveillance systems in HAT endemic countries. The vaccine-induced memory response in HAT is difficult to assess. Firstly, one is limited to vaccines that provide life-long protection and have been administered to the majority of the population and prior to trypanosomiasis infection. Secondly, loss of protection cannot be tested by challenge with the pathogen. Moreover, HAT mainly occurs in remote rural settings where no standard laboratory infrastructure or electricity is available. Although in *T.b. brucei* animal models, immune depression may occur despite intact antibody levels [7], we selected antibody quantification as an initial, though suboptimal, approach to assess immunological memory, taking into account that so far, nothing is known for the human situation. We opted for iso-agglutinins, which are innate antibodies against A and B carbohydrate antigens on red blood cells [11], as well as for measles vaccine antibodies, as this vaccine is part of the standard vaccination programs [12].

We addressed the following questions: (i) does *gambiense* HAT eliminate peripheral blood memory B-cells; (ii) are peripheral blood memory T-cells affected in *gambiense* HAT (iii) does *gambiense* HAT influence iso-agglutinin levels and antibody levels against measles, and; (iv) are these effects reversible upon cure from *gambiense* HAT?

Materials and Methods

Ethics statement

Before enrolment into the study, written informed consent was obtained from adult participants. In the case of minors, an assent was asked for and parents/guardians provided written informed consent. Ethical clearance for the study was obtained from the institutional review board of ITM and the ethical committees of the University Hospital in Antwerp, Belgium (study registration number B30020108363) and of the Ministry of Health of the Democratic Republic of the Congo (DR Congo).

Study population and specimen collection

Trypanosma brucei gambiense infected HAT patients and non-HAT endemic controls were prospectively enrolled (T = 0 months) in the study in DR Congo, Bandundu Province between July and December 2010. Participants were identified during HAT screening activities of the dedicated HAT mobile team of Masi-Manimba, or included at the HAT treatment centres of Masi-Manimba and Bonga-Yasa. Inclusion criteria for HAT patients were the presence of trypanosomes in blood, lymph and/or cerebrospinal fluid (irrespective of disease stage), and being 12 years or older. Exclusion criteria were pregnancy, being previously treated for HAT and being moribund. For each HAT patient, a control was included, fulfilling the following criteria: same gender and age and being and being resident in the same or a neighbouring village. Inclusion criteria for controls were absence of clinical evidence for HAT (no swollen lymph nodes or neurological symptoms), absence of trypanosome specific antibodies in whole blood detected by card agglutination test for trypanosomiasis (CATT) [13]; no trypanosomes in blood detected by the mini anion exchange centrifugation technique [14] and being 12 years or older. Exclusion criteria were identical as for HAT patients.

At enrolment, a crude assessment of the general condition (normal, good, bad, pre-moribund or moribund) was made, based on the participant's ability to eat, walk and take care of himself independently. Participants were questioned for their vaccination history (measles, diphtheria-tetanus-whooping cough, polio, Bacillus Calmette-Guérin (BCG)) and presence of a BCG scar was verified.

Whole blood was collected by venipuncture and collected in 5 ml Cyto-Chex BCT blood collection tubes (Streck, Omaha, NE, USA) and shipped within one week to the Institute of Tropical Medicine (ITM) for phenotyping. From blood sampled on heparin, plasma was prepared that was snap frozen in liquid nitrogen and shipped to ITM where specimens were stored at -70°C until use. Blood taken on EDTA was preserved in an equal volume of GE buffer (6 M guanidine hydrochloride, 0.2 M EDTA,

pH 8.0) at ambient temperature until DNA extraction. Thick and thin blood films were prepared and Giemsa stained for malaria diagnosis.

The participants ABO blood group was determined using Eldoncard 2511 (Eldon Biologicals, Gentofte, Denmark). The HIV status was determined using HIV 1/2 STAT-PAK Assay (Chembio, Medford, NY, USA) which, if positive, was followed by Uni-Gold HIV (Trinity Biotech, Wicklow, Ireland), and if positive, by HIV 1/2 Oraquick ADVANCE (Orasure Technologies, Bethlehem, PA, USA) [15]. In participants positive for all 3 serological tests, HIV infection was confirmed *a posteriori* using PCR, following a nested method in an algorithm of three different primer sets in *pol*, *env* and LTR region [16]. CATT was performed on whole blood taken on heparin, and if positive, the plasma end-titre was determined.

HAT was treated following the guidelines of the National HAT Control Program in DR Congo.

HAT patients were revisited six months after treatment, controls at the corresponding time point (T = 7 months). The participant's general condition was re-assessed. Blood taken on heparin and on Cyto-Chex BCT blood collection tubes was processed as described above. All participants were examined for absence of trypanosomes using the mini anion exchange centrifugation technique, and in controls, CATT was repeated.

Flow cytometry

Whole blood, collected in Cyto-Chex BCT blood collection tubes (Streck, Omaha, NE, USA), was used to study T and B cell subsets by flow cytometry.

B-cells subsets were analysed using mouse anti-human monoclonal antibodies anti-CD45 PerCP (leucocytes), anti-CD20-FITC (B cells), anti-human CD27-APC (IgG1) and anti-human IgM-PE (IgG1) and appropriate IgG1 isotype controls (BD Biosciences, Erembodegem, Belgium). These combinations were used to identify B-cells (CD20), naïve B-cells (CD20+CD27−), memory B-cells (CD20+CD27+), T independent B-cells (CD20+ IgM+) and T dependent B-cells (CD20+IgM−) [17].

T-cells subsets were stained with mouse anti-human monoclonal antibodies anti-CD45 PeCP, anti-CD3 (IgG1)-PE, anti-CD45RO-FITC (IgG2a), anti-CD27-APC (IgG1) and appropriate IgG1 isotype controls (BD Biosciences, Erembodegem, Belgium). These combinations were used to identify T-cells (CD3), naïve T-cells (CD3+CD45RO−CD27+), early effector/memory T-cells (CD3+CD45RO+CD27+) and late effector/memory T-cells (CD3+CD45RO+CD27−) [18]. For the staining of B-cells, 50 µl of fixed blood was pipetted in two test tubes. Blood in both tubes was washed twice with 2 ml of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) to remove serum. Subsequently, a cocktail of anti-CD20/anti-CD27/anti-IgM was added to one tube and anti-CD20/isotype-control cocktail to the other. After 30 minutes of incubation, red blood cell lysing solution was added for 10 minutes, cells were washed and analysed on the flow cytometer (FACSCalibur, BD Biosciences). For the staining of the T-cells the procedure was the same with exception of the washing step with PBS-BSA which was omitted. The cells subsets were analysed using FlowJo software (Tree Star, US).

Prior to the study, the antibody cocktails were tested using whole blood from 3 normal controls. Blood collected in Cyto-Chex BCT blood collection tubes was compared to fresh blood collected in EDTA tubes. Using the above described antibody cocktails, T- and B- cell subsets could be measured in blood collected in Cyto-Chex BCT blood collection stored for at least 14 days.

Determination of IgM and IgG antibody titres against red blood cells (iso-agglutinin end-titers)

Screening for irregular anti- erythrocyte antibodies (antibodies causing agglutination but that are not A and B red blood cell carbohydrate antigen specific) was performed with ID-Diacell I–II–III (Bio-Rad, Cressier, Switzerland) using undiluted plasma. For IgG, 25 µl of plasma and 50 µl of ID-Diacell I, ID-Diacell II or ID-Diacell III cell suspension were incubated for 15 minutes at 37°C on Coombs anti-IgG ID-cards (Bio-Rad, Cressier, Switzerland). For IgM, 25 µl of plasma and 50 µl of each cell suspension were incubated for 15 minutes at 20°C on ID-cards NaCl, enzymetest and cold agglutinins (Bio-Rad, Cressier, Switzerland). After incubation, gel cards were centrifuged (ID-centrifuge, Bio-Rad, Cressier, Switzerland) for 10 minutes and the agglutination reaction was scored. Samples positive for irregular anti-erythrocyte antibodies, implying a risk for false positive iso-agglutinin reactions, were excluded from further analysis.

For assessment of antibody titres against A and B red blood cell carbohydrate antigens, plasma samples of patients with blood group O were tested with A1 and B cells (ID-Diacell ABO, Bio-Rad, Cressier, Switzerland), those from blood group A or B were tested with respectively B or A1 cells only, those from blood group AB were not tested. Two-fold serial dilution series of plasma were prepared in phosphate buffered saline (Yvsolab, Turnhout, Belgium). For IgG iso-agglutinin, 25 µl of diluted plasma and 50 µl of ID-Diacell A1 and/or B cell suspension were incubated for 15 minutes at 37°C on Coombs anti-IgG ID-cards (Bio-Rad, Cressier, Switzerland). For IgM iso-agglutinin, 50 µl of diluted plasma and 50 µl of ID-Diacell A1 and/or B cell suspension were incubated for 15 minutes at 20°C on ID-cards NaCl, enzymetest and cold agglutinins (Bio-Rad, Cressier, Switzerland). After incubation, gel cards were centrifuged (ID-centrifuge, Bio-Rad, Cressier, Switzerland) for 10 minutes and the agglutination reaction was scored. The end-titre was the highest plasma dilution still causing an agglutination reaction.

Measurement of measles antibodies

Quantitative and qualitative determination of specific IgG antibodies to measles virus was performed using Enzygnost anti-measles Virus/IgG ELISA (Siemens, Marburg, Germany), following the manufacturer instructions for the BEP III system (Siemens, Marburg, Germany). Plasma of HAT patients at inclusion and 6 months post-treatment, and corresponding plasma from the respective control were analysed in the same ELISA plate. Based on the reference included in the kit, results were expressed as mIU/ml. Samples with OD$<$0.1 were negative, samples with OD$>$0.2 were positive, samples in the grey zone with 0.1$<$OD$<$0.2 were retested. A measles antibody level of $\geq$200 mIU/ml is assumed to provide protection against infection in a healthy population [12,19].

Statistical analysis

For analysis, only results for which the corresponding matched sample result at the same time point was available were taken into account. Comparisons of quantitative results between controls and HAT patients and between 0 and 7 months were performed with the Wilcoxon Signed Rank Test (SigmaPlot 11). Comparisons of quantitative results between first and second stage patients were performed with the Mann-Whitney Rank Sum test. Data are presented as medians with interquartile range (IQR). Differences in proportions between controls and HAT patients were assessed with McNemar Chi square (STATA 10.0). A p-value of $\leq$0.05 was considered as significant.

Results

Study population

In total, 117 controls and 117 *gambiense* HAT patients were included. Median age was 28 years, 45% of the participants were male. Respectively 9.9% of participants suffered from malaria (13 HAT patients and 9 controls positive/223 thick blood films) and 1 control had HIV. Overall vaccination coverage reported by the study population ranged between 88.4% (183/207) for polio and 100% for BCG, and 80.6% (183/227) of participants had a BCG scar. The general condition for all study participants was judged good to normal. Among the participants, 51.3% had blood group O, 30.2% A, 15.5% B and 3.0% AB. For none of the above parameters, there were significant differences in proportions between HAT patients and controls, except for polio vaccination, reported by 83.3% of controls versus 93.3% of HAT patients ($p = 0.002$).

Among the HAT patients, 97.4% (114/117) were positive in CATT on whole blood (median plasma titre 16, IQR 8–16), 77.4% (48/62) had trypanosomes in the lymph node fluid after successful lymph node puncture, and respectively 43.4% (36/83) and 89.3% (100/112) had trypanosomes in blood detected by the micro-haematocrit centrifugation technique or in the mini-anion exchange centrifugation technique. Cerebrospinal fluid median white blood cell counts were 5/µl (IQR 2–43) and trypanosomes were observed during the cell count in 15.7% (18/115). About half (56/116) of the included HAT patients were in the meningo-encephalitic disease stage (>5 white blood cells/µl or trypanosomes in cerebrospinal fluid).

Respectively 111/117 HAT patients and 105/117 controls were revisited after a median of 211 (IQR 197–241 days, T = 7 months) and 204 days (IQR 178–246) respectively. At revisit, all participants were in good general condition. Although one control had become CATT positive, no trypanosomes were detected in any of the study participants.

Memory B-cells during and after HAT

An overview of the B-cell phenotyping results in HAT patients and controls is presented in Table 1, and an example of a dot plot of CD27 and IgM expression on B-cell subsets (CD20+), in a HAT patient and a control is shown in Figure 1. The percentage of CD20+ B-cells in HAT patients was significantly higher than in controls (median 1.5 times higher, $p<0.001$). Although the percentage of CD20+ cells had decreased 6 months after treatment of HAT, it still remained significantly higher than in controls ($p<0.001$). Within the CD20+ subset, the percentages of CD27+ memory B-cells and IgM+ B-cells were significantly higher in HAT than in controls (increases of the median of respectively 2.3 and 3.6 times, $p<0.001$). After HAT treatment the percentage of CD27+ cells within the B-cell (CD20+) subset still remained significantly higher than in controls ($p = 0.001$), while no significant difference could be observed anymore for the percentage of IgM+ B-cells ($p = 0.7$). The most striking change within the B-cells subset was the more than 6-fold increase of the percentage CD27+IgM+ cells (Q2 in Figure 1) in HAT patients compared to controls ($p<0.001$). After treatment, this subset returned to normal percentages. HAT was associated with only minor differences in the CD27+IgM− subset (Q1 in Figure 1, $p = 0.004$) of B cells. The relative decrease of naive (CD27−) B-cells in HAT was mainly due to a decrease of CD27−IgM− cells (Q4 in Figure 1, $p<0.001$) while the percentage of CD27−IgM+ cells within the B-cell subset had increased (Q3 in Figure 1, $p<0.001$). For none of the B-cell phenotypes studied, significant differences between stage 1 and stage 2 HAT patients were observed ($0.2<p<0.9$).

Memory T-cells during and after HAT

A summary of the T-cell phenotypes is presented in Table 2, and an example of a dot plot of the CD27 and CD45RO expression on T-cell subsets (CD3+), in a HAT patient and a healthy control subject is shown in Figure 2.

The percentage of CD3+ T-cells was significantly lower in HAT than in controls, and returned to normal 6 months after treatment. Within the T-cells subset, memory T-cells were significantly increased (CD45RO+, $p = 0.002$), which was due to a relative increase in early effector/memory (CD45RO+CD27+) T-cells in HAT (1.2 fold increase of the median, $p = 0.003$, Figure 2 Q2). After treatment, the observed differences in memory T-cell subsets between HAT and controls disappeared. No difference was observed in percentage of naïve (CD45RO− CD27+) T-cells between HAT patients and controls ($p = 0.8$) while the percentage of late effector (CD45RO−CD27−) T-cells was significantly lower in HAT than in controls ($p<0.001$, Figure 2 Q4), but normalized after treatment.

No differences were observed in function of the disease stage for any of the measured T-cell subsets ($p>0.08$).

IgG and IgM iso-agglutinin end-titres

Screening for irregular anti-erythrocyte IgG with ID-Diacell I–II–III cells revealed respectively 9/116 and 12/104 reactive controls at T = 0 months and T = 7 months (9 at both time points), and 7/116 and 5/109 reactive HAT patients (4 at both time points). At T = 0 months or T = 7 months, respectively, 7/116 and 4/104 controls (4 at both time points), and 6/116 and 5/109 HAT patients (4 at both time points) reacted for irregular anti-erythrocyte IgM. At inclusion, there was no difference in anti-A or anti-B IgG end titers between controls and HAT patients (table 3). For IgM, at time of inclusion median anti-A1 and anti-B iso-agglutinin end-titres were significantly lower in HAT patients than in controls ($p<0.004$). After treatment, at T = 7 months, the anti-A1 IgM iso-agglutinin end-titre had increased significantly in HAT patients ($p<0.01$), but remained lower for anti-B IgM.

There was no difference in iso-agglutinin end-titres between stage 1 and stage 2 HAT patients (p values>0.1), except for anti-B IgM which was one titer lower in stage 2 ($p = 0.05$).

Antibody levels against measles

Measles antibody concentrations in HAT patients at inclusion and after treatment and in controls at corresponding time points are summarized in Figure 3. At inclusion, the median antibody concentration in HAT patients (1500 mIU/ml, IQR 643–3300) was significantly lower than in controls (2250 mIU/ml, IQR 940–4675). Seven months later, the antibody concentration in the treated HAT patients (1700 mIU/ml, IQR 790–4300) remained significantly lower than in controls (2600 mIU/ml, IQR 1000–5500) although in both groups, the antibody level had increased significantly compared to inclusion ($p<0.001$ and $p = 0.006$ respectively). There was no difference in measles antibody concentration between stage 1 and stage 2 HAT ($p = 0.7$).

A measles antibody level superior to the cut-off assumed to provide protection against infection was present in 94.8% (110/116) of HAT patients and in 98.3% (114/116) of controls, at inclusion and 7 months later. There was no difference in proportions of HAT patients and controls exceeding this cut-off ($p = 0.3$).

No relationship between high measles antibody levels and self-reported vaccination against measles, polio, diphtheria-tetanus-pertussis, BCG or presence of a BCG scar could be observed ($p = 0.6$–1).

Table 1. Peripheral blood B-cell subsets in HAT and in controls.

% B-cell subset		Median (IQR), T = 0 months, n = 84	Median (IQR), T = 7 months, n = 70	*p*
B-cells (CD20+)	Control	11.4 (8.0–14.3)	10.5 (7.7–13.2)	0.9
	HAT	17.0 (12.8–22.4)	14.0 (10.6–19.0)	<0.001
	P	<0.001	<0.001	
Naive B-cells (CD27−)	Control	86.9 (81.5–90.8)	81.2 (73.3–86.5)	0.001
	HAT	69.6 (58.7–79.3)	74.5 (62.7–83.4)	0.4
	P	<0.001	0.003	
Memory B-cells (CD27+)	Control	13.0 (9.2–17.8)	18.3 (12.2–24.3)	0.003
	HAT	30.1 (20.8–40.9)	24.6 (16.2–36.6)	0.5
	P	<0.001	0.001	
T dependent B-cells (IgM−)	Control	86.0 (65.0–94.6)	84.3 (74.4–91.8)	0.03
	HAT	50.3 (22.1–69.3)	83.8 (75.6–94.9)	<0.001
	P	<0.001	0.9	
T independent B-cells (IgM+)	Control	13.7 (4.5–34.8)	14.2 (5.2–23.8)	0.009
	HAT	49.2 (30.7–77.3)	15.8 (3.6–23.9)	<0.001
	P	<0.001	0.7	
T dependent memory B-cells (CD27+IgM−), Q1	Control	10.9 (7.2–14.4)	15.8 (11.7–20.5)	<0.001
	HAT	12.7 (7.9–22.1)	21.4 (14.4–32.0)	<0.001
	P	0.004	0.002	
T independent memory B-cells (CD27+IgM+), Q2	Control	2.0 (0.7–4.5)	2.4 (1.1–3.9)	0.5
	HAT	13.0 (6.3–21.9)	2.5 (0.8–5.9)	<0.001
	P	<0.001	0.1	
T independent naive B-cells (CD27−IgM+), Q3	Control	11.9 (3.0–24.4)	9.11 (2.8–16.1)	0.004
	HAT	35.4 (14.2–47.7)	9.8 (1.6–15.4)	<0.001
	P	<0.001	0.5	
T-dependent naive B-cells (CD27−IgM−), Q4	Control	72.9 (55.7–85.5)	67.9 (62.8–76.6)	0.8
	HAT	31.9 (17.4–49.1)	65.7 (52.7–72.7)	<0.001
	P	<0.001	0.005	

Blood was taken from HAT patients and from non-HAT controls before treatment (T = 0 months) and after treatment or at the corresponding time point (T = 7 months). All comparisons were performed with the Wilcoxon Signed Rank Test. B-cells are expressed as percentage of all lymphocytes and all the B-cell subsets are expressed as percentages of B-cells. Q1–4 refers to the corresponding quarters in Figure 1. Individual data are shown in Fig. S1, and can be obtained from the authors upon request.
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Discussion

Our results suggest that the issue of B-cell dysfunction that troubles mouse models for trypanosomiasis, might not be that severe in human African trypanosomiasis patients infected with *T.b. gambiense*. In *gambiense* HAT patients compared to controls, significantly higher percentages of memory B- and memory T-cells were present in peripheral blood. After treatment, the percentage of memory T-cells normalized and the percentage of memory B-cells did not yet normalize. Iso-agglutinin IgM end-titres were slightly lower in *gambiense* HAT, and normalized only partially after treatment. Although anti-measles antibody levels were, and remained, lower in *gambiense* HAT patients than in controls, no significant difference could be observed in the number of individuals with levels above the international cut-off for protection.

Memory cell populations in experimental *T.b. brucei* infection have exclusively been studied in bone marrow and spleen [5]. For *T. vivax* experimental mice infections, peripheral blood data are available as well [20]. In the human host, only peripheral blood is readily accessible. Until now, data on peripheral blood lymphocyte subsets in HAT are rare, due to the important logistic challenges related to conducting research in settings like DR Congo. The observed relative B-cell increase is consistent with previous findings [10] and in line with polyclonal B-cell activation and proliferation of cells of the B lymphoid series previously described for HAT [21]. The upregulation of Fas (CD95) expression in *gambiense* HAT, measured by Boda *et al.*, led these authors to suggest a poor conversion of B-cells into memory B-cells [10]. In our study, we observed a relative increase of CD27+IgM+ B-cells which are defined as T independent memory B-cells [17] in HAT. In *T. vivax* experimental mice infections, the fall in number of B-cells in the lymphoid organs is similar to experimental *T.b. brucei* infections. In peripheral blood it is accompanied by an increase in the number of transitional IgM+IgD− B-cells and switched IgM−IgD− plasma/memory cells and by a decrease in naive B-lymphocytes [20]. Although the marker combinations used to identify B-cells in these experimental *T. vivax* studies were different from ours, the results for peripheral blood are similar, if we assume that the memory B-cells defined by CD27+ in HAT, are similar to the IgD− population in *T. vivax* infected mice [17].

We confirm the moderate relative T-cell decrease in *gambiense* HAT observed previously by Boda *et al.*, associated to the relative

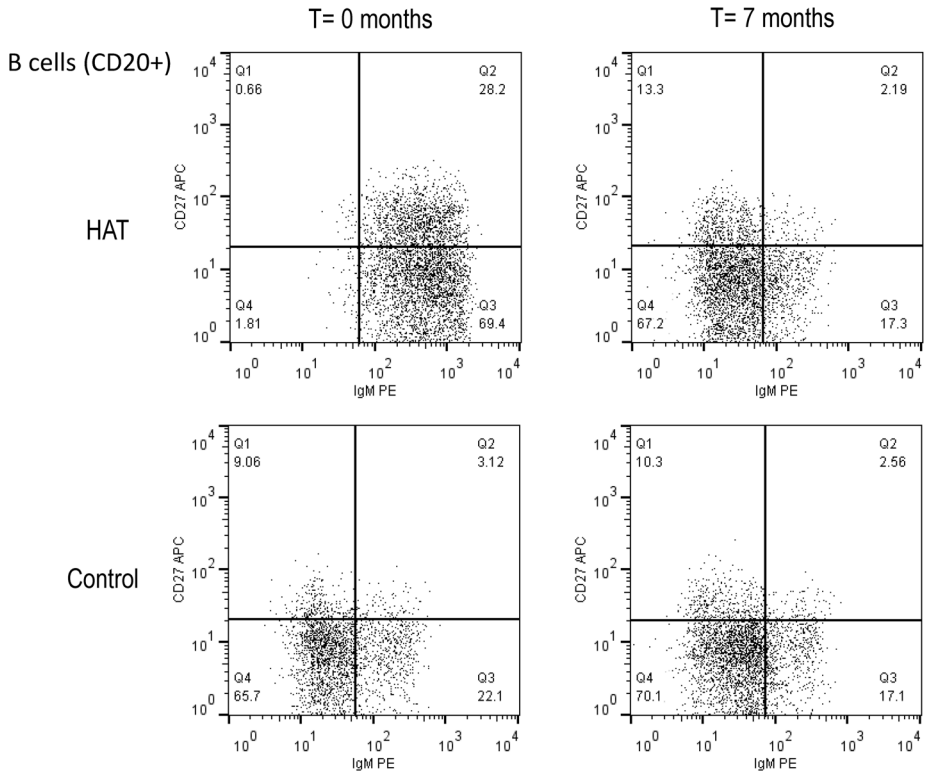

Figure 1. Flow cytometry dot plot of the CD20+ B-cell population in HAT and in a control. B-cell subsets were based on the CD27 and IgM cell surface markers. A HAT patient before and after treatment (T = 0 and 7 months), and a healthy control subject at the same time points are shown. Cut-offs for considering a cell surface marker positive or negative were based on isotype controls and are shown as solid lines, and subdivide the graph into 4 quadrants (Q1–Q4). B-cell subsets in each quadrant are expressed as percentages of CD20+ B-cells.
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expansion of B-cells. The present observation of a relative increase in early memory T-cells (CD45RO+CD27+) seems to corroborate earlier findings of larger numbers of CD4+CD45RA−CD62L+ cells in HAT with CD8+CD45RA−CD62L+ cells remaining constant [10]. As previously suggested [10], there were no differences in lymphocyte subsets according to the disease stage.

HIV also causes a significant increase in the memory (CD45RA−CD45RO+) phenotype CD8 subset [22] and reduces the CD27+ memory B-cell population [23]. The HIV prevalence in our study population was low and is not expected to affect the overall results. Malaria, which is associated with B- and T-cell exhaustion and an increase in an atypical CD19+CD27−CD 21−CD10− memory B-cell population [24], is not expected to account for differences between the control and HAT population since the frequency of occurrence of trophozoites in blood was similar in both groups. However, malaria, or other infections, might account for variation in some cell phenotypes in time, as was observed in the control group. This underlines the importance to sample controls at similar time points as HAT patients and to perform a matched statistical analysis, to maximally eliminate external variation.

The loss of the host's capacity to recall vaccine-induced memory responses, as has been described for laboratory animals [6,7], can in humans, for ethical reasons, not be tested by challenge with a pathogen. Therefore, the Multitest cell-mediated immunity (Pasteur-Mérieux, Lyon, France), an intradermal skin test to measure delayed hypersensitivity as a marker for T-lymphocyte response, was considered but this test was no longer available anymore at time of the project. Neither was it feasible to set-up of facilities for cell culture or ELISPOT. We therefore had to rely on surrogate markers and opted for the quantification of iso-agglutinins and measles antibodies.

Natural IgM antibodies against A and B carbohydrate antigens are T-cell independent, while a T-cell dependent antibody response results in higher affinity IgG1 and IgG3 antibodies [11]. In the presence of an intact immune system, iso-agglutinins to the missing A or B red blood cell carbohydrate antigens are always found, even if there has been no exposure to red blood cells carrying these antigens. These antibodies were therefore used to asses T-cell independent and T-cell dependent humoral immunity. The lower IgM iso-agglutinin titers observed in HAT patients, might indeed point to a moderate effect of *gambiense* HAT on the T-independent antibody response [6], but seems reversible upon treatment.

Measles were selected for antibody quantification for the following reasons. A high proportion of the population is expected to have antibodies against measles since the measles vaccine is part of the standard vaccination programs [12]. Half-life of measles IgG antibodies has been estimated at around 3000 years so they should be measurable in all subjects that have been infected or were successfully immunized [25]. Moreover, in healthy individuals, the absolute level of antibodies needed to fully protect against infection is known, as well as the concentration below which no protection is obtained anymore [26,27]. Vaccine coverage in the last 33 years in DR Congo has been estimated by WHO-UNICEF as 21–95% for BCG and 17–90% for the measles vaccine respectively [28]. Presence of a BCG scar in 80.6% of study participants indicated rather high vaccine coverage, and measles antibody levels above the cut-off were present in 96.6%. Measles antibody levels were comparable to levels observed in pregnant women in Belgium and

Table 2. Peripheral blood T-cell subsets in HAT and in controls.

% T-cell subset		Median (IQR), T = 0 months, n = 84	Median (IQR), T = 7 months, n = 70	*p*
T-cells (CD3+)	Control	73.2 (66.2–76.7)	71.2 (68.0–75.7)	0.02
	HAT	66.7 (61.3–73.1)	70.4 (64.4–75.9)	0.001
	P	<0.001	0.3	
Memory T-cells (CD45RO+)	Control	39.7 (31.1–48.5)	42.2 (33.5–50.5)	0.9
	HAT	44.6 (38.9–51.2)	43.4 (37.0–49.7)	<0.001
	P	0.002	0.2	
Naive T-cells (CD45RO−CD27+), Q1	Control	28.0 (20.7–39.6)	25.5 (14.6–37.1)	0.5
	HAT	28.1 (19.3–39.7)	27.0 (15.4–34.8)	0.3
	P	0.8	0.8	
Early effector/memory T-cells (CD45RO+CD27+), Q2	Control	16.7 (13.0–20.4)	13.4 (8.4–19.1)	0.09
	HAT	19.4 (14.9–24.7)	15.0 (10.2–18.9)	<0.001
	P	0.003	0.5	
Late effector/memory T-cells (CD45RO+CD27−), Q3	Control	23.0 (17.7–27.7)	25.4 (18.2–34.9)	0.05
	HAT	19.5 (16.2–26.1)	27.3 (21.1–34.4)	<0.001
	P	0.09	0.1	
Effector T-cells (CD45RO−CD27−), Q4	Control	23.3 (17.8–32.5)	24.1 (17.2–33.2)	0.7
	HAT	18.1 (14.4–23.9)	23.6 (19.2–32.1)	<0.001
	P	<0.001	0.8	

Blood was taken from HAT patients and non-HAT controls at inclusion (T = 0 months) and after treatment or at the same time point (T = 7 months). All comparisons were performed with the Wilcoxon Signed Rank Test. T-cells expressed as percentage of all lymphocytes. All the T-cell subsets are expressed as percentages of T-cells. Q1–4 refers to the corresponding quarters in Figure 2. Individual data are shown in Fig. S2, and can be obtained from the authors upon request.
doi:10.1371/journal.ppat.1003947.t002

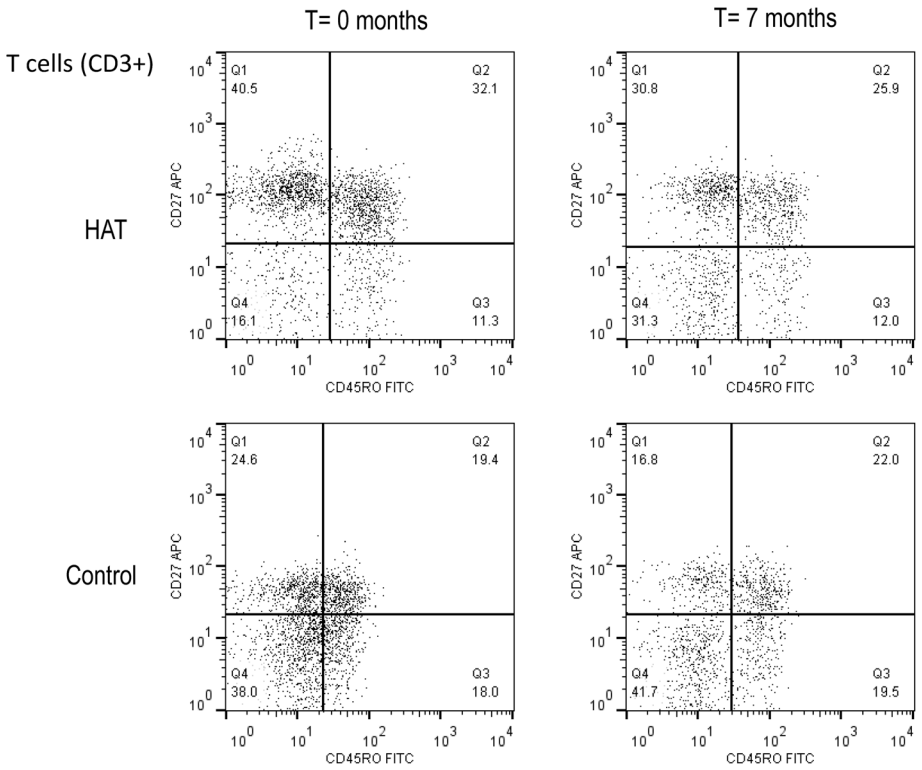

Figure 2. Flow cytometry dot plot of the CD3+ T-cell population in HAT and in a control. T-cell subsets were based on the CD27 and CD45RO cell surface markers. A HAT patient before and after treatment (T = 0 and 7 months), and a control at the same time points are shown. Cut-offs for considering a cell surface marker positive or negative are shown as solid lines, and subdivide the graph into 4 quadrants (Q1–Q4). T-cell subsets in each quadrant are expressed as percentages of CD3+ T cells.
doi:10.1371/journal.ppat.1003947.g002

Table 3. Iso-agglutinin end-titres in HAT and in controls.

Parameter		Median (IQR), T = 0 months	Median (IQR), T = 7 months	*P*
Anti-A1 IgG end-titre	Control	128 (64–256)	128 (64–256)	[a]
	HAT	64 (16–256)	128 (64–256)	[a]
	P	0.1 (n = 47)	0.7 (n = 31)	
Anti-B IgG end-titre	Control	64 (32–256)	64 (32–256)	0.2
	HAT	64 (16–128)	64 (32–128)	0.9
	P	0.6 (n = 64)	0.7 (n = 58)	
Anti-A1 IgM end-titre	Control	128 (64–256)	128 (64–256)	0.5
	HAT	64 (16–128)	128 (32–256)	0.01
	P	0.004 (n = 46)	0.5 (n = 33)	
Anti-B IgM end-titre	Control	64 (32–256)	64 (32–256)	1
	HAT	32 (16–64)	64 (16–128)	0.08
	P	0.002 (n = 67)	<0.001 (n = 63)	

Blood was taken from HAT patients before and after treatment (T = 0 and T = 7 months) and from non-HAT controls at the same time points. n: number of matched pairs used for comparison.

[a]Not calculated due to data loss. Full data can be obtained from the authors upon request.

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in a Swedish volunteer group, in which concentrations were measured with the same ELISA kit [29,30], or in an adult population in Addis-Abeba [31]. The increase in measles antibody concentrations in HAT patients and controls 7 months after inclusion, is unlikely due to a technical bias since samples taken at both time points were analyzed in the same ELISA plate. The rise might have been caused by a natural exposure to measles, as measles outbreaks regularly occur in DR Congo, also during the study period [32]. Although existing data are contradictory, presence of antibodies does not necessarily reflect presence of antibody secreting memory B-cells, as continuous antibody secretion might be due to long-lived plasma cells rather than on-going activation of memory B-cells [30]. Measles vaccine induces both humoral and cellular immune responses comparable to those following natural infection [33]. We withheld from revaccination, since live virus measles vaccination is not recommended for immune-suppressed patients [27], a condition to be expected during HAT, and since revaccination was judged not to be in the patient's best interest, even after treatment for HAT.

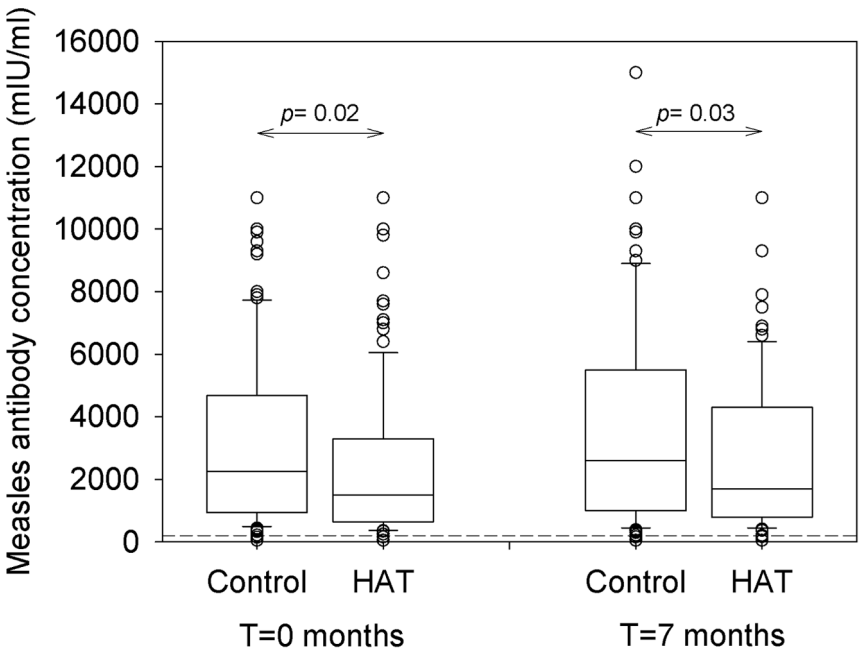

Figure 3. Box plot of measles antibody concentrations in HAT and controls. Blood was taken from HAT patients and non-HAT controls before treatment (T = 0 months, n = 116), and after treatment or at the same time point (T = 7 months, n = 99). The cut-off value for protective immunity of ≥200 mIU/ml is indicated by the dashed line. Full data can be obtained from the authors upon request.

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Overall, our results do not exclude an impairment of humoral and cellular immunity during *gambiense* HAT. Indeed, when given during *gambiense* HAT infection, a reduced response to typhoid vaccine has been observed, as well as diminished reactions to skin test antigens [34]. Similar observations have been made in domestic animals, where the antibody response to and/or efficacy of vaccination against *e.g.* contagious bovine pleuropneumonia [35], foot and mouth disease [36], swine fever [37], antrax spore [38] and *Brucella abortus* [39] were affected when given during infection with various animal trypanosomes. Moreover, in the immunized *T.b. brucei* - *Trichinella spiralis* co-infection experimental model, the anti-*Trichinella* IgG1 response was not affected [7] although protection was partially lost. Due to polyclonal B-cell activation, characteristic for trypanosomiasis infection, specific functional antibodies may be replaced by non-protective, low affinity, cross-reactive antibodies [40]. Although for measles, antibody concentrations remained above the cut-off, we cannot exclude that they have become unfunctional in HAT and their protective capacity may have been lost. The lack of a functionality test is an important difference with previously published experimental mice studies [6,7] and represents the main limitation of the actual study. It might therefore be worth to further assess the protective capacity of the measles antibodies against infection, *e.g.* using a functional assay such as the plaque reduction neutralization test [41]. However, none of the study participants mentioned a measles episode while being questioned for their vaccination history, although as mentioned above, natural exposure might have occurred. Of interest, the agglutinating capacity of the iso-agglutinin antibodies was only moderately affected in our assay.

As discussed above, other limitations inherent to our study are mainly related to research in humans instead of in laboratory animals, and to studying a disease that typically occurs in rural Africa, far from high-tech environments. In this context, blood specimens were collected on a blood stabilizer, intended to preserve peripheral blood samples' qualitative and quantitative leukocyte subset characteristics and allowing collection and storage of blood specimens for immunophenotyping by flow cytometry. Even using this stabiliser, preliminary experiments demonstrated that some lymphocyte subset cell markers (*e.g.* the cell surface marker CCR7, which we had originally selected to be used in combination with CD27 to better identify T memory subsets [18] were not optimally preserved, thus antibody cocktails had to be adapted accordingly. As we did not perform absolute counting of lymphocyte numbers, the observed changes in lymphocyte subpopulations are relative. For the iso-agglutinins, the participants blood group has to be taken into account, but the blood group was not used as a matching criterion at time of collection. In the settings we were working, in practice, it would have been difficult to identify a matched control for the patients with rarer B and AB blood groups. Data for HAT patients and controls that had different blood groups and were not tested against the same red blood cell carbohydrate antigen, were therefore lost. However, similarly lower IgM end-titers in HAT patients were also observed when statistical analysis was performed without matching the results for HAT patients and their corresponding controls (data not shown).

Overall, our results in *gambiense* HAT patients do not suggest trypanosomiasis associated massive memory cell destruction, or loss of antibody levels, although the antibody's protective capacity remains to be confirmed. So far there have never been epidemiological signals/reports that HAT patients, before or after treatment, were at increased risk of having vaccine-preventable diseases (measles or others) compared to the rest of the population. One should however acknowledge that epidemiological surveillance is generally weak in rural Africa and that such occurrences might have been missed. If some degree of immunity loss may exist in HAT patients infected with *T.b gambiense*, it does not seem of clinical relevance. At least for measles, our data indicate that antibody levels remain intact. Some open questions remain. Functionality of measles antibodies should be confirmed to completely ensure that revaccination after gambiense HAT, would not be necessary. It could also be interesting to assess activity of other vaccine-induced antibodies, as the decay of measles antibody concentrations is extremely slow and since we cannot exclude that other vaccines might depend more on memory cell dependent antibody production. Differences in immune-suppression and B-cell apoptosis observed between *gambiense* HAT and experimental infections may be linked to the differences in parasitemia between *T.b. gambiense* HAT and experimental infections [5,34]. As previously suggested [5,34], it might therefore be worth to perform similar investigations in acute *T.b. rhodesiense* HAT, which is characterized by higher parasitemia, and for which no data on peripheral blood memory T- and B-cells or on acquired immunity are available.

Supporting Information

Figure S1 Peripheral blood B-cell subsets in HAT and in controls. Blood was taken from HAT patients and from non-HAT controls before treatment (T = 0 months, n = 84) and after treatment or at the corresponding time point (T = 7 months, n-= 70). B-cells are expressed as percentage of all lymphocytes and all the B-cell subsets are expressed as percentages of B-cells.
(TIF)

Figure S2 Peripheral blood T-cell subsets in HAT and in controls. Blood was taken from HAT patients and non-HAT controls before treatment (T = 0 months, n = 84) and after treatment or at the same time point (T = 7 months, n = 70). T-cells are expressed as percentage of all lymphocytes. All the T cell subsets are expressed as percentages of T cells.
(TIFF)

Figure S3 Measles antibody concentrations in HAT and controls. Blood was taken from HAT patients and non-HAT controls before treatment (T = 0 months, n = 116), and after treatment or at the same time point (T = 7 months, n = 99).
(TIF)

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Author Contributions

Conceived and designed the experiments: VL DMN LK VKB JvG EB JJMT JJ PB. Performed the experiments: VL DMN LB BB. Analyzed the data: VL LK LB BB JvG JJ. Contributed reagents/materials/analysis tools: DMN LK LB VK JJMT JJ PB. Wrote the paper: VL LK JvG EB JJ PB.

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