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Abstract

Human African trypanosomiasis (HAT) is a neglected tropical disease that is endemic in sub-Saharan Africa. Control of the disease has been recently improved by better screening and treatment strategies, and the disease is on the WHO list of possible elimination. However, some physiopathological aspects of the disease transmission and progression remain unclear. We propose a new proteomic approach to identify new targets and thus possible new biomarkers of the disease. We also focused our attention on fluids classically associated with HAT (serum and cerebrospinal fluid (CSF)) and on the more easily accessible biological fluids urine and saliva.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) established the proteomic profile of patients with early and late stage disease. The serum, CSF, urine and saliva of 3 uninfected controls, 3 early stage patients and 4 late stage patients were analyzed.

Among proteins identified, in CSF, urine and saliva, respectively, 37, 8 and 24 proteins were differentially expressed and showed particular interest with regards to their function. The most promising proteins (Neogenin, Neuroserpin, secretogranin 2 in CSF; moesin in urine and intelectin 2 in saliva) were quantified by enzyme-linked immunosorbent assay in a confirmatory cohort of 14 uninfected controls, 23 patients with early stage disease and 43 patients with late stage disease. The potential of two proteins, neuroserpin and moesin, with the latter present in urine, were further characterized.

Our results showed the potential of proteomic analysis to discover new biomarkers and provide the basis of the establishment of a new proteomic catalogue applied to HAT-infected subjects and controls.

Key words: Sleeping sickness, biological fluids, proteomic

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic disease, caused by subspecies of *Trypanosoma brucei*, that mainly affects poor and rural communities in more than 20 sub-Saharan African countries [1]. HAT is one of 20 neglected tropical diseases (NTDs) recognized by the World Health Organization (WHO) [2], and it is estimated that 55 million people live at risk of contracting HAT infection [3]. In the human population, *T. b. gambiense* is responsible for more than 98% of HAT cases in central and western Africa, *T. b. rhodesiense* is responsible of the remaining 2% of cases, which is prevalent in eastern and southern Africa. However, since 2009, fewer than 10,000 cases have been reported each year [3]–[5].

Trypanosomes are inoculated into human hosts by tsetse fly vectors; the trypanosomes then multiply and spread into the blood and lymphatic system. *T. b. gambiense* causes a chronic form of disease that develops over months to years in two stages. The first stage (S1), or hemolymphatic stage, is generally characterized by intermittent fever, lymphadenopathy, hepatosplenomegaly and headaches [5]. These symptoms are discrete and nonspecific for HAT. After a variable duration of time, trypanosomes cross the blood–brain barrier and reach the central nervous system (CNS); this stage is called the second stage (S2) or nervous stage. S2 is characterized by neurological signs and symptoms and usually leads to death if untreated [6], [7].

Currently, several tests adapted to the field are used to diagnose HAT, the card agglutination test for trypanosomiasis (CATT) is still the most widely used screening test although single-format rapid diagnostic tests have been recently validated [8]. When the screening test is positive, the disease must be confirmed by demonstrating the presence of the parasite by microscopy. The tests used include blood concentration techniques such as the capillary tube centrifugation (CTC)

and mini anion exchange centrifugation technique (mAECT) [9], [10] or examination of lymph node aspirates when lymph nodes are swollen. Once diagnosis of the disease is established by one or more of these tests, it is important to stage the disease to guide treatment regimen. The staging criteria defined by the WHO are the absence of trypanosomes in the cerebrospinal fluid (CSF), and fewer than 5 white blood cells (WBC)/μL CSF for patients with S1 disease, and the presence of trypanosomes in the CSF and/or more than 6 WBC/μL CSF for patients with S2 disease. These staging criteria are not always easy to use in settings where the disease occurs; microscopy is not a sensitive enough technique to reliably detect the parasite in the CSF, and the CSF WBC count is not specific for HAT [11]. Without the right diagnosis, S1 disease may progress to S2 disease, and patients may not get the correct treatment. Patients with S1 *T. b. gambiense* HAT are treated with pentamidine. S2 patients require treatments that can cross the blood–brain barrier. ~~Second stage~~ *Trypanosoma b. gambiense* S2 infection can be treated with a nifurtimox–eflornithine combination therapy (NECT), but this treatment is given by infusion which is invasive and difficult to perform in some areas [12]. For *T. b. rhodesiense*, melarsoprol is the only treatment available [3]. A new easy-to-use treatment, named Fexinidazole, is expected to be available in 2018. However, this treatment, even if it appears to be active in both stages of the disease, is proposed as an alternative to NECT, therefore for the treatment of S2. The use of pentamidine to treat S1 remains the current course of action [13]. Even if we can hope for the arrival of a molecule that will allow us to treat both stages of the disease, greatly facilitating the diagnostic and therapeutic plan of patients, the determination of the stage of the disease is still necessary at this time.

The composition of the proteome of different biological fluids change with disease progression and may lead to the generation of biomarkers that could be used in diagnosing and staging of the disease [14]. Currently, proteome mapping is experiencing substantial growth owing to very high-resolution techniques such as liquid chromatography–tandem mass spectrometry (LC-MS/MS). Tandem mass spectrometry analysis allows a large proteome screen compared with more specific techniques such as enzyme-linked immunosorbent assay (ELISA), immunoblotting techniques and others that focus only on a single protein or small part of the proteome. The data obtained with this technique, compared with the current human protein database, allow identification and categorization of the proteins present in a biological sample with extensive proteome coverage. Studies using LC-MS/MS have already discovered new biomarkers in many diseases, such as Duchenne muscular dystrophy, amyotrophic lateral sclerosis, oral cavity squamous cell carcinoma and breast cancer [15]–[18]. To enable the discovery of new biomarkers from the proteome of biological fluids, it is important to characterize the entire proteomes of both healthy and infected individuals. In this study, the serum, CSF, saliva and urine proteomes obtained in patients with S1 and S2 *T. b. gambiense* HAT were compared with uninfected controls. Using a high-resolution mass spectrometer coupled to a nano-ultra-performance liquid chromatography (UPLC) system, several proteins with variable abundance levels between the different groups were revealed by label-free protein quantification analysis.

The objective of this study was to characterize the proteome of serum, CSF, urine and saliva from patients with *T. b. gambiense* HAT and to identify biological markers that could be used in disease diagnosis and staging.

Material and Methods

Subjects and sample collection

The samples used in this study were collected during a prospective cohort study carried out between 2009 and 2011 in three provinces in Angola: Bengo, Uíge and Kwanza Norte. The study enrolled 247 Angolan subjects: 67 were classified as S1 patients, 161 as S2 patients, and 19 as controls. All patients were designated HAT positive by CATT (with or without titration), and trypanosomes demonstration by microscopy in the blood or in lymph node aspiration. Patients in the cohort had *T. b. gambiense* HAT, and were staged before treatment. Patients with no trypanosomes in the CSF and normal WBC counts (≤ 5 WBC/μL CSF) were classified as S1 patients. Patients with a WBC counts of more than 5 WBC/μL CSF and/or presence of trypanosomes in the CSF were classified as S2 patients. Controls were subjects who had tested positive for HAT by CATT but without parasite confirmation in blood, lymph node or CSF and without development of the disease during the whole follow-up period. Every subject was examined clinically and submitted to a questionnaire to document clinical and neurological characteristics. Neurological data included an index of depression, measured with the Hamilton rating scale [19], and the sleep and psychiatric disturbance using the Mini-International neuropsychiatric interview [20]. Main co-infections were excluded in the field by microscopy for common parasitic co-infections (blood smear for malaria, CTC for detection of filariasis, examination of a urine sediment for schistosomiasis when urine was positive for blood). Retrospectively, samples were tested for HIV and syphilis, and all positive samples were excluded from the study.

For both controls and patients, samples of 5 mL of serum, 4 mL of CSF, 2 mL of urine and 2 mL of saliva were stored in liquid nitrogen, transported to Limoges (France), in dry tanks and further

136 stored at -80°C. Samples used for this study were carefully selected among the cohort and
137 common co-infections were excluded.

138 **Ethics statement**

139 Ethical clearance was obtained from the Direccao National de Saude Publica, Ministerio da
140 Saude, Angola. Written informed consent was obtained from all participants and, for participants
141 below 18 years of age, from their parents or guardians, prior to enrolment. Any individual who
142 declined to participate was managed according to the standard procedures of the national HAT
143 control program.

144 **Study design**

145 Potential biomarkers were first screened in serum, CSF, urine and saliva on a small population of
146 controls and patients by mass spectrometry and, second, the most promising biomarkers were
147 then quantified on a larger cohort.

148 ***The screening cohort*** **(n = 10)**

149 LC-MS/MS analysis was done on uninfected controls (n = 3), S1 patients (n = 3) and S2 patients
150 (n = 4). Only adult males were included to avoid variations of the proteome linked to the
151 expression of hormonal proteins.

152 ***Quantitative analysis of the validation cohort by ELISA (n = 80)***

153 Protein quantification was done on samples collected after inclusion for controls (n = 14), S1
154 patients (n = 23) and S2 patients (n = 43). All samples used for the LC-MS/MS analysis were
155 included for protein quantification.
156 Two analyses were done: the first to identify among the detected proteins those that differentiated
157 controls from patients with HAT (n = 66); the second to verify whether these proteins could

158 discriminate S1 from S2 patients. The most promising proteins that could differentiate S1 and S2
159 patients were also quantified at 12 months follow-up to verify their potential to be a marker of
160 cure.

161 **LC-MS/MS analysis**

162 ***Sample preparation***

163 Serum, CSF, saliva and urine samples were used in this analysis. A preliminary step of depletion
164 has been carried out to reduce the quantity of predominant proteins (α1-Acid Glycoprotein, α1-
165 Antitrypsin, alpha 2-Macroglobulin, Albumin, Apolipoprotein A-I, Apolipoprotein A-II,
166 Fibrinogen, Haptoglobin, IgA, IgG, IgM, Transferrin) in these biological fluids. The dynamic
167 range of protein concentrations in different human fluids can be large. Without this step, the high
168 abundance of these predominant proteins would overwhelm the data obtained for the analysis of
169 low abundance proteins. Samples containing 10 µg of protein, after depletion (Proteome purify
170 12 Human, R&D Systems®) and concentration (AMICON ULTRA, Merck Millipore®), were
171 adjusted to 120 µL of the reaction mixture containing 4 M urea, 1.5 M thiourea and 50 mM tris–
172 HCl pH 8.3. Proteins were reduced with 10 mM dithiothreitol for 30 min and then alkylated with
173 55 mM iodoacetamide for 20 min. Alkylated proteins were first digested with 500 ng of
174 endopeptidase lys-C (Wako®) for 3 h at room temperature (RT). Then the mixture was diluted
175 with 3 volumes of MilliQ-water and treated with 500 ng of trypsin (Sequence Grade Trypsin,
176 Promega®) for 16 h at RT. Enzymatic activity was stopped by addition of formic acid to a final
177 concentration of 3%. Samples were stored at -20 °C until use. In the case of saliva, a preliminary
178 acetone (100%) precipitation step at -20 °C was carried out. The saliva and urine samples were
179 not depleted to avoid a loss of proteins as the sample volume was low (1.5mL).

180 ***Sample analysis***

Peptide mixtures (10 corresponding to patients) were analyzed by a Q-Exactive Plus coupled to a Nano-LC Proxeon 1000 equipped with an easy spray ion source (all from Thermo Scientific). Peptides were separated by chromatography with the following parameters: Acclaim PepMap100 C18 pre-column (2 cm, 75 µm i.d., 3 µm, 100 Å), Pepmap-RSLC Proxeon C18 column (50 cm, 75 µm i.d., 2 µm, 100 Å), 300 nL/min flow rate, gradient from 95 % solvent A (water, 0.1% formic acid) to 35% solvent B (100 % acetonitrile, 0.1% formic acid) over a period of 98 minutes, followed by a column regeneration for 23 min, giving a total run time of 2 hours. Peptides were analyzed in the Orbitrap cell, in full ion scan mode, at a resolution of 70,000 (at *m/z* 200), with a mass range of *m/z* 375–1500 and an AGC target of 3 $x10^6$. Fragments were obtained by high collision-induced dissociation (HCD) activation with a collisional energy of 30%, and a quadrupole isolation window of 1.4 Da. MS/MS data were acquired in the Orbitrap cell in a Top20 mode, at a resolution of 17,500, with an AGC target of $2x10^5$, with a dynamic exclusion of 30 seconds. The most intense precursor ions were acquired first by MS/MS. Peptides with unassigned charge states or monocharged were excluded from the MS/MS acquisition. The maximum ion accumulation time was set to 50 ms for MS acquisition and 45 ms for MS/MS acquisition.

Peptide and protein identification

For the peptide and protein identification step, all MS and MS/MS data were processed with Proteome Discoverer software (Thermo Scientific, version 2.1) and with Mascot search engine (Matrix Science, version 5.1). The mass tolerance was set to 6 ppm for precursor ions and 0.02 Da for fragments. The maximum number of missed cleavages was limited to two for the trypsin protease. The SwissProt database with the *Homo sapiens* and the *T. b. gambiense* taxonomies was used for the MS/MS identification step. The following variable modifications (2 maximum per peptide) were allowed: oxidation (M) and carbamidomethylation (C). Peptide identification in all

fluids analyzed were validated using a 1% FDR (false discovery rate) threshold calculated with the Percolator algorithm [21].

Peptide and protein abundance quantification and statistical validation

Progenesis QI for Proteomics software (version 4.0, Waters) was used for the relative quantification of the protein abundances by using co-detection to eliminate missing values. No filters based on peptide by protein occurrences were applied for the protein quantification. The relative quantitation of proteins according to the three groups (Control, S1 and S2 patients) was performed using a between subject analysis and a Hi-3 method for which the three most abundant peptides were used for protein quantification. Abundance variations of proteins with an ANOVA *p*-value under 0.05 were further considered. The protein fold change makes it possible to show a difference of this protein abundance according to the groups when it is greater than 2. The potential biological role of each selected protein was verified and completed by a complete bibliographic review to confirm its interest.

Validation of protein abundance variations by ELISA

Selection of ELISA assays

Human neuroserpin ELISA, BioVendor® (LOD-Limit of Detection: 0.03 ng/mL), human NEO1 ELISA Kit, Mybiosource® (LOD: 5.00 pg/mL) and human SCG2 ELISA Kit, Elabscience Biotechnology® (LOD: 0.75 ng/mL) were selected to detect levels of neuroserpin, neogenin and secretogranin 2 in CSF, respectively. The level of neuroserpin detection on diagnosis samples allowed further detection at 12 months follow-up. Human moesin ELISA kit, Abbexa Ltd® (LOD: 0.054 ng/mL) and human ITLN2 ELISA kit, Elabscience® (LOD: 3.75 ng/mL) were used to detect levels of moesin in urine and levels of ITNL2 in saliva, respectively. For each ELISA

227 test, the protocol provided by the manufacturer was followed. Optical densities (OD) were
228 measured with a Multiskan go (Thermo Scientific®) spectrophotometer.

229 ***Statistical analysis***

230 Using the data obtained by ELISA, cut offs and receiver operating characteristic (ROC) curves
231 were made to determine the best sensitivity/specificity ratio for each marker. The significance has
232 been evaluated using Kruskal–Wallis test and Fisher's exact test for testing the null of
233 independence. The level of agreement with cut offs found in the literature was estimated using
234 Cohen's kappa coefficient and generalized linear models were used to make logistic regressions.
235 Tests were performed using R Core Team [22]. The following packages were used: ROCR for
236 drawing curves [23] and PMCMR for pairwise comparisons of Kruskal–Wallis test [24].

237 .

238

Results

Population characteristics

Demographic, clinical and biological data are summarized in Table 1. All screening cohort selected patients and controls were male. Biological, neurological and parasitological examination showed that no controls suffered from sleep or neurological disorders, no trypanosomes were found in their blood or CSF, and their average WBC count was 4.61 WBC/µL CSF. S1 patients had similar clinical characteristics to the control group but with presence of trypanosomes in blood and an average of 1.33 WBC/µL CSF. S2 patients had confirmed neurological signs and sleep disorders; their average WBC count was 195.79 WBC/µL CSF.

The protein quantification cohort included 14 controls (sex ratio: 0.56), 23 S1 patients (sex ratio: 0.92) and 43 S2 patients (sex ratio: 0.54). Biological, neurological and parasitological examination showed that no controls had trypanosomes in the blood or CSF. The CSF examination of one of these controls revealed the presence of 8 WBC/µL, but no co-infection was detected for this patient, and the neurological examination did not reveal any disorders. Among the controls only 2 patients had sleep disorders. All S1 patients had trypanosomes in the blood and a WBC count between 0 and 4 WBC/µL CSF. None of the S1 patients had trypanosomes in the CSF, and the neurological examination showed that 6 patients had neurological disorders, and 10 had sleep disorders. All S2 patients had trypanosomes in the blood, and 23 out of 43 patients had trypanosomes in the CSF. The WBC count of these patients ranged from 6 to 597 WBC/µL CSF. Neurological examinations showed that 70% (30/43) of these patients had neurological disorders, and 67% (29/43) had sleep disorders.

For the quantification of proteins in urine and saliva, samples were not available for all controls and patients. For urine samples, the analysis was done on 13 controls, 21 S1 patients and 38 S2 patients. For saliva samples, the analysis was done on 13 controls, 22 S1 patients and 38 S2 patients.

Global LC-MS/MS analysis

Searching the LC-MS/MS data for semi-tryptic peptides in the protein database allows the identification of any possible protein degradation of the samples caused by the method and duration of freezing. There were no major differences between the samples of occurrences of peptides derived from non-tryptic endogenous proteases. This analysis was carried out on the screening cohort samples and showed that the biological samples of these patients had not been degraded.

The samples were tested for the presence of peptides described in the *T. b. gambiense* protein database; however, although parasite proteins were identified in each of the 4 biological fluids tested, they were not studied further owing to a lack of validation of protein identification. However, they are present in the database published in supplementary files.

Analysis of the total number of different proteins identified in serum, CSF, saliva and urine for each group of individuals (controls, S1 patients and S2 patients) (Figure 1) showed that S2 patients had a decrease in the number of different proteins expressed in the CSF compared with control and S1 patients. For saliva, the mean total number of different proteins identified in S2 patients (472 different proteins) was less than the total number of different proteins identified in S1 patients (594 different proteins) and by the control group (627 different proteins). The total number of proteins expressed in sera was similar in the different groups. For urine, the lowest total number of proteins identified was found in the controls.

Figure 1: Distribution of the number of proteins identified in the serum, CSF, saliva and urine of the screening cohort with respect to disease stage.

Captions: The total number of different proteins identified by LC-MS/MS analysis in serum, cerebrospinal fluid (CSF), urine and saliva in boxplots ~~and a table~~. Boxplots show the maximum and minimum number of proteins identified by LC-MS/MS, the means and quartiles for each fluid analyzed. For serum, CSF and urine data there are 3 controls (C), 3 patients with stage 1 (S1) disease and 4 patients with Stage 2 (S2) disease. For saliva there are 2 controls, 3 patients with S1 disease and 4 patients with S2 disease. The mean number of different proteins identified by LC-MS/MS analysis in serum, cerebrospinal fluid (CSF), urine and saliva are available in supplementary data.

LC-MS/MS analysis and protein quantification in sera

The proteomic analysis of the 10 screening cohort serum samples showed the presence of 269 proteins. The 10 best-scored proteins are presented in table 2. These data are heavily skewed owing to the very high levels of some serum proteins, such as albumin and immunoglobulins, despite the use of depletion protocols. This is highlighted by the presence of proteins with high identification scores, which are normally depleted, in table 2. As such, any changes in protein levels specific to HAT are not statistically pertinent.

LC-MS/MS analysis and protein quantification in CSF

The proteomic analysis of the screening cohort CSF samples showed the presence of 491 proteins. The relative abundances of these proteins were estimated by label-free quantification with Progenesis QI software. Among these proteins, 159 were found differentially abundant (ANOVA *p*-value lower than 0.05 and Fold Change higher than 2) between controls, S1 or S2 patients. After analysis of these 159 proteins on the basis of their statistical data (ANOVA *p*-value and fold change), their differences attributable to disease stage, and their biological role, 37 proteins presented potential interest in HAT characterization (Table 3). Neogenin, secretogranin 2 and neuroserpin were chosen for protein quantification in CSF samples as their results showed the best combination of our defined criteria. Briefly, the ANOVA *p*-value calculated for neogenin is 3.82×10^{-5}, and the fold change is close to infinity; the average normalized abundance (ANA) calculations showed an expression of this protein only in controls and S1 patients (2.72×10^{4} and 2.17×10^{4}, respectively) (Table 3). The ANOVA *p*-value calculated for secretogranin 2 is 5.45×10^{-5} and the fold change is 11.27 (Table 3). More secretogranin 2 is found in the CSF of healthy controls (ANA: 2.49×10^{6}) and S1 patients (ANA: 2.01×10^{6}) than in S2 patients (ANA: 2.21×10^{5}).

318 The most significant ANOVA result was obtained for neuroserpin at $2.61x10^{-8}$, and the Fold
319 Change was close to infinity (Table 3). This protein is present in the CSF of healthy controls
320 (ANA: $2.5x10^{4}$) and S1 patients (ANA: $2.32x10^{4}$) but not in S2 patients.
321 ~~Protein quantification by ELISA was performed for the 3 proteins meaningful results were not~~
322 ~~obtained for neogenin and secretogranin 2. For neogenin, the amount was too low to be detected~~
323 ~~by ELISA, and for secretogranin 2, similar concentrations were found for controls, S1 and S2~~
324 ~~patients. Mean values obtained were 0.27 ng/mL, with a standard deviation of 0.003. It was thus~~
325 ~~not possible to use secretogranin 2 as a possible marker of differentiation for patients or controls.~~
326 Protein quantification by ELISA was performed for the 3 proteins, but for neogenin an
327 secretogranin 2, the amount was too low to be detected by ELISA. It was therefore not possible to
328 use them as a possible marker of differentiation for patients or controls. Only the neuroserpin
329 ELISA results showed significant differences (Figure 2) and allowed statistical tests to be
330 performed for further analysis. There was no difference in neuroserpin CSF concentration
331 between the control and patients at inclusion when analyzed by the Kruskal–Wallis test; however,
332 neuroserpin levels were significantly different ($p < 0.05$) between S1 and S2 patients. ROC curve
333 analysis was used to determine the threshold level of neuroserpin for discriminating S2 patients
334 from S1 patients (area under the curve (AUC): 0.72). It was determined that a level of CSF
335 neuroserpin below 4.99 ng/mL was indicative of a patient having S2 disease (sensitivity: 0.94 and
336 specificity: 0.58). Logistic regression analysis showed that age and sex of individuals have no
337 effect on CSF neuroserpin concentration; however, there was a correlation between neuroserpin
338 concentration and presence of sleep disorders (odds ratio: $2.1x10^{-02}$ (CI: $1.0x10^{-3}$; $1.47x10^{-1}$), $p <$
339 0.01) and neurological disorders (odds ratio: $6.3x10^{-2}$ (CI: $0.9x10^{-2}$; $2.32x10^{-1}$), $p < 0.001$).
340 Similarly, a link could be made between the presence of trypanosomes in the CSF and the
341 concentration of neuroserpin (odds ratio: $5.09x10^{-4}$ (CI: $1.93x10^{-4}$; $4.22x10^{-2}$), $p < 0.001$).

342 Figure 2: Changes in the neuroserpin concentration in CSF of controls and patients with stage 1

343 and stage 2 HAT at inclusion and after 12 months

344 Captions: Neuroserpin concentrations in the CSF for 14 healthy controls, 23 S1 patients and 43

345 S2 patients; the concentrations for 5/14 of the controls 16/23 of the S1 patients and 28/43 of the

346 S2 patients after 12 months of treatment are also shown.

347

348 Neuroserpin levels were then tested for association with sleep and neurological disorders and the
349 presence of trypanosomes in the CSF. The Kruskal–Wallis test revealed that CSF neuroserpin
350 could act as a marker for these disease-associated pathologies ($p < 0.05$). A ROC curve was used
351 to determine the threshold of 5.09 ng/mL, below which patients had sleep disorders (AUC: 0.79;
352 sensitivity: 0.72 and specificity: 0.67). The ROC curve analysis also allowed us to determine a
353 threshold of 4.97 ng/mL of CSF neuroserpin (AUC: 0.77), below which the patients had
354 neurological disorders (sensitivity: 0.9 and specificity: 0.59). The threshold of CSF neuroserpin
355 determined by ROC curve analysis for discriminating the presence of trypanosomes in the CSF
356 (AUC = 0.95) is 4.83 ng/mL, below which is indicative of CSF invasion by trypanosomes
357 (sensitivity: 0.98 and specificity: 0.91). The discriminating power of CSF neuroserpin was tested
358 against the parasitological examination of CSF (gold standard) with an agreement of 0.88
359 (Cohen's kappa coefficient), which is an excellent concordance.
360 At follow-up, at 12 months following treatment, the neuroserpin concentration was quantified in
361 the CSF in 5/14 of the controls, 16/23 of the S1 patients and 28/43 of the S2 patients. Kruskal–
362 Wallis analysis showed that there was no difference in neuroserpin levels between the 3 groups
363 after 12 months post-treatment, and the concentrations at 12 months were close to those found in
364 the control and S1 patients at inclusion. The mean neuroserpin concentration in the CSF in all the
365 groups is 5.13 ng/mL (Figure 2).
366
367 **LC-MS/MS analysis and protein quantification in urine**
368 The proteome analysis of urine samples showed the presence of 664 proteins, and 32 proteins
369 were differentially abundant (ANOVA *p*-value lower than 0.05 and Fold Change higher than 2)
370 between the control and the S1 or the S2 patients. From these 32 proteins, 8 were chosen as
371 interesting (Table 4). Moesin appeared to be the most promising protein for quantification by

372 ELISA at inclusion. The ANOVA *p*-value for moesin analysis was $9.38x10^{-3}$ and its fold change
373 was 47.11 (Table 4). Moesin is increased in S1 patients (ANA: $7.12x10^{4}$) and S2 patients (ANA:
374 $1.57x10^{4}$) compared with the controls (ANA: 1511.77). Quantification of moesin at 12 months
375 follow-up was not possible owing to the low number of urine samples available.
376 The moesin ELISA was performed on samples from 59 patients from both S1 and S2 patients and
377 13 uninfected controls. The Kruskal–Wallis test revealed a significant difference in moesin levels
378 ($p < 0.05$) between controls and patients with HAT. The threshold for discriminating controls
379 from patients was determined with ROC curve analysis (AUC = 0.70). A level of moesin above
380 0.448 ng/mL (sensitivity: 0.64 and specificity: 0.61) in urine is indicative of HAT disease.
381 Further characterization of moesin levels between S1 (n = 21) and S2 patients (n = 38) compared
382 with uninfected controls (n=13) was performed. No statistical difference in moesin abundance
383 levels was observed between uninfected controls and S1 patients and no significant difference
384 was observed between S1 and S2 patients. However, when comparing the uninfected controls and
385 S2 patients, a significant difference in moesin levels was found ($p < 0.05$). ROC curve analysis
386 (AUC: 0.72) was used to determine a urine moesin threshold of 0.60 ng/mL (sensitivity: 0.63 and
387 specificity: 0.65) above which patients had S2 HAT. Using logistic regression, it was shown that
388 the moesin concentrations were not influenced by sex, age, presence of trypanosomes in CSF, or
389 sleep and neurological disorders.
390
391 **LC-MS/MS analysis and protein quantification in saliva**
392 The screening was carried out on the screening cohort but with only 2 controls (owing to an error
393 in tube labelling). The proteome screening of saliva allows the identification of 954 proteins.
394 Among these 954 proteins, 137 were found to be differentially abundant (ANOVA *p*-value lower
395 than 0.05 and fold change higher than 2) between the control and the S1 or the S2 patients, and

24 were chosen for their particular interest (Table 5). Among them intelectin 2 (ITLN2) was chosen for quantification by ELISA. ITLN2 had an ANOVA of $1.23x10^{-4}$ and a Fold Change of 120.23 as determined by quantitative proteomics; ANA, determined by Progenesis QI software analysis, showed lower ITLN2 concentration in the saliva of patients in both stages (S1 ANA: 258.69; S2 ANA: 350.60) than controls (ANA: $3.11x10^{4}$).

Saliva ITLN2 concentrations measured by ELISA of controls (n = 13) and those of patients (n = 60) were compared with the Kruskal–Wallis test. There was no significant difference between the two populations (p = 0.40). Saliva ITLN2 concentrations were then compared between S1 patients (n = 22), the S2 patients (n = 38) and uninfected controls (n = 13) using the Kruskal–Wallis test. There was no significant difference in ITLN2 levels between controls, S1 and S2 patients (p = 0.06). Therefore, it was not possible to determine a threshold for this protein (AUC = 0.59).

Discussion

This is, to our knowledge, the first simultaneous proteomic characterization of 4 biological fluids (serum, CSF, saliva and urine) from the same HAT patients. The proteome screening analysis by LC-MS/MS has revealed 69 proteins (37 proteins in CSF, 8 proteins in saliva and 24 proteins in urine) that could be interesting for diagnosis or staging of the disease. The potential of 5 proteins (3 proteins in CSF, 1 protein in urine and 1 protein in saliva) were further verified as biomarkers in diagnosis or staging. These assays are a preliminary step in the identification of the entire proteome of HAT patients.

The semi-tryptic peptide analysis of the database showed that the method and the freezing duration of our samples did not alter the proteome quality. However, very few proteins or degraded fragments from the parasite were identified owing to poor Mascot scores. This may be due to the low quantity of parasitic proteins relative to the amount of human proteins and/or to limitations or absence of *T. b. gambiense* proteins in the existing database. This needs to be further investigated, which is why our study was focused on the human proteome and the detection of biomarkers for S2 patients and not on parasite proteins.

In sera, the label-free quantification did not identify any protein of notable interest (ANOVA: $<$ 0.05 and Fold Change: $>$ 2). The quantification by LC-MS/MS of an exhaustive set of proteins in serum is difficult; this is partly due to the complexity of the serum, which contains a large number of different proteins with an extremely large range of expression [25], [26]. In this case, despite the depletion step carried out, the presence of a large quantity of proteins (mainly albumins and globulins) impedes quantification of other proteins as shown in other studies [27].

In the CSF, the protein composition showed a clear decrease in the diversity and number of identified proteins in patients with S2 disease. This result is surprising but can be explained by

two physiopathological mechanisms that may act together. The first is trypanosome-induced immunosuppression [28]–[30], which is a marked weakening of the humoral response owing to massive B cell death [31], [32]. Furthermore, it has been described that the release of trypanosome suppression immunomodulating factor by the parasite triggers the development of suppressive M1 macrophages [33] and blocks T cell proliferation [34]. This immunosuppression could explain the decrease in the number of proteins found in the CSF of the patients with S2 disease because the disappearance of the immune cells implies a decrease of the proteins related to them. The second explanation is a leakage or resorption of nervous system proteins owing to the permeabilization of the blood–brain barrier [35], [36]. The loss of nervous system proteins and increase in plasma-associated protein, such as complement system proteins, albumin, transthyretin and apolipoproteins has been described by others in CNS infections altering the blood–brain barrier [37]–[39] and was corroborated in the CSF analysis of our study.

In CSF, the analysis allowed the identification of 491 proteins, of which many had been previously associated with HAT, such as vascular cell adhesion protein 1 (VCAM1) [40], [41] and osteopontin (SPP1) [42]. The study focused on 37 proteins that were differentially expressed between the uninfected controls, S1 and S2 patients, and 3 proteins — namely neogenin, secretogranin 2 and neuroserpin — were chosen for further analysis by ELISA [43]–[50]. Neogenin is a protein associated with the nervous system, and it has already been proposed as a biomarker in other pathologies [45], [50]. In the brain, neogenin is a receptor for many proteins, including repulsive guidance molecule (RGM) family members, which is involved in axon guidance, apoptosis and neuronal differentiation [46], [50]. The use of the RGMa–neogenin signaling pathway as a therapeutic target to overcome inflammatory and neurodegenerative diseases has already been proposed [50]. Secretogranin 2 is a soluble member of the granin

protein family, which are widely distributed in neuroendocrine and nervous system tissues. ~~Granins are stored in large dense-core vesicles within neurons; secretogranin 2 can also be detected in glial cells [43]. Secretogranin 2 has numerous functions, including neurite outgrowth and chemoattractive effects on monocytes, eosinophils and endothelial cells [43]. In 2013, Jakobsson *et al* proposed secretogranin 2 as a CSF marker for severe forms of bipolar disorder. Patients with bipolar disorder have defects in the regulatory secretory pathway; one of the altered proteins is secretogranin 2 [48], and reduction is linked to severe forms. Reduced levels of secretogranin 2 and secretogranin 3 were observed in serum and CSF samples of patients with multiple sclerosis [45], [47], [49]. In our HAT sample collection, neogenin and secretogranin 2 were highlighted by LC-MS/MS analysis; however, the ELISA assay used seemed unable to detect these proteins, which may be due to the detection limit of the commercial ELISA test being too high for the samples in this study.~~ In our HAT sample collection, neogenin and secretogranin 2 were highlighted by LC-MS/MS analysis; however, our patients had neogenin and secretogranin 2 concentrations under the ELISA assay limit of detection.

Finally, among the tested proteins, only neuroserpin allowed the discrimination of disease stage with a decrease in neuroserpin concentrations with disease progression. This is the first study showing a link between neuroserpin and HAT. Neuroserpin is a protein belonging to the serine protease inhibitor superfamily, which is expressed throughout the nervous system but more particularly at the latter stages of the development of neuronal cells [44], [51], [52]. Neuroserpin inhibits the activation of tissue plasminogen and has an essential role in axogenesis, synaptic plasticity [53], memory and brain development [54], [55]. Neuroserpin is linked to familial encephalopathy with neuroserpin inclusion bodies (FENIB) [56]. This dementia is due to point mutations favoring the polymerization of neuroserpin and its retention in endoplasmic reticulum of neurons [56]–[58]. This protein is also associated with Alzheimer disease, but its role is not yet

clearly identified [59]–[61]. To date, we have no knowledge of work studying the relationship between neuroserpin and HAT. The decrease in late stage disease could be due to the fact that neuroserpin is predominantly expressed in the neurons of the hippocampus, caudate and cerebral cortex [62], [63]. The hippocampus and the caudate are close to the choroid plexus, which is described in the literature as the first route of entry of the parasite into the brain owing to the absence of the blood–brain barrier at this location [64]–[66]. It is probable that these regions are the first to be affected by the presence of the trypanosome. ELISA quantification allowed us to determine a cut-off point for neuroserpin, below which HAT patients may be considered to be in S2 (4.99 ng/mL; sensitivity: 0.94 and specificity: 0.58) at diagnosis. Patients with a neuroserpin concentration greater than 4.99 ng/mL are considered in S1 of the disease. Levels had returned to normal at 12 months follow up, suggesting that the decrease is directly linked to the presence of the parasite or the elicited immune reaction. It was also possible to determine that the onset of sleep disorders was associated with a CSF neuroserpin concentration of less than 5.09 ng/mL. This last cut-off could explain how some patients with S1 disease present symptoms of sleep disorders [67], [68]. Similarly, the presence of trypanosome in the CSF was correlated with a CSF neuroserpin concentration of less than 4.84 ng/mL. Each of these cut-offs correlate with classical stage markers, indicating that neuroserpin may be considered as a good additional or unique marker if its concentration is easy to quantify.

The identification of biomarkers in alternative fluids such as urine and saliva is extremely interesting for field screenings or use in resource-limited countries. Access to these fluids is noninvasive, and collection can be done by non-qualified people, or they can even can be used for self-diagnosis [69], [70]. Currently, saliva is a source of biomarkers for neurological diseases [71], HIV [72], diabetes [73]–[75] and cancer [76], [77]; urine is used for the diagnosis of

503 osteoclastogenesis [78], urological cancer [79], and proteins in the urine have been identified as
504 potential biomarkers for breast cancer [80], Alzheimer disease [81] and Parkinson disease [82].
505 In urine, proteins linked to infectious processes have been highlighted. Moesin was the most
506 interesting according to our identification criteria. This protein belongs to the family of ezrin–
507 radixin–moesin (ERM) proteins, and it is involved in angiogenesis. In fact, the phosphorylation
508 of moesin allows the mediation of endothelial angiogenesis [83]. This protein also participates in
509 the connection between the cytoskeleton and the plasma membrane of human cells. This property
510 allows this protein to limit infection with herpes simplex virus 1 [84]. It has been shown to have a
511 role in invasion of *Trypanosoma cruzi* amastigotes into human cells [85]. Moesin is also involved
512 in the regulation, proliferation and adhesion of human lymphoid cells [86]. A mutation of this
513 protein leads to primary immunodeficiency [87]. The involvement of moesin in kidney lesions
514 was established by Chen *et al* [88]; however, its role is not clear. The ERM complex intervenes in
515 the cellular dynamics by taking part in the mobility of cancer cells described in oral cancers and
516 carcinomas [89], [90]. In our study, moesin levels were elevated in urine samples from S1 and S2
517 patients compared with uninfected controls. This result was corroborated by the ELISA
518 quantification. ELISA quantification shows that there is a significant difference between moesin
519 concentrations in uninfected controls compared with patients. Furthermore, a difference in
520 moesin concentration was observed between the uninfected controls and S2 patients, which could
521 make it possible to discriminate stage when the presence of the parasite is established in the
522 blood. It was subsequently impossible to carry out the analysis at 12-months follow-up as not
523 enough samples were available.
524
525 In saliva, 24 potential interesting proteins were identified that are differentially expressed, and
526 ITLN2 was chosen as a potential candidate biomarker owing to its physiopathological

527 background. However, ITLN2 levels detected by ELISA could not discriminate controls from
528 patients, nor S1 from S2 patients (p = 0.06). It does not appear aberrant to find this protein in an
529 inflammatory pathology such as HAT [91], [92]. The intelectin families, and particularly ITLN2,
530 are proteins expressed principally in the small intestine. ITLN2 is involved in host–pathogen
531 interactions [93] and iron metabolism [94] and allergic inflammation [91]. Studies are currently
532 determining any potential antimicrobial and anti-parasitic activity of ITLN2 [91], [92], [95].
533 However, the presence of ITLN2 and its role in saliva remains unexplained. The quantification of
534 this protein on a larger sample could make it possible to identify the pathophysiological role of
535 this protein in HAT.
536
537 There can be some bias in saliva and urine analysis. In case of saliva, intra- and inter-individual
538 composition variations can be influenced by many parameters, such as salivary flow, sex, age, the
539 subject's emotional state, season, nycthemeral rhythm and medication [96]. In the same way,
540 external elements to the salivary system, such as nasal and bronchial secretions, the gingival
541 fluid, epithelial cells, microbial flora and food debris, can modify its composition [97], [98]. It is
542 recommended to perform the saliva collection before brushing the teeth and at least 30 minutes
543 after any ingestion of food [99]. For urine, the time of sample collection was not calibrated
544 (between 7 am and 1 pm) and thus may lead to variations in urine composition and concentration.
545 In this study, these variations could not all be controlled and may constitute a bias.
546 One weakness of this study is the number of patients that were selected for the screening by LC-
547 MS/MS analysis. Indeed, for practical reasons 10 patients were selected among the 247 included
548 in the cohort for the analysis of screening, for which there is homogeneous data (age, sex and no
549 co-infection detected). Despite the low number of patient samples analyzed by LC-MS/MS, the
550 patient sampling in each group (controls, S1 and S2) is statistically homogenous. Another

possible criticism is the lack of uniformity in the sampling times, which is especially important in the case of urine and saliva.

Conclusion

In conclusion, our work allowed the characterization of a large number of proteins presenting an interest in the understanding of HAT pathophysiology and diagnosis. The high sensitivity of new technologies such as LC-MS/MS allow the discovery of new candidate disease marker and open the path to other more accessible biological fluids like urine or saliva. Our work identified two potential new markers, neuroserpin and moesin, the latter of which is found in urine, which could simplify diagnostic strategies in the field.

Graphical abstract

~~Human samples of sera, CSF, saliva and urine from screening cohort were prepared for LC-MS/MS analysis.~~ Human African trypanosomiasis samples (sera, CSF, saliva and urine) from a screening cohort were prepared for characterization of disease biomarkers by LC-MS/MS analysis. Bottom-up identification of proteins allows to categorize 269 proteins in the serum, 491 proteins in the CSF, 954 proteins in the saliva and 664 proteins in the urine. Among these proteins identified, 37 proteins in the CSF, 24 proteins in the saliva and 8 proteins in the urine were differentially abundant between the control, the S1 or the S2 patients and were of interest for HAT diagnosis. Among these proteins, 5 were assayed by ELISA in the protein quantification cohort: neogenin, secretogranin 2 and neuroserpin in the CSF; intelectin 2 in the saliva and moesin in the urine.

*For saliva screening cohort: controls (n = 2), S1 patients (n = 3) and S2 patients (n = 4).

Supplementary data

Table represents the average number of different proteins identified by LC-MS/MS analysis in serum, cerebrospinal fluid (CSF), urine and saliva. For serum, CSF and urine data there are 3 controls (C), 3 patients with stage 1 (S1) disease and 4 patients with Stage 2 (S2) disease. For saliva there are 2 controls, 3 patients with S1 disease and 4 patients with S2 disease.

Highlights

LC-MS/MS analysis allow the discovery of new candidate disease markers for HAT.
Neuroserpin and moesin are two potential new markers that could simplify diagnostics.
CSF protein composition showed a ~~clear~~ decrease of identified proteins in S2 patients.
Easily accessible biological fluids, like urine, could be used for HAT diagnosis.

Ethics approval and consent to participate

Ethical clearance was obtained from the Direccao National de Saude Publica, Ministerio da Saude, Angola. The authorization for automated processing of personal data relating to the study was obtained from the Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé (CCTIRS) (N° 08.228bis)

Consent for publication

Written informed consent was obtained from all participants and, for participants below 18 years of age, from their parents or guardians, prior to enrolment. Any individual who declined to participate was managed according to the standard procedures of the national HAT control program.

Availability of data and material

The complete data sets are available in the PRIDE partner repository [100] under the identification number : PXD007842 as .raw files, Proteome Discoverer 2.1 .pdResult file, associated pep.xml and xlsx files, and label-free report generated by Progenesis QI.

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Supporting Information Legends: Identification and relative quantification of all peptides/proteins from the proteomics experiments and according to the different experimental conditions are publicly available via ProteomeXchange and the PRIDE partner repository under the identification number: PXD007842 as.raw files, Proteome Discoverer 2.1. pdResult file, associated pep.xml and xlsx files, and label-free report generated by Progenesis QI.

Competing interests

The authors declare that they have no competing interests

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Table 1: Characteristics of the screening and protein quantification cohort

Screening cohort		Control	S1	S2
n		3	3	4
Sex-ratio (M/F)		3/0	3/0	4/0
Age (SD)		36.67 (27.15)	43.33 (21.13)	31.25 (18.14)
Neurological disorders		0	0	4
Sleep disorders		0	0	4
CSF WBC/µL (SD)		4.61 (3.05)	1.33 (0.58)	195.79 (147.44)
Trypanosome per µL blood		0	3	4
Trypanosome per µL CSF		0	0	4
Protein quantification cohort		**Control**	**S1**	**S2**
n *		14	23	43
Sex ratio (M/F)		5/9	11/12	15/28
Age	Mean (SD)	38 (20.14)	39.09 (14.17)	34.55 (12.93)
	Min	12	15	14
	Max	70	58	58
Neurological disorders		0	6	30
Sleep disorders		2	10	29
CSF WBC/µL	CSF WBC/µL (SD)	2.64 (1.90)	1.34 (0.93)	137.82 (165.73)
	Min	0	0	6
	Max	8	4	597
Trypanosome per µL blood		0	23	43
Trypanosome per µL CSF		0	0	23

This table represents the epidemiological and biological data of 3 controls, 3 patients with stage 1 (S1) disease and 4 patients with stage 2 (S2) disease who constituted the screening cohort, as well as the 14 controls, the 23 patients with S1 disease and the 43 patients with S2 disease enrolled in protein quantification cohort. For urine and saliva analysis, some samples were not present in the protein quantification cohort. *For urine (n = 72): 13 Controls, 21 S1 and 38 S2. For saliva (n = 73): 13 controls, 22 S1 and 38 S2.

915 Table 2: Top-10 best scored proteins identified in serum

Accession	Description	Score	ANOVA	Max Fold Change	Average normalized abundances		
					Control	S1	S2
CO3_HUMAN	Complement C3	8016.88	0.82	1.31	1.91e+007	1.74e+007	1.46e+007
A2MG_HUMAN	Alpha-2-macroglobulin	5874.13	0.94	1.37	1.52e+007	1.67e+007	1.22e+007
VTDB_HUMAN	Vitamin D-binding protein	5127.27	0.87	1.11	9.21e+006	8.29e+006	8.67e+006
APOB_HUMAN	Apolipoprotein B-100	4819.10	0.35	2.68	2.89e+006	1.08e+006	1.08e+006
TRFE_HUMAN	Serotransferrin	4375.11	0.67	1.54	8.19e+006	1.26e+007	1.18e+007
CERU_HUMAN	Ceruloplasmin	4249.73	0.18	1.47	3.09e+007	2.30e+007	2.10e+007
HPT_HUMAN	Haptoglobin	3150.25	0.36	4.37	9.20e+006	1.34e+007	4.02e+007
A1AT_HUMAN	Alpha-1-antitrypsin	3085.22	0.63	1.22	3.80e+007	4.45e+007	3.64e+007
IGHM_HUMAN	Ig mu chain C region	2899.80	0.89	2.05	4.60e+007	3.58e+007	2.25e+007
IGKC_HUMAN	Ig kappa chain C region	2880.99	0.63	2.20	8.53e+007	7.25e+007	3.88e+007
IGHG1_HUMAN	Ig gamma-1 chain C region	2813.11	0.33	3.20	1.29e+007	4.14e+007	1.72e+007

916

917 S1: stage 1 disease; S2: stage 2 disease. Score: mascot protein score

918

Table 3: Most notable proteins in the CSF with altered abundance levels between control, S1 and S2 patients (classified by the increase in ANOVA *p*-value)

Accession	Description	Score	ANOVA	Max Fold Change	Average normalized abundances		
					Control	S1	S2
NEUS_HUMAN	Neuroserpin *	33.73	2.61e-008	Infinity	2.50e+004	2.32e+004	0.00
MCH_HUMAN	Pro MCH	18.23	2.53e-006	51.23	2.67e+004	2.31e+004	520.50
VGF_HUMAN	Neurosecretory protein VGF	768.70	6.20e-006	17.49	1.46e+006	1.59e+006	9.10e+004
NDRG2_HUMAN	Protein NDRG2	26.38	9.85e-006	Infinity	3.65e+004	2.53e+004	0.00
NEO1_HUMAN	Neogenin *	27.68	3.82e-005	Infinity	2.72e+004	2.17e+004	0.00
PTPRS_HUMAN	Receptor type tyrosine protein phosphatase S	28.48	4.39e-005	Infinity	2.31e+004	1.55e+004	0.00
SCG2_HUMAN	Secretogranin 2 *	732.53	5.45e-005	11.27	2.49e+006	2.01e+006	2.21e+005
NCAM2_HUMAN	Neural cell adhesion molecule 2	123.80	2.49e-004	12.57	1.50e+005	1.43e+005	1.19e+004
AT1B1_HUMAN	Sodium/potassium transporting ATPase subunit beta 1	37.94	2.62e-004	6864.45	1361.14	2311.93	0.34
DHPR_HUMAN	Dihydropteridine reductase	112.47	3.39e-004	212.16	2.41e+004	2.52e+004	118.73
NFASC_HUMAN	Neurofascin	106.58	3.98e-004	19.75	4.44e+004	4.74e+004	2400.82
SCG3_HUMAN	Secretogranin 3	1068.39	4.07e-004	9.94	1.20e+006	1.46e+006	1.47e+005
SCG1_HUMAN	Secretogranin 1	4373.28	4.41e-004	6.14	1.59e+007	1.30e+007	2.58e+006
PEBP4_HUMAN	Phosphatidylethanolamine binding protein 4	23.40	4.51e-004	151.57	3.44e+004	1.89e+004	227.04
PCDH9_HUMAN	Protocadherin 9	42.21	4.54e-004	473.46	6.07e+004	6.17e+004	130.22
TICN3_HUMAN	Testican 3	111.74	1.24e-003	5.13	3.32e+004	2.87e+004	6478.72
NRX3A_HUMAN	Neurexin 3	37.45	1.33e-003	745.63	4182.12	9806.72	13.15
PTMA_HUMAN	Prothymosin alpha	78.55	1.40e-003	38.11	1.07e+005	6.51e+004	2.48e+006
PENK_HUMAN	Proenkephalin A	268.45	2.12e-003	4.34	4.07e+006	3.54e+006	9.39e+005
COL12_HUMAN	Collectin 12	134.26	3.30e-003	7.21	1.18e+005	7.12e+004	1.64e+004
SPON1_HUMAN	Spondin 1	77.80	3.35e-003	11.26	8.09e+004	4.53e+004	7181.93
CNTN2_HUMAN	Contactin 2	347.42	3.99e-003	78.73	6.75e+004	6.28e+004	857.46
TICN2_HUMAN	Testican 2	119.88	6.92e-003	9.75	5.79e+004	3.79e+004	5938.73
NCAM1_HUMAN	Neural cell adhesion molecule 1	709.91	8.60e-003	4.76	4.33e+005	3.25e+005	9.09e+004
CSTN1_HUMAN	Calsyntenin 1	293.93	9.35e-003	28.52	1.17e+005	1.24e+005	4346.68
CNTN1_HUMAN	Contactin 1	469.45	0.01	5.46	2.21e+005	1.60e+005	4.04e+004
CAD13_HUMAN	Cadherin 13	144.25	0.01	386.59	3.18e+004	4.65e+004	120.35
TIMP2_HUMAN	Metalloproteinase inhibitor 2	135.16	0.01	7.69	2.31e+005	1.47e+005	3.01e+004
L1CAM_HUMAN	Neural cell adhesion molecule L1	67.55	0.01	8.76	1.97e+004	1.95e+004	2254.34
TETN_HUMAN	Tetranectin	473.62	0.02	6.41	2.20e+005	2.53e+005	3.95e+004
OMGP_HUMAN	Oligodendrocyte myelin glycoprotein	296.85	0.02	3.60	3.26e+005	2.25e+005	9.03e+004
NEUM_HUMAN	Neuromodulin	129.92	0.02	2.67	1.58e+005	2.67e+005	9.99e+004
CBLN4_HUMAN	Cerebellin 4	24.53	0.02	2214.50	5630.78	4124.35	2.54
ECM1_HUMAN	Extracellular matrix protein 1	136.66	0.03	2.25	1.08e+006	1.16e+006	5.15e+005
PEBP1_HUMAN	Phosphatidylethanolamine binding protein 1	268.07	0.03	12.41	1.54e+005	1.94e+005	1.56e+004
TICN1_HUMAN	Testican 1	124.56	0.05	11.39	5.19e+004	5.17e+004	4556.89
MIF_HUMAN	Macrophage migration inhibitory factor	56.43	0.05	13.45	1.82e+004	2.73e+004	2031.17

All shown proteins passed thresholds of a fold change ≥ 2 and a *p*-value ≤ 0.05. Score: mascot protein score. * Protein quantified by ELISA. S1: stage 1 disease; S2: stage 2 disease.

Table 4: Most notable proteins in the urine with altered levels between control, S1 and S2 patients (classified by increase in ANOVA *p*-value)

	Description	Score	ANOVA	Fold Change	Average normalized abundances		
					Control	S1	S2
P51688	N-sulphoglucosamine sulphohydrolase	89.58	1.51e-004	Infinity	0.00	5.23e+004	1856.39
P16870	Carboxypeptidase E	23.90	1.61e-004	Infinity	0.00	2.06e+004	8488.37
P25774	Cathepsin S	151.85	5.80e-003	371.97	37.12	1.38e+004	621.63
P26038	Moesin *	319.43	9.38e-003	47.11	1511.77	7.12e+004	1.57e+004
P59190	Ras-related protein Rab-15	47.97	0.02	Infinity	0.00	1.74e+005	2088.10
P29622	Kallistatin	120.72	0.02	170.86	5936.72	4.28e+004	250.56
Q9H8L6	Multimerin 2	52.32	0.05	Infinity	0.00	8618.93	4.37e+004
P02766	Transthyretin	78.07	0.05	17.94	1.36e+004	1.42e+005	2.44e+005

All shown proteins passed thresholds of a fold change ≥ 2 and a *p*-value ≤ 0.05. Score: mascot protein score. * Protein quantified by ELISA. S1: stage 1 disease; S2: stage 2 disease.

933 Table 5: Most notable proteins in the saliva with altered levels between control, S1 and S2

934 patients (classified by increase in ANOVA *p*-value)

935

Accession	Description	Score	ANOVA	Fold Change	Average normalized abundances		
					Control	S1	S2
P19878	Neutrophil cytosol factor 2	26.41	1.21e-005	Infinity	0.00	1207.94	8375.66
Q8WWU7	Intelectin 2 *	44.75	1.23e-004	120.23	3.11e+004	258.69	350.60
Q15643	Thyroid receptor-interacting protein 11	25.42	3.24e-004	Infinity	0.00	0.00	1.06e+005
P22392	Nucleoside diphosphate kinase B	36.48	1.87e-003	7.52	2.34e+004	4.61e+004	1.76e+005
Q04446	1,4-alpha-glucan-branching enzyme	40.31	0.01	28.76	515.25	7843.70	1.48e+004
P35241	Radixin	357.98	0.02	2.48	3.30e+005	3.79e+005	8.19e+005
P27348	14-3-3 protein theta	106.60	0.02	11.21	2444.76	1.19e+004	2.74e+004
Q04323	UBX domain-containing protein 1	101.23	0.02	408.77	27.88	5286.01	1.14e+004
P49913	Cathelicidin antimicrobial peptide	93.06	0.02	13.27	1.99e+004	3.28e+004	2.65e+005
O00602	Ficolin-1	84.63	0.02	43.65	620.60	1512.69	2.71e+004
Q9BQI0	Allograft inflammatory factor 1-like	41.75	0.02	Infinity	0.00	914.27	7081.79
P02753	Retinol-binding protein 4	24.27	0.02	3.59	2.92e+004	3.97e+004	1.05e+005
O60437	Periplakin	353.95	0.02	16.22	6164.29	1.30e+004	1.00e+005
Q9HC84	Mucin-5B	2457.44	0.03	11.30	2.98e+007	2.64e+006	5.98e+006
P05120	Plasminogen activator inhibitor 2	255.32	0.03	25.13	2266.87	3.00e+004	5.70e+004
P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	130.27	0.03	23.02	1150.30	1.80e+004	2.65e+004
Q15631	Translin	49.17	0.03	20.39	722.18	2713.99	1.47e+004
P51888	Prolargin	22.23	0.03	17.73	3887.41	2.59e+004	6.89e+004
Q13813	Spectrin alpha chain, non-erythrocytic 1	257.23	0.04	11.90	2035.25	4051.54	2.42e+004
P99999	Cytochrome c	231.53	0.04	4.55	2.80e+004	4.21e+004	1.27e+005
P04632	Calpain small subunit 1	220.74	0.04	5.69	9299.74	2.29e+004	5.29e+004
P31997	Carcinoembryonic antigen-related cell adhesion molecule 8	60.26	0.04	26.63	3450.76	1.15e+004	9.19e+004
P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	52.04	0.04	6.29	1660.25	6513.70	1.04e+004
Q15907	Ras-related protein Rab-11B	43.85	0.04	6.84	4755.71	2.47e+004	3.25e+004

936

937 All shown proteins passed thresholds of a fold change ≥ 2 and a *p*-value ≤ 0.05. Score: mascot

938 protein score. * Protein quantified by ELISA, S1: stage 1 disease; S2: stage 2 disease.

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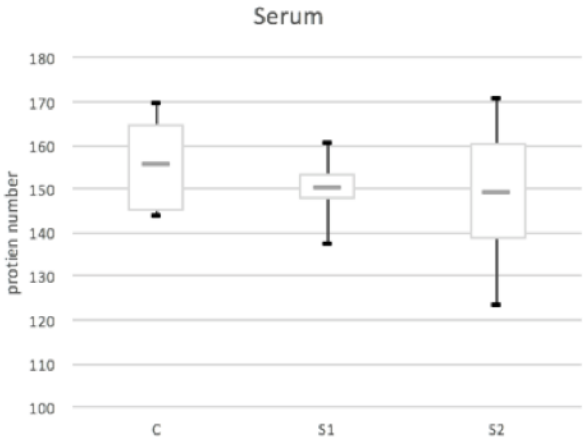
Serum
protien number
180
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100
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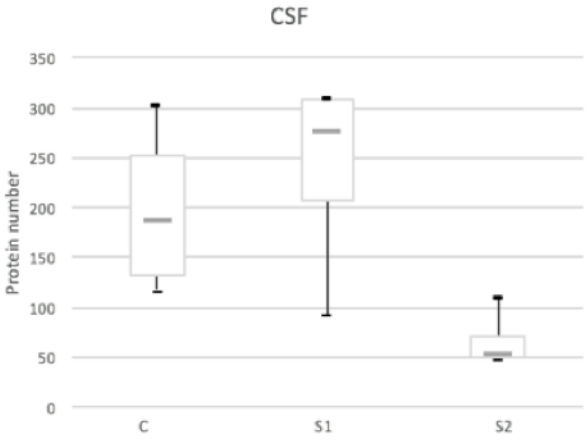
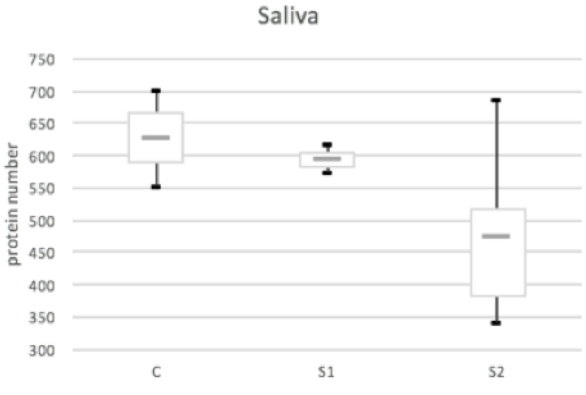
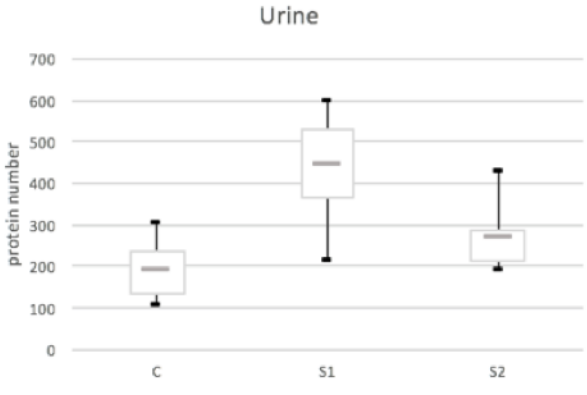
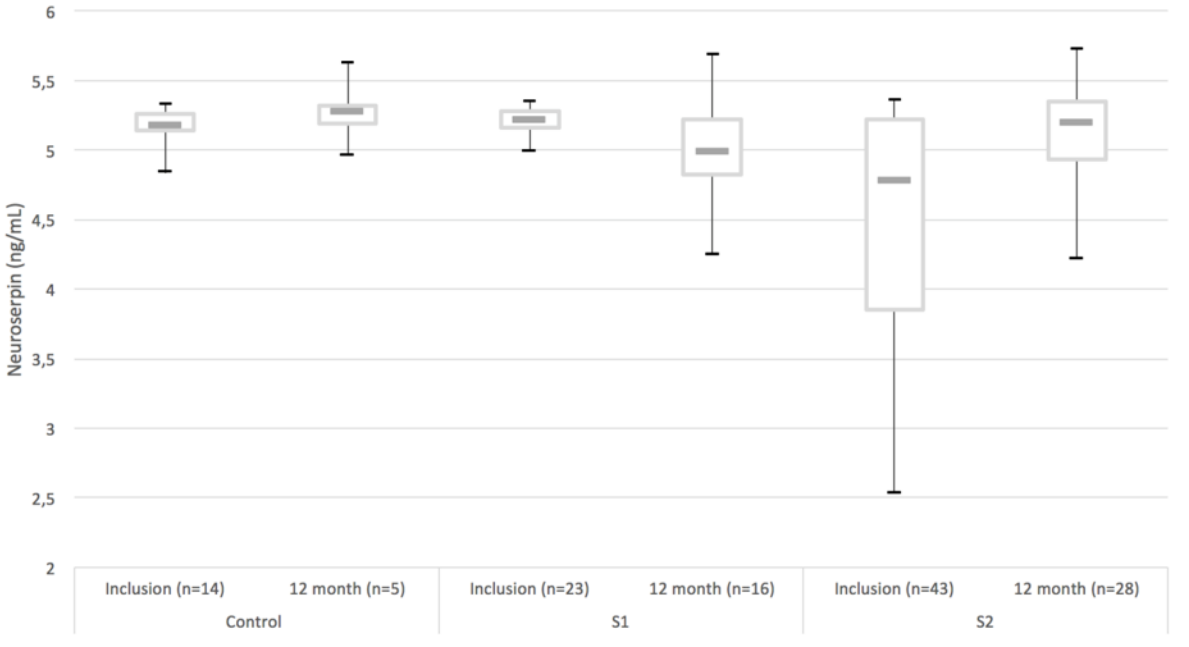
S1
S2


CSF
Protein number
350
300
250
200
150
100
50
0
C
S1
S2


Saliva
protein number
750
700
650
600
550
500
450
400
350
300
C
S1
S2


Urine
protein number
700
600
500
400
300
200
100
0
C
S1
S2


Neuroserpin (ng/mL)
6
5,5
5
4,5
4
3,5
3
2,5
2
Inclusion (n=14)
12 month (n=5)
Control
Inclusion (n=23)
12 month (n=16)
S1
Inclusion (n=43)
12 month (n=28)
S2

Screening cohort

Controls (n = 3), S1 patients (n= 3) and S2 patients (n = 4)

Serum

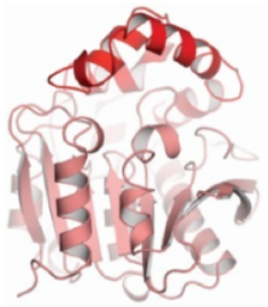
CSF

Saliva*

Urine

Bottom-up protein identification by MS/MS

Proteome



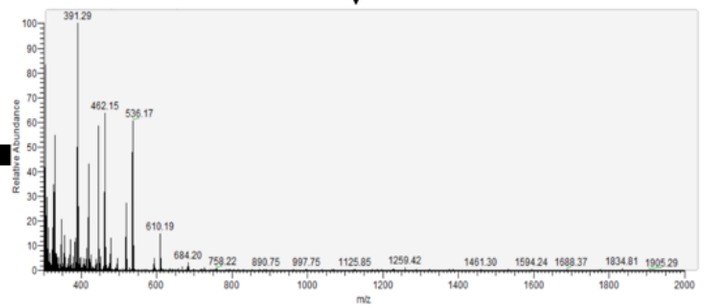
Sample preparation & Enzymatic digestion

Peptides

Liquid Chromatography

MS/MS

Q Exative plus® mass spectrometer



Proteins identification

Identified protein

Serum	CSF	Saliva	Urine
269 proteins	491 proteins	954proteins	664 proteins

Protein quantification cohort

Controls (n = 14), S1 patients (n= 23) and S2 patients (n = 43)

	CSF	Saliva	Urine
proteins differentially abundant between groups	159 proteins	137 proteins	32 proteins
Proteins of interest	37 proteins	24 proteins	8 proteins
Protein dosed by ELISA	Neogenin Secretogranin 2 Neuroserpin	Intelectin 2	Moesin