Increased acute immune response during the meningo-encephalitic stage of Trypanosoma brucei rhodesiense sleeping sickness compared to Trypanosoma brucei gambiense

Natalia Tiberti a,1, Veerle Lejon b, Dieudonné Mumba N’goi c, Enock Matovu d, John Enyarue e, Nadia Walter a, Catherine Fouda a, Pascal Lutumba f, Krister Kristensson g, Sylvie Bisser h,i, Joseph Mathu Ndung’u j, Philippe Büscher k, Jean-Charles Sanchez a,*

a Translational Biomarker Group, Department of Human Protein Sciences, University of Geneva, Geneva, Switzerland
b Institut de Recherche pour le Développement, Unité Mixte de Recherche UMR 177 IRD-CIRAD, Montpellier, France
c Department of Parasitology, Institut National de Recherche Biomedicale, Kinshasa, Democratic Republic of the Congo
d Department of Biotechnical and Diagnostics Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda
e Department of Biochemistry, College of Natural Sciences, Makerere University, Kampala, Uganda
f Department of Tropical Medicine, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
g Department of Neuroscience, Karolinska Institute, Stockholm, Sweden
h INSERM UMR1094, Tropical Neuroepidemiology, Limoges, France
i Institute of Neuroepidemiology and Tropical Neurology, School of Medicine, CNRS FR 3503 GEIST, University of Limoges, Limoges, France
j Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland
*k Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

A R T I C L E   I N F O

Article history:
Received 9 July 2014
Received in revised form 3 October 2014
Accepted 10 November 2014
Available online 15 December 2014

Keywords:
Human African trypanosomiasis
T. b. gambiense
T. b. rhodesiense
Cerebrospinal fluid
Pathway analysis
C-reactive protein
Orosomucoid 1

A B S T R A C T

The host central nervous system (CNS) response to infection with Trypanosoma brucei (T. b.) gambiense or T. b. rhodesiense parasites, the causing agent of human African trypanosomiasis (HAT), is a poorly explored area. The two parasites are responsible for respectively a chronic and an acute form of HAT. In both cases, however, the disease progresses from a haemolymphatic first stage (S1) to a meningo-encephalitic second stage (S2) due to the penetration of parasites into the CNS.

In the present study, we investigated and compared the cerebrospinal fluid (CSF) from S2 patients affected by either T. b. gambiense or T. b. rhodesiense HAT, using a mass spectrometry quantitative proteomics approach. Gene ontology and pathway analyses on the 222 quantified human proteins revealed a predominant activation of the innate immune and the acute phase responses in rhodesiense HAT patients. These results were further confirmed through the verification of the over-expression of two proteins involved in these mechanisms, C-reactive protein (CRP) and orosomucoid 1 (ORM1), in 126 S2 HAT patients suffering from either the chronic or the acute form of HAT. Both proteins were significantly increased (p < 0.0001) in the CSF of rhodesiense HAT patients.

These findings contribute in better understanding the pathophysiological mechanisms of late stage HAT caused by T. b. gambiense or T. b. rhodesiense and pave the way for further investigations on the clinical significance of CRP and ORM1.

Mass spectrometry data are available via ProteomeXchange (identifier PXD001082).

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-SA license (http://creativecommons.org/licenses/by-nc-sa/3.0/).

1. Introduction

Human African trypanosomiasis – HAT or sleeping sickness – is a parasitic disease endemic in sub-Saharan Africa. The disease is caused by two subspecies of Trypanosoma brucei (T. b.): T. b. gambiense and T. b. rhodesiense, which are both transmitted through the bite of tsetse flies of the genus Glossina [1]. The two parasites are responsible for two forms of HAT that are geographically separated and clinically different. T. b. gambiense...
occurs in West and Central Africa and causes a chronic form of HAT corresponding to 98% of all reported cases [2]. T. b. rhodesiense is found in East and South-East Africa, where it causes an acute infection [3,4].

Sleeping sickness is characterized by the progression from a first haemolymphatic stage (S1) of infection to a second meningo-encephalitic stage (S2). The first stage is associated to the proliferation of parasites in the host's bloodstream and lymphatic system. If not treated, S1 disease most likely progresses to the second stage as a consequence of the penetration of the parasite into the central nervous system (CNS) across the blood-brain barrier (BBB) [5]. The speed of progression from S1 to S2 differs according to the infecting parasite: it occurs within weeks after infection in the case of T. b. rhodesiense, while it can take months or years for T. b. gambiense [6]. Consequently, T. b. gambiense HAT is considered as a chronic disease, while T. b. rhodesiense is seen as an acute condition. In both cases, however, early stage patients present unspecific clinical symptoms and signs, while late stage patients are associated to the appearance of neurological disorders, including sleep disturbances [1]. Neurological manifestations have been reported to be more marked in the gambiense form [7,8], while cardiac involvement is considered more important in the rhodesiense one [1,8–11].

The treatment of the two forms of HAT also differs. Pentamidine is used to treat gambiense S1 patients, while suramin is used for rhodesiense S1 patients [12]. S2 patients can all be treated with melarsoprol, which is however highly toxic. In the case of T. b. rhodesiense, while it can take months or years for T. b. gambiense [6]. Consequently, T. b. gambiense HAT is considered as a chronic disease, while T. b. rhodesiense is seen as an acute condition. In both cases, however, early stage patients present unspecific clinical symptoms and signs, while late stage patients are associated to the appearance of neurological disorders, including sleep disturbances [1]. Neurological manifestations have been reported to be more marked in the gambiense form [7,8], while cardiac involvement is considered more important in the rhodesiense one [1,8–11].

Regardless of the well-known differences in clinical presentation between both forms of HAT, to date, the investigation of the host response to the two sub-species of parasite in clinical samples has hardly been addressed. A number of studies aiming to discover diagnostic and staging biomarkers have, directly or indirectly, compared the concentration of immunoglobulins and immune-mediators in plasma and/or cerebrospinal fluid (CSF) of patients suffering either from gambiense or rhodesiense HAT [16–23]. However, to our knowledge, no study has been performed so far aiming at comparing biological samples from gambiense and rhodesiense HAT patients. In the present work, we investigated the CSF from S2 gambiense and rhodesiense patients using a quantitative proteomics approach in order to highlight differences in their proteome and to evaluate them in the context of HAT pathophysiology.

2. Material and methods

2.1. Ethical statement

HAT patients investigated in the present study were enrolled in the Democratic Republic of the Congo (D.R.C.), Angola, Uganda and Malawi as part of prospective studies already published elsewhere [16,17,21,24]. The relevant Institutional and National Ethics Committees of the D.R.C., Uganda, Angola, Malawi and Belgium approved the respective studies.

CSF control samples were obtained at the University Clinics of Kinshasa (D.R.C.) from subjects being hospitalized for health problems other than HAT and needing a lumbar puncture as a required procedure for their care. The study protocol was approved by the Kinshasa School of Public Health Ethical Committee and the Ministry of Public Health of the D.R.C.

All participants signed a written informed consent and accepted to be enrolled in the studies. For children, or for patients whose mental state was altered, a signature from a parent or a guardian was obtained. All patients had the possibility to withdraw from the study at any moment.

2.2. Population description

Patients suffering from sleeping sickness were diagnosed based on the parasitological detection of trypanosomes in blood and/or in lymph. HAT stage was then determined through the detection of parasites and the counting of white blood cells (WBC) in CSF, according to the guidelines of the National Sleeping Sickness Control Programs of the countries of sample collection. For the present study, patients were classified as stage 1 when no parasite was detected in the CSF and the number of CSF WBC was ≤5/μL, while stage 2 patients were defined by the presence of trypanosomes in the CSF and/or CSF WBC >5/μL, following WHO guidelines [15]. All CSF samples here collected were investigated before treatment administration.

All control patients included in the present study were negative for the presence of trypanosomes in blood and CSF, and had CSF WBC count <5 cells/μL. None of these patients presented neurological disorders and their detailed demographic description is reported in Supplementary Table S1.

2.2.1. Discovery cohort

CSF samples (n=6) obtained from 6 HAT patients were investigated using a quantitative mass spectrometry (MS) proteomics approach. Among them, 3 patients suffered from S2 T. b. gambiense HAT and originated from the D.R.C. [24], and 3 patients suffered from S2 T. b. rhodesiense and originated from T. b. rhodesiense endemic regions in Uganda – Serere district (FINDTRYP study) [17]. The demographic description of the patients is reported in Supplementary Table S2.

2.2.2. Verification cohort

The verification cohort comprised n = 185 CSF samples obtained from S1 (n = 39) and S2 (n = 126) HAT patients affected either by the gambiense or the rhodesiense form of HAT, and from non-infected control subjects (n = 20) (Table 1).

In addition to CSF, n = 29 plasma samples obtained from S2 T. b. gambiense (n = 15) and S2 T. b. rhodesiense patients (n = 14) were investigated as well (Supplementary Table S3). These samples were obtained from a sub-group of patients also tested for CSF.

A graphical summary of the experimental design – from the discovery to the verification – applied in the present study, is reported in Fig. 1.

2.3. Proteomics investigation

2.3.1. Sample preparation

For each patient (n=6), 60 μL of CSF were reduced with 50 mM tris(2-carboxyethyl)phosphine (TCEP), alkylated with 400 mM iodoacetamide and digested overnight at 37°C with trypsin (Promega, Madison, WI, USA) after having spiked 0.5 μg per sample of bovine beta-lactoglobulin (Sigma–Aldrich, St. Louis, MO, USA) as an internal control. Samples were then labelled with the TMT® 6-plex tagging reagents (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. T. b. gambiense samples were labelled with the tags TMT-126, TMT-127 and TMT-128, while T. b. rhodesiense samples were labelled with the tags TMT-129, TMT-130 and TMT-131. After tagging, samples were pooled, desalted with C18 Macro Spin Columns (Harvard Apparatus, Holliston, MA, USA) and dried under vacuum. The pooled sample was then fractionated by off-gel electrophoresis (OGE – Agilent, Santa Clara, CA, USA) into 12 fractions using a linear
IPG strip (13 cm, GE Healthcare, Uppsala, Sweden). Each OGE fraction was desalted with C18 Micro Spin Columns (Harvard Apparatus, Holliston, MA, USA), dried under vacuum and analysed by tandem mass spectrometry [25,26].

2.3.2. Mass spectrometry analysis
MS analyses were performed on an electrospray ionization (ESI) LTQ Orbitrap (OT) velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were

---

Table 1
Verification cohort; demographic description of HAT patients and control subjects whose CSF was tested in the present study.

<table>
<thead>
<tr>
<th></th>
<th>T. b. gambiense</th>
<th>T. b. rhodesiense</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1 (n = 20)</td>
<td>S2 (n = 63)</td>
<td>S1 (n = 19)</td>
</tr>
<tr>
<td><strong>Demography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex, F (n)³</td>
<td>11</td>
<td>28</td>
<td>9</td>
</tr>
<tr>
<td>Age, years [mean ± SD]¹</td>
<td>37 ± 14.4</td>
<td>32.5 ± 11.8</td>
<td>32.2 ± 19.3</td>
</tr>
<tr>
<td><strong>Geographical origin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D.R.C., (n)</td>
<td>20</td>
<td>38</td>
<td>–</td>
</tr>
<tr>
<td>Angola, (n)</td>
<td>–</td>
<td>25</td>
<td>–</td>
</tr>
<tr>
<td>Uganda, (n)</td>
<td>–</td>
<td>–</td>
<td>16</td>
</tr>
<tr>
<td>Malawi, (n)</td>
<td>–</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td><strong>CSF examination</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypanosome positive, n</td>
<td>0</td>
<td>56</td>
<td>0</td>
</tr>
</tbody>
</table>

² Chi-squared test, non significant differences.
³ Kruskal–Wallis test, non significant differences.
⁴ Missing information for one patient.
⁵ Missing information for one patient who, however, was CATT negative in blood.

---

Fig. 1. Graphical representation of the experimental design applied in the present study.
trapped on a home-made 5 μm 200 Å Magic C18 AQ (Michrom) 0.1 x 20 mm pre-column and separated on a home-made 5 μm 100 Å Magic C18 AQ (Michrom) 0.75 x 150 mm column with a gravity-pulled emitter. The analytical separation was run for 65 min using a gradient of H2O/formic acid (FA) 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B), run as follows: 0–1 min 95 A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60,000 and the ion population was set to a × 10⁶ with an m/z window from 400 to 2000. A maximum of 3 precursors were selected for both collision-induced dissociation (CID) in the LTQ and high-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 7000 (isolation width of 2 m/z) while for MS/MS detection in the OT, it was set to 2 × 10⁵ (isolation width of 2.5 m/z), with resolution of 7500, first mass at m/z = 100, and maximum injection time of 750 ms. The normalized collision energies were set to 35% for CID and 60% for HCD [26].

2.3.3. Protein identification and quantification

Protein identification was done using EasyProt platform v2.3 [27]. Peak lists were generated from raw data using ReadW software. After peak list generation, the CID and HCD spectra were merged for simultaneous identification and quantification [26]. Peptide spectral matches were searched against Swiss–Prot/UniProt database (Version 13 June 2012, 536 489 entries), by choosing Homo sapiens taxonomy. All other searching parameters were set as follows: (i) oxidized methionines as variable modifications; (ii) carboxymethylation of cysteines, TMT six-plex amino-termini and TMT six-plex lysines as fixed modifications; (iii) trypsin was selected as the digestion enzyme allowing for CID and HCD [26]; (iv) only peptides with a minimum of 6 residues were selected for identification; (v) the precursor ion tolerance was set to 10 ppm.

A second search against the UniProt/TrEMBL database (Version 11 December 2013, 49 453’151 entries), choosing the Trypanosomatidae taxonomy (ID5654) was performed, in order to check for the presence of parasite proteins. All other settings were kept as reported above.

For all identified human proteins, relative protein quantification was obtained with Isobar quantification tool (version 1.76) [28] embedded in EasyProt. Only peptides specific for a unique entry in the job were taken into account for protein quantification, and a unique quantified peptide was considered sufficient to obtain protein quantification. The protein ratio T. b. rhodesiense/T. b. gambiense was computed according to the tagging design, i.e. 129 + 130 + 131/126 + 127 + 128, after isotopic purity correction (according to the algorithm given by the manufacturer) and Isobar default normalization [28].

The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) [29] via the PRIDE partner repository [30] with the dataset identifier PXD001082.

2.4. Gene ontology and pathway analysis

Significant differentially expressed CSF human proteins were submitted to gene ontology (GO) analysis to evaluate their association with specific biological processes (BP) GO terms. The analysis was performed with bioCompendium, available at http://biocompendium.embl.de.

The whole list of identified and quantified CSF human proteins was further investigated with Ingenuity® Pathway Analysis – IPA (Ingenuity® Systems, Redwood City, CA, USA, http://www.ingenuity.com) [31–33]. Briefly, a list containing the IDs of each identified and quantified protein, the corresponding fold-change for the rhodesiense/gambiense ratio, as well as the quantification ratio p-value and sample p-value generated with Isobar, were uploaded in the IPA analysis tool.

A “Core Analysis” was then performed to highlight important biological pathways associated to the dataset. The following settings were applied: Ingenuity knowledge base (genes + endogenous chemicals) was the reference set, both direct and indirect relationships were included and no filter at the level of the cut-off or p-value on experimentally observed data were applied to keep all the entries for the analysis. Finally, human was selected as the species.

By using the canonical pathway tool of IPA Ingenuity, the proteins of the experimental dataset have been displayed within well-established signalling or metabolic pathways. For each pathway, the following parameters were calculated: p-value, indicating the association between a specific pathway and the experimental dataset; and ratio, indicating the percentage of genes in a pathway also found in the experimental dataset. This ratio was computed by dividing the number of genes belonging to a specific pathway found in the experimental data set, by the total number of genes of that pathway. To highlight those pathways most likely associated to the dataset, the threshold for the p-value was set to 1E-04.

2.5. Verification by immunoassays

The concentration of C-reactive protein (CRP), orosomucoid 1 (ORM1) and complement component 9 (C9) was measured using commercially available immunoassays on the verification cohort (Table 1). CRP was measured by bead suspension assay (mBSA – Millipore, Billerica, MA, USA), ORM1 was measured by competitive ELISA and C9 by sandwich ELISA (Abcam, Cambridge, UK). All samples were tested in simplicate and the assays were performed following manufacturers’ instructions. The technical performance of the experiments was validated using commercial (for CRP) or home-made (for ORM1 and C9) quality controls.

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS v20.0 (IBM, NY, USA). All statistical tests were two-tailed and the p-value for significance was set to 0.05. Comparisons between two un-paired groups were done using the Mann–Whitney U test. Comparisons between more than two unpaired groups were done with the Kruskal–Wallis test followed by post-test paired comparisons. When multiple comparisons were done, the Bonferroni’s correction was applied, multiplying the p-value by the number of comparisons performed. Box plot graphs were obtained with Aabel v2.4.2 (Gigawiz Ltd. Co., Tulsa, OK, USA).

3. Results

3.1. Quantitative proteomics analysis

The investigation using quantitative proteomics of 6 CSF samples from HAT S2 patients (n = 3 T. b. gambiense and n = 3 T. b. rhodesiense) allowed identifying 239 CSF human proteins with at least 2 unique peptides and <1% false discovery rate (FDR). Ninety three percent of the identified peptides were correctly tagged with the TMT labels, demonstrating the efficient technical performance of the experiment. The technical variability among the 6 tags was evaluated through the relative intensity of the peptides identifying the bovine beta-lactoglobulin – internal control – which showed a coefficient of variation (CV) of 17%. After isobar default normalization and removal of proteins corresponding to keratins (n = 3) and those with multiple entries
(n = 7) (i.e. having all peptides shared with more than one entry), the final list of quantified human proteins corresponded to 222 entries (Supplementary MS information).

According to Isobar statistics, 11 proteins resulted to be significantly differentially expressed between *rhodesiense* and *gambiense* samples. Eight of these proteins were over-expressed in the *rhodesiense* form of sleeping sickness, while 3 were under-expressed (Table 2).

No parasite protein was identified (FDR < 1%, 2 unique peptides) when the search for the *Trypanosomatidae* taxonomy was performed, probably as a consequence of CSF centrifugation prior to examination for parasite detection, storage and inclusion in the specimen bank, as a common laboratory practice. To investigate parasite proteins, the sediment of the centrifugation should be analysed.

### 3.2. Gene ontology and pathway analyses

Among the 11 differentially expressed proteins, 8 were successfully associated to four BP GO terms. Two of these terms were significantly represented (adjusted p-value < 0.05) among the 8 proteins: the acute inflammatory response (GO: 0002526) and the humoral immune response (GO: 0006959) (Supplementary Fig. S1). Interestingly, the acute inflammatory response involved 7 out of the 8 proteins over-expressed in *rhodesiense* samples compared to *gambiense* samples.

To go deeper into the obtained data and to investigate whether specific pathways were represented in the dataset, an analysis with IPA Ingenuity was performed. Among the 222 entries uploaded, 198 were successfully mapped against the Ingenuity knowledge database and thus used for the subsequent analyses. By applying the 1E-04 p-value threshold, 13 pathways were selected as significantly represented in our dataset, with the most represented being the “acute phase response signalling” (p-value = 1.09E-44), the “liver X receptors/retinoid X receptors (LXR/RXR) activation” (p-value = 1.05E-39) and the “complement system” (p-value = 3.05E-25) (Table 3 and Supplementary Table S4). The first 4 of these pathways, the acute phase response signalling, had 42 of its total 173 genes (24%) represented in our dataset. Of these 42 entries, 30 had a positive fold change (i.e. >1.0) for the *rhodesiense/*gambiense ratio and comprised 6 of the significantly over-expressed proteins (Table 3). The second most represented pathway was the LXR/RXR activation. Among the 34 proteins of our dataset involved in this pathway, 22 had a positive fold-change, including 6 significantly expressed proteins. Finally, the complement cascade had 17 out of its 33 components (52%) represented. Despite the majority of these components had a positive fold change (14/17), only one – complement component C9 – was significantly differentially expressed between *T. b. rhodesiense* and *T. b. gambiense*, with a *rhodesiense/gambiense* ratio of 1.78.

CRP, ORM1 and C9 were selected for further verification, based on their TMT ratios as well as on their appearance in the pathways of interest. CRP showed the highest ratio (*rhodesiense/gambiense* = 9.30), and was part of the most represented pathway, the acute phase response signalling (Tables 2 and 3). ORM1 was highly significantly differentially expressed between the two forms of parasite with a 2.69 ratio and appeared in the acute phase response signalling and in the LXR/RXR activation pathways. Finally, the complement component 9 (C9) had a *rhodesiense/gambiense* ratio of 1.78 and appeared in the three most enriched pathways.

### 3.3. Protein verification

To confirm the results obtained from the proteomics discovery experiment, we compared the concentration of CRP, ORM1 and C9

<table>
<thead>
<tr>
<th>#</th>
<th>AC</th>
<th>Protein</th>
<th>Gene</th>
<th>Peptide count</th>
<th>Spectral count</th>
<th>Ratio</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P06311</td>
<td>Ig kappa chain V-III region IARC/BLA1</td>
<td>na</td>
<td>2</td>
<td>2</td>
<td>0.69</td>
<td>0.007</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>O00584</td>
<td>Ribonuclease T2</td>
<td>RNase T2</td>
<td>3</td>
<td>3</td>
<td>0.69</td>
<td>0.006</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Q8NPW2</td>
<td>Semaphorin-4B</td>
<td>SEMA4B</td>
<td>2</td>
<td>2</td>
<td>0.72</td>
<td>0.048</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P04114</td>
<td>Apolipoprotein B-100</td>
<td>APOB</td>
<td>4</td>
<td>5</td>
<td>1.36</td>
<td>0.041</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>P19652</td>
<td>Alpha-1-acid glycoprotein 2</td>
<td>ORM2</td>
<td>5</td>
<td>10</td>
<td>1.49</td>
<td>0.005</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Q03591</td>
<td>Complement factor H-related protein 1</td>
<td>CFHR1</td>
<td>1</td>
<td>1</td>
<td>1.52</td>
<td>0.023</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>P02748</td>
<td>Complement component C9</td>
<td>C9</td>
<td>8</td>
<td>10</td>
<td>1.78</td>
<td>0.006</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Q14624</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H4</td>
<td>ITIH4</td>
<td>10</td>
<td>18</td>
<td>1.80</td>
<td>0.005</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>P18428</td>
<td>Lipopolysaccharide-binding protein</td>
<td>LBP</td>
<td>2</td>
<td>2</td>
<td>2.31</td>
<td>0.046</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>P02763</td>
<td>Alpha-1-acid glycoprotein 1</td>
<td>ORM1</td>
<td>6</td>
<td>18</td>
<td>2.69</td>
<td>0.001</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>P02741</td>
<td>C-reactive protein</td>
<td>CRP</td>
<td>3</td>
<td>5</td>
<td>9.30</td>
<td>0.001</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Only proteins having both p-values < 0.05 were considered significantly differentially expressed. Peptide count and spectral count correspond to the number of peptides and the number of spectra used for the quantification, respectively.

---

### Table 2

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Ratio</th>
<th>p-value</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute phase response signalling</td>
<td>0.243</td>
<td>1.09E-44</td>
<td>C-reactive protein, Alpha-1-acid glycoprotein 2, Lipopolysaccharide-binding protein, Inter-alpha-trypsin inhibitor heavy chain H4, Complement component C9, Alpha-1-acid glycoprotein 2</td>
</tr>
<tr>
<td>LXR/RXR activation</td>
<td>0.278</td>
<td>1.05E-39</td>
<td>Alpha-1-acid glycoprotein 1, Lipopolysaccharide-binding protein, Inter-alpha-trypsin inhibitor heavy chain H4, Complement component C9, Alpha-1-acid glycoprotein 2</td>
</tr>
<tr>
<td>Complement system</td>
<td>0.515</td>
<td>3.05E-25</td>
<td>Complement component C9</td>
</tr>
</tbody>
</table>

For each pathway, the complete list of identified proteins is available in Supplementary Table S4.

---

* a: Proportion of genes of the dataset belonging to the pathway/total number of genes of the pathway.

* b: Association between the pathway and the experimental dataset.

* c: Significantly differentially expressed proteins of the dataset involved in the pathway.
in the CSF of T. b. gambiense (n = 63) and T. b. rhodesiense (n = 63) S2 HAT patients, measured by immunoassay. CRP and ORM1 showed a highly significant difference (Mann–Whitney U test, p-value < 0.0001), with a rhodesiense/gambiense ratio of 31.59 and 2.79, respectively (Fig. 2 and Supplementary Table S5). The CSF concentration of C9 showed a lower difference (but still significant) with a p-value = 0.044 and a rhodesiense/gambiense ratio of 1.41, suggesting a less marked accuracy of this molecule in discriminating between the two forms of HAT in the brain.

Since the investigated patients originated from different countries (i.e. D.R.C. and Angola for T. b. gambiense; Uganda and Malawi for T. b. rhodesiense), we evaluated the levels of the 3 molecules with respect to their geographical origin. Nevertheless, no statistical difference was observed between samples collected in countries endemic for the same form of HAT, i.e. between D.R.C. and Angola, or between Uganda and Malawi (Supplementary Fig. S2). With this patients’ classification, a significantly higher concentration in S2 T. b. rhodesiense compared to S2 T. b. gambiense CSF was confirmed for CRP, independently from the country of sample collection. A similar profile was observed for ORM1 but, interestingly, no significant difference was observed between Congolese and Malawian S2 patients (Supplementary Fig. S2 and Supplementary Table S5). As expected, C9 showed the less pronounced differences and only Angolan and Ugandan patients showed different CSF levels of the protein, further sustaining its limited potential to differentiate the two forms of sleeping sickness.

To try to put our findings into the context of HAT progression, the levels of CRP, ORM1 and C9 were also measured in the CSF of a small number of S1 patients (T. b. gambiense – n = 20; T. b. rhodesiense – n = 19). CSF from control subjects (n = 20) were included as well (Fig. 3).

In gambiense patients, the levels of the 3 molecules were not different from those measured in controls, except for ORM1, which was increased in S2 T. b. gambiense compared to controls.

On the contrary, in rhodesiense patients, the concentration of the 3 molecules measured in the CSF of S2 patients was always significantly higher than in control subjects. Moreover, CRP was also increased in S1 T. b. rhodesiense compared to controls.

When the levels of the 3 molecules were compared between S1 and S2 patients, the only differences observed were for ORM1 and C9 for the comparison S1 vs. S2 T. b. rhodesiense; in all other cases no difference was observed between S1 and S2 patients infected by the same sub-species of trypanosome.

Finally, the 3 proteins were also measured in the plasma samples from a small number of S2 HAT patients (T. b. gambiense n = 15; T. b. rhodesiense n = 14). Interestingly, plasma CRP levels discriminated between gambiense and rhodesiense S2 HAT with a p-value < 0.0001. The two other molecules did not (Fig. 4).

4. Discussion

T. b. gambiense and T. b. rhodesiense HAT have been, for long time, considered two distinct forms of disease from both clinical and geographical point of view. To try to contribute in understanding the molecular mechanisms characterizing the host immune response against the two parasites, we compared the cerebrospinal fluid of patients suffering from S2 HAT caused by either T. b. gambiense or T. b. rhodesiense parasites.

The global evaluation of CSF human proteins identified by MS, showed that pathways associated with the immune response – acute phase response signalling, LXR/RXR activation and complement system – were significantly enriched in the CSF of HAT late stage patients, in agreement with the known inflammatory response occurring in the brain of S2 HAT patients. The central role of the neuro-inflammatory response in HAT pathology has, in fact, already been highlighted [34–39], even if the mechanisms of CNS invasion by the parasites and of development of neurological disorders are still not completely understood.

The acute phase response represents the first rapid mechanism of protection against invading microorganisms and acts through non-specific processes such as the induction of pro-inflammatory cytokines, the activation of the complement systems and the opsonization [40]. The increased expression of proteins involved in this pathway in the CSF of late stage T. b. rhodesiense patients is thus in agreement with the acute nature of this form of disease compared to the gambiense one. These results were further confirmed through the verification of CRP and ORM1 on clinical samples, both resulting to be highly significantly differentially expressed (p < 0.0001) between the 2 forms of HAT.

CRP, the most known acute phase protein, is a member of the pentraxin family and plays an important role in the innate immunity against a number of stimuli including invading pathogens [41,42]. Despite non-specific for a determined infection, the variations of CRP plasma concentration can be very useful when considered in the clinical context. Strong increases in plasma concentration have been observed, among other conditions, during parasitic diseases, including malaria and leishmaniasis [41]. Considering the CSF compartment, CRP has been widely investigated in the context of bacterial meningitis and it has been proposed that the presence of gram negative bacteria could enhance CRP permeability across the blood brain barrier (BBB) [43]. Despite mainly known of hepatic origin, many cell types have been suggested to be able to produce CRP, including neurons

---

**Fig. 2.** Box plots of the concentration of CRP, ORM1 and C9 measured in the CSF of T. b. gambiense (n = 63) and T. b. rhodesiense (n = 63) S2 patients. Whiskers represent the 10th and the 90th percentile, circles represent outliers. Mean and median are represented as a diamond and a solid line on the graphs, respectively. Statistical comparisons have been done with the Mann–Whitney U test. *** Corresponds to p-value < 0.0001 and * corresponds to p-value < 0.05. T.b.g. = T. b. gambiense; T.b.r. = T. b. rhodesiense.
and macrophages [44]. The specific origin of CRP measured in the CSF of HAT patients is not known and the increased CSF concentration in S2 rhodesiense could be a consequence of diffusion from plasma or could be of intrathecal origin. Interestingly, in a mouse model of obesity, it has been highlighted that high doses of blood CRP increase BBB permeability [45]. Even so, the elevated concentration of CRP measured in the CSF of T. b. rhodesiense patients, both S1 and S2, seems to suggest an early involvement of this protein in brain inflammation. Since it has been proposed that the passage of the trypanosomes across the blood-CSF and the BBB could be a multistep process [23,39], deeper investigations will help in determining whether CRP represents an early response to this invasion, or an enhancer for parasite brain penetration. Independently from its mechanisms of production and action, the increased concentration of CRP seems to be associated to the rhodesiense infection rather than to the gambiense one, indicating that different immune responses are elicited by the two trypanosomes, with a predominant involvement of the innate immunity in rhodesiense HAT. These results are further sustained by the observation of significantly increased concentrations of CRP in the plasma of S2 T. b. rhodesiense compared to S2 T. b. gambiense.

<table>
<thead>
<tr>
<th>Post-test, p value</th>
<th>CRP [ng/mL]</th>
<th>ORM-1 [µg/mL]</th>
<th>C9 [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL vs. S1 T.b.g.</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CTRL vs. S2 T.b.g.</td>
<td>ns</td>
<td>0.016</td>
<td>ns</td>
</tr>
<tr>
<td>CTRL vs. S1 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CTRL vs. S2 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.008</td>
</tr>
<tr>
<td>S1 T.b.g. vs. S2 T.b.g.</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>S1 T.b.g. vs. S1 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>S1 T.b.g. vs. S2 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>S2 T.b.g. vs. S1 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>0.010</td>
</tr>
<tr>
<td>S2 T.b.g. vs. S2 T.b.r.</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>ns</td>
</tr>
<tr>
<td>S1 T.b.r. vs. S2 T.b.r.</td>
<td>ns</td>
<td>0.004</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Fig. 3. Box plots reporting the concentration of CRP, ORM1 and C9 measured in the CSF of control subjects (CTRL, n = 20); S1 T. b. gambiae (S1 T.b.g., n = 20), S2 T. b. gambiae (S2 T.b.g., n = 63); S1 T. b. rhodesiense (S1 T.b.r., n = 19), and S2 T. b. rhodesiense (S2 T.b.r., n = 63). Whiskers represent the 10th and the 90th percentile, circles represent outliers. Mean and median are represented as a diamond and a solid line on the graphs, respectively. Statistical comparisons have been done using the Kruskal–Wallis test followed by paired comparisons (post-test with Bonferroni’s correction). For the three molecules, the Kruskal–Wallis p-value was ≤0.0001, the p-value of the paired comparisons are reported in the table.

Fig. 4. Box plots of the concentration of CRP, ORM1 and C9 measured in the plasma of S2 T. b. gambiae (n = 15) and S2 T. b. rhodesiense (n = 14) HAT patients. Whiskers represent the 10th and the 90th percentile, circles represent outliers. Mean and median are represented as a diamond and a solid line on the graphs, respectively. Statistical comparisons have been obtained through the Mann–Whitney U test. *** = p-value <0.0001; ns = non significant.
Similar to CRP, ORM1 is an acute phase protein significantly over-expressed in the CSF of T. b. rhodesiense patients compared to T. b. gambiense. Even though the specific function of ORM1 has not been clearly described yet, this protein seems to act as a modulator of the inflammatory response [46]. ORM1 is mainly released by hepatocytes, but other cell types such as monocytes, leukocytes and endothelial cells have shown similar abilities [47]. Among the proposed functions for this protein, two are particularly interesting in the context of sleeping sickness: its participation in inflammatory signalling between the immune system and the CNS, and its ability to directly interact with microbial products [46]. In our study, the CSF concentration of this protein was significantly increased in T. b. rhodesiense patients, while S1 T. b. rhodesiense CSF showed the same concentration as control subjects. This result might indicate a different response of CRP and ORM1 to the infection. A different time delay of the two proteins has already been reported, with CRP representing an earlier response in T. b. rhodesiense [48]. Deeper investigations, also involving experimental models of HAT, are required to better understand the specific role of CRP and ORM1 during HAT infection as well as their mechanisms of production.

ORM1 is also involved in the LXR/RXR activation, the second most enriched pathway in our study. LXR – liver X receptors – and RXR – retinoic X receptors – are transcriptional factors that participate, as a heterodimer, in the regulation of the expression of genes involved in cholesterol metabolism and sterol absorption [49]. LXRα are highly expressed in the intestine, however, their activity at the BBB level has been demonstrated as well [49,50].

Interestingly, the same pathway was found to be significantly differentially activated in an expression microarray study comparing the response, in animal models, to different T. brucei strains: the LXR/RXR activation pathway was found to be predominant in mice showing more severe pathological effects [51], in agreement with our findings.

Another important mechanism associated to the innate immune response is the activation of the complement cascade [40]. In our study, the complement cascade was highly significantly represented in the CSF of S2 HAT patients, but without particular association with one form of the disease, as confirmed by the small differences of C9 concentration on the verification cohort. This result might suggest that the presence of trypanosomes in the brain of HAT patients is associated with the activation of the complement system in the attempt to clear parasites, but without specificity for one of the two forms.

Globally, our results indicate that different host responses are elicited by the two parasites. Similar differences had already been shown within the T. b. rhodesiense subspecies [52]. CRP and ORM1, the most interesting proteins in our study, did not show any statistical difference between patients affected by the same form of HAT but enrolled in different countries. However, it should be stated that a limited number of samples was tested in the present study and deeper investigations including a new discovery phase should be performed to address this question.

The present study has some limitations that should be taken into account. First, despite the very promising results obtained for CRP and ORM1, a larger number of clinical samples should be analysed to better evaluate their clinical interest. These further investigations should also include a larger number of plasma samples, both S1 and S2, as they might help in better understanding the role of CRP and ORM1 in HAT pathophysiology. Another important aspect that should be underlined is the lack of clinical information on patients’ neurological conditions. The correlation between the degree of the neurological involvement and the CSF concentration of CRP and ORM1 could have helped in better understanding their role in T. b. gambiense and T. b. rhodesiense HAT progression. Finally, important additional information could be obtained by the investigation of CRP and ORM1 in CSF samples from patients suffering from other pathologies endemic in HAT regions, such as HIV, malaria and meningitis. This kind of analysis would help in better evaluating CRP and ORM1 potential in terms of disease biomarkers.

In conclusion, in the present study we demonstrated that the proteomics investigation of rhodesiense and gambiense CSF from S2 patients, combined with bioinformatics tools and verification strategies, highlighted a prevalent activation of the innate immune and acute phase responses in the CSF of patients suffering from T. b. rhodesiense HAT. These results pave the way for further investigations from both the pathophysiological and the clinical point of view to determine CRP and ORM1 role in disease progression and their mechanisms of production. Based on the promising results reported here, CRP and ORM1 should be further investigated on a larger number of plasma samples, also including S1 patients, to extensively evaluate their potential for differentiating between rhodesiense and gambiense clinical samples. The clinical differentiation of the two forms of disease might become important in the light of their potential future overlap in Uganda, following the spread of T. b. rhodesiense infection, due to infected livestock movement [52]. Since for the moment the two parasites can only be distinguished by PCR, blood and CSF markers, such as CRP, might find an application in the future. Additionally, the validation of CRP and ORM1 on a larger number of CSF and plasma samples might reveal other clinical applications, for example for the early detection of T. b. rhodesiense S2 relapses after treatment.

Acknowledgments

The authors thank the Foundation for Innovative New Diagnostics (FIND), for financial support in sample collection in Uganda, D.R.C. and Angola; and the Belgian Directorate General for Development Cooperation, for financial support in patients’ sample collection in D.R.C. Part of the specimen collection in Malawi was supported by a grant from the European Union [FP6-2004-INCO-DEV-3 032334; NEUROTROP]. The authors also acknowledge the PRIDE team for their support for MS data deposition to ProteomeXchange Consortium (identifier PXD001082).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.trpfort.2014.11.001](http://dx.doi.org/10.1016/j.trpfort.2014.11.001).

References
