Master’s in Proteomics and Bioinformatics

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Potential biomarker expression and variation during the course of infection in experimentally infected Vervet Monkeys by *T. b. rhodesiense*

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Abstract

African trypanosomiasis is a parasitic disease of human (Human African trypanosomiasis) and livestock (Animal African Trypanosomiasis). HAT is caused by two subspecies of *Trypanosoma brucei*, *T. b. gambiense* & *T. b. rhodesiense* and transmitted by tsetse fly. There are two distinct stages during the disease progression: the haemolymphatic phase (restriction of the trypanosomes to the blood and lymph system) and the neurological phase (presence of the parasites in the cerebrospinal fluid-CSF). As a fact, clinical signs of HAT are nonspecific; therefore, there is an urgent need for a reproducible biomarker for the disease indication and the stage determination. In addition, it is more interesting to investigate a potential biomarker expression during the disease progression and follow its level in the infection course and some treatments. Vervet Monkeys are appropriate models for protozoan parasitic diseases. When they are infected by *T. b. rhodesiense*, they show clinical, pathological and immunological changes similar to those occurring in humans. In this study, by using different methods of immunoassay, some potential biomarkers such as GSTP1, H-FABP, CXCL13, Neopterin, B2MG, CXCL10, IL10, CXCL8, MMP-9, VCAM1 and ICAM1 in serum and CSF samples of experimentally infected Vervet Monkeys were examined to know whether there are any cross-reaction between antibodies against human antigens and monkeys’ antigens. In addition, the variations of GSTP1 concentration was investigated during the disease progression in experimentally infected Vervet Monkeys by *T. b. rhodesiense*. 
I. Introduction

1.1 African Trypanosomiasis

Throughout history, African trypanosomiasis which is a parasitic disease of human and livestock has seriously suppressed the economic and civilized organisation of Africa especially in the central parts. It is caused by trypanosomes which are single-celled protozoan haemoflagellate parasites of the order Trypanosomatida and the genus Trypanosoma. They live and multiply extra-cellularly in the mammalian host bloodstream and tissue fluids. They have a special mitochondrion which is called kinetoplast. It contains up to 25% of the total cell DNA and links to the basal body of the flagellum. The trypanosome thoroughly needs glucose in order to obtain energy (ATP). In the vertebrate host, it only uses inefficient glycolysis due to the abundance of the nutrients. In the insect vector, the parasite processes a cytochrome chain and tricarboxylic acid (TCA) cycle. There are a large number of membrane-associated transport proteins in the surface of the trypanosome in order to get glucose and other small molecular weight nutrients. These proteins never react with antibodies because they are covered by variant surface glycoprotein (VSG) coat (1). In the T. brucie genome, about 2,000 different genes encode variant surface glycoproteins (2, 3). Due to this fact, VSGs can hinder the mammalian host immune system from accessing the plasma membrane of the parasite by switching during the infection course; therefore, the number of trypanosomes irregularly varies in the patient’s blood (4). In addition, the parasites secret the expressed/secreted proteins (ESPs), which cause different aspects of pathogenesis.

1.1.1 Tsetse fly

Tsetse flies are the vectors of the trypanosomes. In the feeding procedure, tsetse fly obtains the parasites and then transmits them from infected vertebrate hosts to uninfected animals. 31 species and subspecies of tsetse flies exist under the order Diptera, the family Glossinidae and the genus Glossina (Fig. 1). Only eleven of them are important for the disease transmission to humans. They are abundantly classified into three species based on the combination of distributional, behavioral, molecular and morphological characteristics: The savannah flies (Morsitans, occasionally named Glossina), the riverine flies (Palpalis previously named Nemorhina) and the forest flies (Fusca previously named Austenina). In their life cycle, there are no seasonal intermissions. However, during the dry seasons especially in savannah, tsetse populations diminish (1).

Figure 1: Tsetse fly (genus Glossina).
1.1.2 Trypanosoma

There are many species of *Trypanosoma*. Three species, *T. congolense*, *T. vivax* and *T. brucei* cause animal African trypanosomiasis or nagana disease. Only two subspecies of *Trypanosoma brucei* (Fig.2), *T. brucei gambiense* and *T. brucei rhodesiense*, which are morphologically indistinguishable, but have different epidemiological features and measuring 20-30 $\mu$m by 1.5-3.5 $\mu$m are infectious to humans. They can be differentiated by molecular methods not parasitological ones. The third subspecies, *T. brucei brucei* is not infectious to humans or a subset of catarrhine primates. Because of their innate protection, which is provided by trypanosome lytic factors (TLFs) in serum (6–7), consequently, the parasite is lysed by human apolipoprotein L1 (apoL1). In tsetse flies, *Trypanosoma* infection rates are various. *T. vivax* species has the highest and *T. brucei* species has the lowest infection rates (1).

![Figure 2: Trypanosoma brucei in a blood smear.](image)

1.1.3 Human African Trypanosomiasis (HAT)

Human African Trypanosomiasis (HAT), commonly known as sleeping sickness, is a so-called neglected tropical parasitic disease. It had been omitted from Africa during the 1960s, but it has returned as a disease, which is a major threat to human health and it is invariably fatal if untreated (Kennedy et al. 2002). Nowadays, it is endemic in some parts of Sub-Saharan Africa, covering about 36 countries and 60 million people.

1.1.4 Animal African Trypanosomiasis (AAT)

Animal African Trypanosomiasis, known as nagana pest or nagana, is a disease of vertebrate animals. It is caused by a larger number of *Trypanosoma* species, transmitted by a greater number of *Glossina* species and has the greater epidemic through the African continent. Generally, trypanosome infections in livestock are up to 100-150 folds higher than in human (Jordan 1976).

Trypanosomes cause relatively moderate infections in wild animals. However, they cause a severe and mostly fatal disease in domestic animals. The signs of nagana, which causes the disease in all domestic animals, are weight loss, weakness (64), enlarged lymph nodes (63),
fever, languid (inactive), eyes discharge, oedema, anemia and paralysis (65). The animals become weak and unsuitable for work as the disease progresses, due to this fact, they called the animal disease "N'gana" which is a Zulu word and it means "powerless/useless" (8). Because of the animal disease, agriculture and livestock industry are very difficult inside of the tsetse belt. Wild and domestic animals may be the main reservoirs for human trypanosomes infections (9-10).

1.1.5 HAT transmission elements

The three absolutely necessary elements for African trypanosomiasis transmission are the tsetse flies (several species of *Glossina*), the trypanosomes and the mammalian hosts. These three elements, all together at the same place and time, are not sufficient. Exposure of human to tsetse fly must be regular and frequent. This exposure depends on human behaviour and a variety of hazardous circumstances: the "chance occurrence"(11).

The ability of tsetse fly species to develop infections is different, for example, female tsetse flies usually have higher infection rates than males, it could be the fact that females live longer than males and therefore have a higher infection probability. The sensibility of tsetse flies to *T. brucei* infection is related to intracellular rickettsia-like organisms (RLOs) (1). Tsetse flies with RLOs in their midgut can be six times more infected with trypanosomes than others without (Maudlin et al. 1990).

Instead of the classic transmission cycle of HAT (man to man), there are three more cycles such as domestic cycle (involving man & domestic animals), wild cycle (wild animal to wild animal) and mix cycle (involving human, domestic & wild animals).

1.1.6 Trypanosome life cycle

After ingestion of a blood meal from an infected host, tsetse fly becomes infected. In the insect vector midgut, the trypanosomes develop into procyclic trypomastigotes and divide for approximately 10 days and then they obtain a functional cytochrome system and TCA cycle. After division cycles, the parasites migrate to the salivary glands and transform into epimastigotes. These forms divide and transform into metacyclic trypomastigotes, the infective stage for mammalian hosts. The life cycle of trypanosome in the insect is 25-50 days, which depends on the fly species, the trypanosome strain and the temperature (Fig.3). If tsetse flies infected by more than one strain of trypanosome, genetic exchange between the two strains happens and it increases genetic diversity.
1.1.7 Two distinct stages of sleeping sickness

When the tsetse fly injects the infective stage, known as the metacyclic stage, into the human host, the parasites rapidly transform into bloodstream trypomastigotes (long & slender forms) and replicate by binary fission in the interstitial spaces at the site of infection. The metabolic wastes and cell debris cause the local inflammatory reaction and the formation of a chancre.

Figure 3: *T. b. gambiense* and *T. b. rhodesiense* life cycle in human and tsetse fly. Copyright Alexander J. da Silva and Melanie Moser, Centers for Disease Control Public Health Image Library. Reprinted with permission from the Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Ga.

Figure 4: The site of the tsetse fly bite in HAT patient (WHO).
1.1.7.1 Early stage (the haemolymphatic phase) and the symptoms

There are two distinct stages during sleeping sickness. The first stage of the disease, also known as the haemolymphatic phase, starts when the trypanosomes are exposed to the blood and lymph system (9). The symptoms and signs of this stage are nonspecific including interrupted fever, headache, fatigue, malaise (weakness), anaemia, arthralgia (joint pains), pruritus (itching), oedema and swollen lymph nodes on the back of the neck (T. b. gambiense) (65, 66). Generally, the number of parasites in the blood is low; therefore, diagnosis by microscopic examination is often negative. From there, the parasites distribute throughout the host, invade all organs of the body including the heart and central nervous system (CNS).

1.1.7.2 Second stage (the neurological phase) and the symptoms

The second stage of the disease, also known as the neurological phase (meningoencephalitic stage), is characterized by trypanosome invasion of meninges and the infiltration of the leptomeninges and perivascular areas by lymphocytes, plasma cells, macrophages and finally Mott cells (Mattern 1964; Poltera 1980). Generally, the parasites spread through the brain and spinal cord and it causes abnormal cerebrospinal fluid (CSF) with serious pathological effects in human. The precise time of the earliest penetration of the parasites to the blood–brain barrier is still unknown. At this stage, trypanosomes penetrate to the central nervous system (CNS) and can be seen in the cerebrospinal fluid. During the meningoencephalitic stage, varied specific signs and neurological symptoms can be present. They can be summarized as follows: difficulty walking and talking, weight loss, extreme lethargy (being drowsy), poor condition, disturbed sleep pattern (Hence, it is called sleeping sickness), daytime somnolence, sporadic fever, headache, sensory disturbances (hyperpathia), extreme tendon reflexes, pruritus (itching), primitive reflex presence including palmomental reflex and sucking reflex, tremor (involuntary shaking of the body), apathy and convulsion. Personality changes and psychiatric disorders are frequent with alternative moods, confusion, agitation, aggressive behavior and euphoria (feeling of happiness & confidence). In addition, absent stare, mutism (inability to speak), epileptic seizures and finally coma occur (65, 66). The evolution of the disease causes death if untreated (12, 13) within weeks-months (infected with T. b. rhodesiense) or within months-years (infected with T. b. gambiense).

Figure 5: Late stage of HAT (72)
The disease is linked to the high level of immunoglobulin M (IgM) antibodies in CSF (Mattern 1964; Greenwood & Whittle 1980) and increase of cytokine concentrations such as Interleukin 10 (IL-10) (Rhind et al. 1997; MacLean et al. 2001, 2006), IL-1b (Courtieux et al. 2006), etc.

The Rhodesian or East African form of sleeping sickness, caused by *T. b. rhodesiense*, is more severe and has acute symptoms, which can progress within weeks. It is a zoonotic infection that the game animals are its reservoir. The Rhodesian disease is considered as a compressed Gambian form because most of the clinical signs and the neuropathological changes are similar in both forms. For *T. b. rhodensiense*, the chancre, a leathery swelling at the site of the infection is usually the first sign of the disease and the patients experience irregular alternative fevers related to the parasite invasion. Oedema of the face and anemia are frequent signs of infection. The disease is more virulent and acute; it can rapidly develop into late stage sleeping sickness within weeks. Less than 10% of the reported cases of the sleeping sickness are related to this form of parasite.

*Trypanosoma brucei gambiense* causes the Gambian or West African form, which develops and progresses slowly, in several years. Its main reservoir is human. For *T. b. gambiense*, lymphadenopathy occurs more frequently. The enlargement of the spleen and liver are others symptoms. The second stage development of HAT may not happen for decades and the patients may only suffer from fatigue because of the alternative parasites increase in the blood. It causes more than 90% of sleeping sickness cases.

1.1.8 Geographical distribution of sleeping sickness

Sleeping sickness is only found in the tropical parts of Africa because of the ecology of the insect vector. A north–south line can be drawn through present-day Africa to restrict the distributions of Gambian and Rhodesian sleeping sickness (Welburn et al. 2001a). *T. b. gambiense* causes a chronic form of the disease, which is found in the lowland rain forest of West and Central Africa. Whereas, *T. b. rhodesiense* causes a more acute condition, which is found in the savannah woodlands of East and South Africa. In addition, this line separates the Francophone and Anglophone areas in the colonial era (Although Ghana and Nigeria are included in the Francophone region) (14) (Fig. 6).

Uganda is the only country, which hosts both forms of sleeping sickness: Rhodesian in the south–east and Gambian in the north–west. Although these two forms of disease have remained apart since records began, however, they have been moving gradually towards each other in recent years (14).
1.1.9 Treatment and drugs

Currently active drugs available for treatment of human African trypanosomiasis are few and limited. Because of the toxicity of drugs, hospitalization and continues follow-up examinations to check for relapse are required. Treatment is stage specific: pentamidine and suramine are used for the hemolymphatic stage of Gambian and Rhodesian forms, respectively, and have some side effects. The drug available for the late stage, Melarsoprol (arsenic compound) is used due to its ability to penetrate the blood-brain barrier. This drug is highly toxic and causes encephalopathy in 5-10% of treated patients and the fatality in 50% of patients afflicted. Therefore, reliable early diagnosis of the disease stage is crucial. Only for both stages of T. b. gambiense infection, Eflornithine is effective, not against T. b. rhodesiense (Iten et al. 1995). The patients treated by Melarsoprol and Eflornithine need long period hospitalization and monitoring. NECT (Nifurtimox- Eflornithine Combination Therapy) is a new, effective and safe treatment for second stage of Gambian form, which is low-cost with less hospitalization. Since it is not possible to develop an effective vaccine because of the VSG coat on the surface of parasites in the near future, more research should be performed to develop more effective chemotherapy. In table 1, some drugs of HAT are mentioned.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>year of the first use</th>
<th>Structure</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atoxyl</td>
<td>Stage1, T.b.g.*</td>
<td>1905</td>
<td>Pentavalent</td>
<td>Optical nerve Atrophy</td>
</tr>
<tr>
<td>Tryparsamide</td>
<td>Stage1&amp;2, T.b.g.</td>
<td>1921</td>
<td>Pentavalent</td>
<td>Optical nerve Atrophy</td>
</tr>
<tr>
<td>Orsanine</td>
<td>Stage2, T.b.g.</td>
<td>1925</td>
<td>Isomer of Stovarsol</td>
<td></td>
</tr>
<tr>
<td>Melarsen</td>
<td>Stage1&amp;2, T.b.g.</td>
<td>1939</td>
<td>Pentavalent</td>
<td></td>
</tr>
<tr>
<td>Melarsen-oxide</td>
<td>Stage1&amp;2, T.b.g &amp;T.b.r**</td>
<td>1941</td>
<td>Trivalent</td>
<td></td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>Stage2, T.b.g&amp;T.b.r</td>
<td>1949</td>
<td>Trivalent</td>
<td>rate of relapses increase</td>
</tr>
<tr>
<td>Non-arsenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suramin sodium</td>
<td>Stage1, (T.b.g)T.b.r</td>
<td>Beginning of 20s</td>
<td>Naphtylamine polysulphone</td>
<td></td>
</tr>
<tr>
<td>Pentamidine</td>
<td>Stage1, T.b.g.</td>
<td>1940</td>
<td>Diamidine aromatic</td>
<td></td>
</tr>
<tr>
<td>Isethionate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>Stage2, T.b.g(T.b.r)</td>
<td>1977</td>
<td>5-nitrofurane</td>
<td>***</td>
</tr>
<tr>
<td>Efornithine</td>
<td>Stage 2, T.b.g.</td>
<td>1981</td>
<td>DL-α-difluoromethylornithine</td>
<td></td>
</tr>
</tbody>
</table>

*T.b.g. = *Trypanosoma brucei gambiense* **T.b.r = *Trypanosoma brucei rhodesiense***

***No official indication for the HAT side effects and action on T.b.r.

Table 1: General information on the treatments of HAT (15).

1.2 Biomarker for the stage determination and test of cure

The effective diagnosis is essential, not only for the trypanosome detection, but also the stage determination of sleeping sickness, because failure to treat patients in the late stage will always lead to death. On the other hand, the diagnosis must be made as early as possible in order to prevent parasite transmission towards the brain, because if neurological signs increase, treatment procedures become difficult, toxic and unsatisfactory (Bouteille et al. 2003) and also the treatment chances decrease. The lacks of sensitive and specific methods for early diagnostic remain a major problem for sleeping sickness surveillance and control (16, 17, 18).

In the early stage of disease, because of nonspecific and variable clinical diagnostic signs, serological tests play an important role such as antibody-detecting card agglutination trypanosomiasis test (CATT/*T. b. gambiense*) (19, 20). In addition, the existence of the parasites in chancre fluid, blood, lymph node aspirates and bone marrow is diagnosed by
microscopic examination, which is unreliable at low parasite rate (23). However, this stage is easy to cure. Disease stage determination is based on presence of neurological signs and CSF examination after lumbar puncture, which is unsuitable for much extended screening goal (21).

New treatments for the second stage of HAT are scarce and the procedure from a modern drug to clinical tests is still a struggle (Barrett et al. 2007). Therefore, improving diagnostic strategies of CNS involvement is essential to restrict the use of toxic drugs. As a consequence, cerebrospinal fluid (CSF) white blood cell count and the presence of trypanosomes remain the main diagnostic procedures in the field with disagreement due to the CSF cell count threshold (Chappuis et al. 2005).

Because of nonspecific clinical signs of sleeping sickness (22), it is necessary to find a reproducible and substitute biomarker for the disease presence and the stage determination: such a biomarker must be robust, manifest high sensitivity and selectivity and change with the disease stage (23).

By using “omics” technologies, the infectious diseases diagnosis have been possible. Both genomic and proteomic methods have been used for HAT diagnosis (24, 25). The utility for diagnosis of some biomarkers are still investigated in detail. Most of the studies have been on the West African form (Lejon et al. 1998, 2002, 2003, 2007). Little information is available on the more severe and acute form caused by T. b. rhodesiense. Identification of clinical markers of disease stage would help using the safe available treatment and diminish the use of more toxic late stage drugs (27).

Only at later time points the parasites penetrate across the blood-brain barrier into the brain. The penetration of leukocytes and parasites into the brain probably indicate the prevalence of the late stage of infection (30). Chemokines mediate the holding of immune cells in inflamed tissues. Individual chemokines play different roles in neuroinflammation in various experimental models of infectious disease. It is difficult to predict whether chemokines are protective by involving immune eradication of the parasitic attack or are causing more inflammatory damage and disease (32).

Although an association between levels of some chemokines in the CSF of HAT patients has been described (33, 34), a systematic analysis of chemokines and other molecules expression during trypanosome infections are still needed.

It is essential to use animal models to confirm and determine the usefulness of diagnostic markers and their potential contribution to treatment. In this filed, it is profitable to monitor the variation of molecules during the course of disease and follow the changes after different treatments and to investigate efficiency of the treatments.
1.3 Vervet Monkey as a model

For studying human African trypanosomiasis, different animal models have been proposed including mouse, rat, dog, sheep and non-human primates such as monkey. They were infected by using three pathogenic strains (human: *T. b. gambiense* and *T. b. rhodesiense*; animal: *T. b. brucei*). Due to the high toxicity of second stage drugs, development of sleeping sickness models is necessary. Although, using animal models have some limitations because of differences between animals and humans such as complexity of the human nervous system compared to animals, different response of immune systems, etc. However, the advantages of using animal models for sleeping sickness outweigh its defects because obtaining physiological data is easy (body temperature, food consumption, etc). In addition, investigating the clinical signs of the patients during the time course of the disease are not possible and frequent sampling especially of CSF is unethical.

Among all animal models, Vervet Monkey (*Chlorocebus aethiops*, African green monkey) old world monkey in the family *Cercopithecidae* is an appropriate model for protozoan parasitic diseases specially *T. b. rhodesiense* (Fig.7). This small, black-faced monkey is common in East Africa as it adapts easily to different environments and is widely distributed. When they are infected with parasites, they show clinical, pathological and immunological changes similar to those occurring in humans. Vervet Monkeys infected with *T. b. rhodesiense* survive for a short time without treatment and significant CNS signs can be obtained only by prolonging survival with an ineffective trypanocidal treatment (Schmidt & Sayer. 1982a, 1982b).

Vervet Monkeys experimentally infected with human infective trypanosomes develop a disease, which clinically mimics the disease in humans (Farah et al. 2004; Ouwe-Missi-Oukem-Boyer et al. 2006; Thuita et al. 2008). Therefore, they provide an excellent opportunity to investigate controlled laboratory studies on serum and CSF samples and identification of potential biomarkers of the disease stages. In this case, it is possible to follow a potential biomarker expression during the disease progression and consider its level before infection, after infection and after specific treatments.

![Figure 7: Vervet Monkey (*Chlorocebus aethiops*).](image)
1.4 Research Goal

The study was performed to follow the variations of potential biomarkers in CSF and serum samples of experimentally infected Vervet Monkeys by means of human immunoassay kits and to indicate the changes during the course of disease. We hypothesized that before infection, each target level is in a normal value and after infection and the presence of the parasites in the body fluid, the target expression changes towards increase and finally after different cures, it reflects the variations according to the administrated treatments in serum and CSF samples of animal models.

1.4.1 Specific aim 1: CXCL13, Neopterin, IP10, IL10, CXCL8, MMP-9, B2MG, VCAM1, ICAM1, H-FABP & GSTP1: antibody recognition

In this present study, one of the objectives was to know whether it was possible to measure monkeys' serum and CSF molecule expression with human immunoassay kits. In other words, whether there are cross-reaction between antibodies against human antigens and animal models' antigens. The second purpose was to know which dilution was suitable for measuring some molecule concentrations, which were considered as a potential biomarker such as GSTP1, H-FABP, CXCL13, Neopterin, B2MG, CXCL10, IL10, CXCL8, MMP-9, VCAM1 and ICAM1. To reach this goal, different dilutions and different time points of CSF and serum samples were considered (before infection, after infection & treatments).

GSTP1 expression in the brain is still not well known; however, its concentration was evaluated in the second stage of HAT patients and it has been demonstrated to be a potential biomarker for the staging of sleeping sickness. H-FABP has been validated as a biomarker of stroke (37). CXCL13 concentration significantly increased in CSF during the infection course of HAT (49). CXCL8 and IL-10 levels have been elevated in CSF of the second stage patients (38, 39). Neopterin is a sensitive indicator of cellular immune activation and its diagnostic validity helps in differential diagnosis of inflammatory diseases (42). In addition, the high level of Neopterin was observed in the second stage patient CSF of sleeping sickness (44). B2MG is known as a major protein element in the human CSF (43). CXCL10 were evaluated in both serum and CSF of cerebral malaria patients (40) and mostly distinguished the first and second stage of HAT (infected by *T. b. gambiense*) (51). MMP-9 is an enzyme that promotes tumor invasion (45) and CSF MMP-9, which increases in other neurological conditions, could provide an additional biomarker for the separation of patients with Vascular Dementia and Alzheimer Disease (46). Finally, the expression of the adhesion molecules ICAM1 and VCAM1 increased in the presence of trypanosome (48).

1.4.2 Specific aim 2: Variation of GSTP1 concentration in infected Vervet Monkeys

The main objective of the study was to monitor the treatment efficiency of animal models, which were experimentally infected by *T. b. rhodesiense*. For this purpose, GSTP1 was selected for further investigations. We performed a systematic comparison of the GSTP1 expression in the serum and CSF of experimentally infected Vervet Monkeys by *T. b. rhodesiense* during the disease progression, before and after some treatments. To achieve this
goal, serum and CSF samples of experimentally infected Vervet Monkeys were considered
and GSTP1 concentration was measured in all available samples.
II. Material and Methods

2.1 Animals: Vervet Monkeys

Vervet Monkeys were guarantied singly in cages, fed on commercial pellets and fresh vegetables, protected for zoonotic diseases and treated for all parasitic infections. Nine monkeys were infected intravenously with approximately $10^4$ trypanosomes (T. b. rhodesiense, isolate KETRI 2537) (50). Two non-infected animals were considered as the control group. The animals were anaesthetized at weekly intervals, weighted and clinical tests performed (50). Blood and CSF samples were obtained at regular periods of time. All infected monkeys were treated sub-curatively with diminazene aceturate (DA) 28 days post infection (dpi) to remove all parasites from the blood and to accentuate the second stage of the disease. If they had not been treated, they would have died around 50 days. Monkeys remaining at 98 dpi were treated curatively with Melarsoprol (MelB) between 140-145 dpi and kept for 300 days.

2.2 Samples: CSF & serum of Vervet Monkeys

Serum and CSF samples of eleven Vervet Monkeys were sent from Institute of Primate Research in Kenya to Biomedical Proteomics Research Group (BPRG). Both CSF and serum samples were obtained at 24 different time points from -14 to 273 dpi\(^1\). All samples have been stored at -80\(^\circ\)C.

GSTP1: CSF \(^2\) and serum samples of all available dpi for monkeys 559, 589, 561, 553, 563, 580, 592, 528, 526, 569 and 508.

H-FABP: CSF and serum samples of all available dpi for monkeys 559 and 569 and some time points for monkey 553 were tested.

CXCL13, B2MG, Neopterin, IL10, CXCL10, CXCL8, ICAM1, VCAM1 & MMP-9: CSF samples of three infected monkeys: monkey 561 at three time points: 0, 28, and 91 dpi, monkey 553 at five time points: pool -14 & 0, 28, 49 and 91 dpi and monkey 563 at three dpi: 0, 28 and 91 dpi and also one non-infected monkey: monkey 508 at four dpi: pool 28 & 42 and pool 84 & 98 dpi were tested.

2.3 Immunoassay

An immunoassay is a biochemical test which measures the substance concentration in a biological liquid, the specific reaction of an antibody to its antigen. Usually, they use monoclonal antibodies, which bind to one site of a particular antigen, and therefore, provide a more specific test, which is less interfered by the presence of other proteins.

\(^1\) 24 days post infection for CSF and serum samples of all monkeys were not available.

\(^2\) The results of 35 dpi in monkeys 569 & 563 and 35 & 42 dpi in monkey 580 were not considered as they were contaminated with blood.
In addition, it has an important role in the diagnosis of many infectious diseases such as HAT.

2.3.1 ELISA

ELISA (Enzyme-linked immunosorbent assay), also known as EIA (enzyme immunoassay), is used to evaluate the presence of specific antigens in a sample. In other words, an unknown amount of antigen is attached to a solid support (usually a polystyrene microplate) and then a specific antibody is added so that it can bind to the antigen and forming a complex. The detection antibody can be linked to an enzyme or can be detected by another antibody, which is attached to an enzyme. Between each step, some washes are performed with a mild detergent solution to remove any unbound analytes or antibodies. At the end, a substrate solution is added that the enzyme can convert to a detectable and visible signal, which presents the antigen concentration in the sample. ELISA Types: Direct ELISA, Indirect ELISA, Sandwich ELISA, Competitive ELISA and Reverse ELISA (Fig.8).

Figure 8: Different types of ELISA (71).
2.3.2 Multiplex assay

A multiplex assay is a type of laboratory procedure based on Fluorescent Bead Arrays (FBA) that simultaneously measures up to one hundred different analytes in a single assay (Fig.9). It is easy to carry out automatically in order to save time and money compared to a single parameter measurement by using traditional methods. The basic principle of FBAs is similar to ELISA. It is based on microscopic spherical Polystyrene particles, microspheres, which serve as a solid phase for molecular detection reactions. Upon incubation of the microspheres with the sample, antigens bind to the monoclonal antibodies (conjugated with beads) in a specific manner. The microspheres are currently available in one hundred different fluorescent color tones of red and infrared, which emit light in different regions of the optical spectrum. A specific detection antibody is used to label antigens, which are bound to the microspheres. It carries an additional fluorescent dye, which emits in the green region of the spectrum (Fig.10).

It is possible to use mix bead sets with different conjugated antibodies to simultaneously tests many antigens in a sample. Analysis and evaluation of Fluorescent Bead Arrays is performed with the Luminex analysis system. It is based on the technology of flow cytometry, which use two different lasers. Since the spectral region of the second antibody is different from the regions of the internal dyes of beads; it is possible to perform the classification of different microspheres and the quantitation of bound antigens at the same time. In other words, a red laser illuminates the dyed beads; the system can distinguish different antigens in the assay. At the same time, a green laser excites fluorescent dye, which binds to the second antibody. The amount of this fluorescence is proportional to the amount of captured antigen. The concentration of each analyte in the sample is automatically calculated using corresponding standard curves obtained from recombinant protein standards.
2.3.3 Homemade ELISA (GSTP1)

GSTP1 concentration was determined by homemade ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) sandwich. Ten plates were designed to do the experiments. CSF and serum samples were measured separately. They were performed in duplicate. Biotinylated anti-GSTP1 human antibodies (2 μg/mL) (Biosite, California, USA) were coated 1 hour at 37°C on a Reacti-Bind™ NeutrAvidin™, Coated High Binding Capacity (HBC) Black 96-well Plates with SuperBlock R Blocking Buffer (Pierce, Rockford, IL). After three washes, CSF samples (diluted 1:4), serum samples (diluted 1:10), quality controls and standards (recombinant Sigma GSTP1 at the concentration ranging from 0 to 100 ng/mL) \(^1\) were incubated for 1 hour at 37°C. After some washes, Alkaline phosphatase conjugated antibodies against human GSTP1 (2 μg/mL) (Biosite, California, USA) were added and incubated for 1 hour at 37°C. After the last three washes, Attophos® AP Fluorescent substrate (Promega, Madison, WI) was added. Plates were read immediately on a SpectraMax GEMINI-XS (Molecular Devices Corporation, Sunnyvale, CA, USA). Plate reader uses the kinetic mode. Vmax values were automatically calculated by the instruments based on relative fluorescence units RFU (λ\text{excitation} = 444 nm and λ\text{emission} = 555 nm). GSTP1 concentration in CSF and serum was back calculated using 5PL and 4PL, respectively, calibration curves based on measured standards values.

2.3.4 Commercial ELISA

H-FABP

H-FABP concentration was determined by a commercial available ELISA kit (Hycult Biotechnology, Uden, Netherlands) according to the manufacturer's instructions.

\(^1\) In CSF experiment: From the third experiment, the concentrations of standards were changed (ranging 0 to 10 ng/mL) because most of the concentration of samples were close to the limit of detection.
The Hbt human H-FABP ELISA kit was a solid-phase enzyme-linked immunosorbent assay (ELISA) sandwich. CSF samples (non-diluted) in duplicate, serum samples (diluted 1:5) in duplicate, standards and controls were incubated together with peroxidase conjugated second antibodies in microtiter wells coated with antibodies recognizing human H-FABP for 1 hour. Unbound materials in the wells were removed by washing and substrate, tetramethylbenzidine (TMB) was added for 15 minutes and the color developed proportionally to the amount of H-FABP in the sample. The enzyme reaction was stopped by adding stop solution (citric acid) and the absorbance was measured with the spectrophotometer. A standard curve was obtained by plotting the absorbances versus the corresponding concentrations of the standards. H-FABP concentration in CSF and serum was back calculated using 5PL calibration curve based on measured standards values.

CXCL13

CXCL13 concentration was determined by a commercial ELISA kit (R & D systems, Inc, Minneapolis, USA) according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. CSF samples (diluted 1:2, 1:4 & 1:20) in simplicate, standards and controls were pipetted and incubated together with Assay Diluent RD1S for 2 hours in microplate wells coated with mouse monoclonal antibodies against CXCL13. After washing away any unbound substances, CXCL13 conjugate (mouse monoclonal antibodies against CXCL13 conjugated to horseradish peroxidase) was added for 2 hours incubation. Following some washes to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells for 30 minutes and the color developed in proportion to the amount of CXCL13 bound. The color development was stopped by adding stop solution (Sulfuric acid). The intensity of the color was measured with the spectrophotometer. CXCL13 concentration in CSF was back calculated using 5PL calibration curve based on measured standards values.

B2MG

B2MG (Beta-2 Microglobulin) concentration was determined by a commercial ELISA kit (Calbiotech, Inc, CA, USA) according to the manufacturer's instructions. The B2MG ELISA kit is based on the principle of a solid-phase enzyme-linked immunosorbent assay. CSF samples (diluted 1:3, 1:10 &1:50) in simplicate, standards ¹ and controls were incubated together with Sample Diluent at 37°C for 30 minutes in microplate wells coated with mouse monoclonal antibodies against Beta-2 Microglobulin molecule. After washes, enzyme conjugate reagent (sheep anti-B2MG antibodies conjugated to horseradish peroxidase) was incubated at 37°C for 30 minutes, resulting in the B2MG molecules being sandwich between the solid phase and enzyme-linked antibodies.

¹One concentration was added to the end of standards.
After some washes and removing unbound-labeled antibodies, a solution of TMB Reagent was added and incubated for 20 minutes at room temperature and the color development was stopped with the addition of stop solution. The intensity of the color was measured with the spectrophotometer. B2MG concentration in CSF was back calculated using 5PL calibration curve based on measured standards values.

**Neopterin**

Neopterin concentration was determined by commercial ELISA kit (B.R.A.H.M.S Aktiengesellschaft, Hennigsdorf) according to the manufacturer's instructions. The B.R.A.H.M.S Neopterin EIA is a competitive enzyme immunoassay for the quantitative determination of Neopterin. CSF samples (pure, diluted 1:2 &1:4) in simplicate, standards and controls were added together with enzyme conjugate (neopterin/alkaline phosphatase conjugate) to uncoated premixing microplate. The aliquot of this mixture was transferred to the microplate wells coated with anti-neopterin antibodies (polyclonal, sheep) and incubated for 2 hours in dark. The Neopterin of the samples competed with the neopterin/alkaline phosphatase conjugate for the binding sites of these antibodies. After subsequent washes and the removal of all unbound components, substrate solution (4-nitrophenyl phosphate) was added for 30 minutes. The enzymatic reaction was stopped by stop solution (sodium hydroxide). The intensity of the color depended on the quantity of enzyme bound and was inversely proportional to the Neopterin level. Therefore, high Neopterin values corresponded to a low optical density. The optical density was measured by means of the spectrophotometer. Neopterin concentration in CSF was back calculated using 5PL calibration curve based on measured standards values.

**2.3.5 Bead suspension array**

**IL10, CXCL8 & CXCL10**

The levels of three cytokine and chemokines (IL10, CXCL8 & CXCL10) were determined by using the Bioplex® bead suspension array according to manufacturer's instructions (Bio-Rad, Hercules, CA). The principle of bead-based assays is similar to a sandwich immunoassay. Three sets of polystyrene dyed beads were conjugated separately with the antibody against the desired molecules. The coupled beads could react with a sample containing the target molecules. All these sets were mixed together by the supplier and delivered ready-to-use. An equal amount of beads was added to each well. After some washes, CSF samples (pure, diluted 1:2 & 1:4) in simplicate, standards and controls were incubated for 30 minutes at room temperature. After washing to remove unbound molecules, a mix of the corresponding three biotinylated detection antibodies was incubated 30 minutes at room temperature. It caused the formation of a sandwich of antibodies around the analyte. After washing, streptavidin-phycoerythrin (streptavidin-PE) was added to bind the biotinylated detection antibodies on the bead surface for 10 minutes. After last washes, beads were re-suspended in the assay buffer. The content of each well was aspirated in to the Bio-Plex system, a dual-laser and flow-based microplate reader system. The lasers detected the internal fluorescence of each dyed beads as well as the fluorescent reporter signal on the bead surface. Fluorescence intensity indicated the relative quantity of target molecules in the sample. In
other words, each bead was identified and the related analyte was simultaneously quantified based on bead color and fluorescence, respectively. The concentration of each target was calculated by the Bio-Plex Manager™ software using corresponding standard curves (5-PL and linear) obtained from recombinant protein standards.

**ICAM1 & VCAM1**

The levels of adhesion molecules including intercellular cell adhesion molecule (ICAM1) and vascular cell adhesion molecule (VCAM1) were determined by using the Fluorokine® MAP Human Adhesion Molecule MultiAnalyte Profiling Base Kit (R & D System, Inc, Minneapolis, USA) according to the manufacturer's instructions. Fluorokine® MAP multiplex kits are designed for use with different analyzers such as BioRad BioPlex™ analyzer, a dual laser, flow-based and detection platform manufactured by Luminex Corporation.

The dyed microparticles were conjugated with analyte-specific antibodies. An equal amount of microparticles was added to each well. CSF samples (pure, diluted 1:2 & 1:4) in simplicate, standards and controls were pipetted and incubated into microplate wells for 2 hours and the immobilized antibodies bound the desired analytes. Then, biotinylated antibodies specific to the analytes were added to each well for 1 hour. Following the washes to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the captured biotinylated antibody, was added to each well for 30 minutes. The final washes removed unbound Streptavidin-PE and the microparticles were re-suspended in buffer and read using the BioRad BioPlex™ analyzer. One laser was determined which analyte was being detected. The other laser determined the amount of analyte. Each target concentration was calculated by the Bio-Plex Manager™ software using corresponding standard curve 5-PL for VCAM1 and 4-PL for ICAM1 obtained from recombinant protein standards.

**MMP-9**

MMP-9 concentration was determined by the Fluorokine® MAP Human MMP MultiAnalyte Profiling Base Kit (R & D Systems, Inc, Minneapolis, USA) according to the manufacturer's instructions. Fluorokine® MAP multiplex kits are designed for use with different analyzers such as BioRad BioPlex™ analyzer, a dual laser, flow-based and detection platform manufactured by Luminex Corporation.

The dyed microparticles were conjugated with analyte-specific antibodies. An equal amount of microparticles was added to each well. CSF samples (pure, diluted 1:2 & 1:4) in simplicate, standards and controls were pipetted into wells for 2 hours and the immobilized antibodies bound the analytes. After washing away any unbound substances, biotinylated antibodies specific to the analytes were added to each well for 1 hour. Following the washes to remove unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds the biotinylated detection antibodies, was added to each well for 30 minutes. The final washes removed unbound Streptavidin-PE and the microparticles were re-suspended in buffer and read using the BioRad BioPlex™ analyzer. One laser was determined which analyte was being detected. The other laser determined the amount of
analyte. Each target concentration was calculated by the Bio-Plex Manager™ software using corresponding standard curve 5-PL obtained from recombinant protein standards.

2.4 Data Analysis

The collected data were statistically analyzed using Microsoft office Excel 2007 and the SPSS (PASW statistics 18) software. Statistical analysis of differences between groups was performed using non-parametric tests; Mann-Whitney U test (comparison between two groups) because the values didn’t present a normal distribution. Statistical significant was threshold 0.05 (P<0.05) for these tests. Some data were presented as median and error bars (confidence interval).
III. Result

3.1 B2MG, CXCL13, Neopterin, MMP-9, VCAM1, ICAM1, IP10, IL10 & CXCL8 in CSF and H-FABP in serum & CSF: antibody recognition

B2MG: In monkeys 561 and 553, B2MG concentration was below the limit of detection except 98 dpi in monkey 561 with the lowest dilution. In contrast, the cross-reaction between monkeys' protein and antibodies against human B2MG were clear in monkeys 563 and 508. Except 0 dpi of monkey 563 and 2 pooled dpi of monkey 508 with the highest dilution, the rest were distinctly above the limit of detection. The protein level in 0 dpi of monkey 563 and both 2 pooled dpi of monkey 508 (control) was approximately the same. The time points diluted 1:3 were more detectable. As a conclusion of this assay, it was possible to measure monkey's B2MG concentration with this human kit and the best dilution was 1:3. Clearly, B2MG level of infected monkey increased continuously in the course of infection (Fig.11).

![Figure 11: B2MG concentration of monkey 563 and mean 2 pooled dpi of control in CSF samples diluted 1:3.](image)

CXCL13: CXCL13 concentration, in three infected monkeys, was increased from before infection to after infection. 49 dpi in monkey 553 decreased afterward it showed upward movement. In addition, except dpi before infection in most dilutions of three infected monkeys and 28 dpi diluted 1:20 in monkey 561, all the results were above the limit of detection. In non-infected monkey 508, the level of this protein was low and closer to the limit of detection. The best dilution was considered 1:4. This protein had the high concentration in 91/98 dpi and it approximately had the same level in before infection and control (Fig.12).
Neopterin: The molecule concentration at most dpi of three dilutions in four monkeys was above the limit of detection. In infected monkeys, it showed high concentration in 28 dpi compared to before infection and after 28 dpi its expression slightly decreased in monkeys 561 and 563 but in monkey 553 showed the huge decrease until 49 dpi and partially changes until 91 dpi. Two pooled dpi in monkey 508 approximately had the same concentration as before infection. The non-diluted concentration in all monkeys was detectable and above the limit of detection. That's why, it was considered as the best dilution for this molecule in CSF samples. The expression of this molecule became weak in 91/98 dpi compared to 28 dpi and there were the same level before infection and control (Fig. 13).

MMP-9: All the results were below the limit of detection except 28 dpi (non-diluted & 1:2 diluted) and 91 dpi non-diluted in monkey 563. The protein level was higher in 28 dpi compared to 91 dpi in monkey 563. Although the results were below the limit of detection
but there were slightly changes between two pooled dpi in monkey 508 and in monkeys 561, in addition, the concentration of protein increased during the course of disease. Therefore, they should be elucidated with caution. In spite of poor result, the best dilution could be non-diluted as presented in figure 14.

**Figure 14:** MMP-9 concentration of monkey 563 and mean 2 pooled dpi of control in CSF samples non-diluted.

ICAM1: all the results were below the limit of detection and it was not possible to determine the best dilution in this case. In other words, it was not suitable to measure this kind of protein in monkeys' CSF by this kind of human kit.

VCAM1: In VCAM1 case, it was completely different. Except 0 dpi diluted 1:4 in monkey 561, all the results were above the limit of detection which was indicative of the cross-reaction of monkeys' protein and antibodies against human VCAM1. In addition, the protein concentration after infection was clearly higher than before infection. After 28 dpi, its level increased in monkey 561 but decreased in monkeys 553 and 563. Approximately, the same level of two pooled dpi in non-infected monkey observed and they were higher than before infection levels. In this case, non-diluted was considered as the best one (Fig.15).

**Figure 15:** Mean VCAM1 concentration of monkeys 563, 553 & 561 and mean 2 pooled dpi of control in CSF samples non-diluted.
IP10, IL10 & CXCL8: For these three cytokine and chemokines, all the results were below the limit of detection except one dpi in monkey 508 (for IL10). As a conclusion, it was not possible to measure these proteins by this kind of kit.

H-FABP: Only the concentration of few time points was above the limit of detection, therefore, it was not enough to investigate H-FABP level during disease progression for both serum and CSF of considered monkeys. For instance, in serum of monkey 559, after 77 dpi H-FABP level increased until 106 dpi and then its expression became low. However, in monkey 569, there were cross-reactions in one time point before and one after infection (Fig.16).

![Figure 16: H-FABP concentration in serum samples of monkeys 559 & 569.](image)

<table>
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<tr>
<th>Protein</th>
<th>B2MG</th>
<th>CXCL13</th>
<th>NEOPTERIN</th>
<th>MMP-9</th>
<th>ICAM1</th>
<th>VCAM1</th>
<th>IP10</th>
<th>IL10</th>
<th>IL8</th>
<th>H-FABP</th>
</tr>
</thead>
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<td>YES</td>
<td>YES</td>
<td>NO</td>
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<td>YES</td>
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<td>NO</td>
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<td>NO</td>
</tr>
<tr>
<td>Cross-reaction in CSF samples</td>
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<td>n/a</td>
<td>n/a</td>
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<td>n/a</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>NO</td>
</tr>
</tbody>
</table>

Table 2. Summary of the cross-reaction results in considered molecules.

3.2 Variation of GSTP1 concentration in infected Vervet Monkeys

In each monkey (infected and non-infected), the variations of GSTP1 concentration in both serum and CSF were different. In other words, if following GSTP1 concentration from -14 to 273 dpi in each infected monkey, the level of GSTP1 concentration variations in different monkeys were not the same. In addition, if comparing GSTP1 concentration at the same time
point between different infected monkeys, it showed different concentrations as well. On the other hand, in non-infected monkeys, different GSTP1 concentrations were measured from -14 to 273 dpi. These facts might be related to physiological differences between these animals. In this case, to have a better view of protein variation, all the concentrations were normalized by the division of all available dpi with mean three dpi before infection. Therefore, the normalized median of each time point in serum and CSF samples of infected monkeys and the normalized median of all control samples in serum and CSF were considered (Fig. 17 & 18).

In serum of infected monkeys, GSTP1 Level was lower than non-infected until 63 dpi. In other words, before 63 dpi GSTP1 level was lower than controls and after that time point, it changed toward increasing during the disease progression. However, up and down movements were observed. From 0 dpi to 14 dpi the protein level increased. Then, its expression became weak until 49 dpi. After this time point, it started to increase until 84 dpi and after that dpi it showed the decrease until 106 dpi and again started to increase with up and down movements. 152 dpi presented the highest concentration and from that time point the protein concentration started to diminish (Fig.17).

![Serum graph](image)

**Figure 17:** Median GSTP1 level in serum samples of infected monkeys at each time point.

GSTP1 level in CSF samples was higher in infected than non-infected monkeys. Except one dpi which showed the high protein level when comparing with other dpi, the rest were changing in a low level (Fig.18). During the course of disease, there were up and down movements. From 0 dpi to 21 dpi, its level decreased, after that time point it expressed
strongly until 56 dpi, and then it came down in 70 dpi. After the increase until 98 dpi, it started to diminish.

Three time points were considered as critical days post infection: 1) 0 dpi was the day of infection by trypanosome, 2) 28 dpi when the monkeys were treated sub-curatively with diminazine aceturate (DA) to remove all parasites from the blood and to accentuate the second stage of the disease and 3) between 140-145 dpi, the monkeys were treated curatively with Melarsoprol (MelB). To have a better view by considering these three critical dpi, some time points of serum and CSF samples of infected monkeys from -14 to 273 were grouped in seven classes and all controls in one group. Here, normalized GSTP1 level for both serum and CSF samples in all infected monkeys were presented (Fig.19 & 20).

Before infection (BI) -7 dpi, GSTP1 had the higher level in CSF and lower in serum in comparison to controls, which was not significant. After infection (AI) 14 dpi, in serum samples, GSTP1 level was increased if compared to BI but it was approximately the same as controls, which was not expected. After diminazine aceturate treatment (ADAT1) 42 dpi, the protein expression became weak. However, after some time in (ADAT2) 84 dpi, it significantly showed higher concentration than before and in (ADAT3) 140 dpi, it changed to decrease. In addition, after Melarsoprol treatment (AT1) 152 dpi, GSTP1 level was still increased; it presented the highest concentration comparing to other time points. Finally, after more than one hundred days after MelB treatment in (AT2) 273 dpi, the protein level diminished (Fig.19).
In CSF, after infection, there was a change compared to BI and controls as well. After DA treatment, if considering (ADAT1) 42 & (ADAT2) 84 the protein expression had slightly changes than after infection which were not significant and in (ADAT3) 140 dpi it showed the downward movement. Finally, after MelB treatment (AT1 & AT2), protein level still showed lower expression (Fig.20).

In the Figures 19 & 20, ⭐️ corresponds to p<0.05 and ⭐⭐️ corresponds to p<0.01.
IV. Discussion & Conclusion

Serum and CSF of nine experimentally infected Vervet Monkeys by *T. b. rhodesiense*, which causes sleeping sickness, were evaluated for this research. Two non-infected Vervet Monkeys were considered as controls.

The main objective of this study was to investigate variation of potential biomarkers during the course of infection and follow the changes before infection, after infection and after some treatments. In other words, the study was performed to confirm the changes of considered targets during the progression of disease. The potential biomarkers were selected based on their characteristics as brain damage biomarkers (GSTP1 & H-FABP), inflammation related proteins (CXCL8, IP10, CXCL13 & IL10) and other potential markers (B2MG, Neopterin, MMP-9, VCAM1 & ICAM1). To achieve this goal, it was necessary to know whether human kits were suitable to measure the molecules concentration in serum and CSF of experimentally infected monkeys. Due to the lack of monkey kits and antibodies for these molecules, it was mandatory to use the human ones.

As indicated in the results, the antibodies against human antigens were able to recognize and bind to the monkey antigens. However, some results were below or close to the limit of detection. In this case, it could be useful to perform the test with other available kits or other kinds of antibodies to improve the sensitivity.

Considering the results, B2MG, β-2 Microglobulin is generally expressed on nucleated cells. It is a component of the MHC class I proteins and its level increases in inflammatory and pathogenic diseases. B2MG indicated concentration above the limit of detection in monkeys 563 (with an increase during the disease progression) and 508. There were cross-recognitions between monkeys B2MG and antibodies against human B2MG. In this case, dilution 1:3 was the best because it presented more detectable results in different monkeys' time points and the results were in the middle of standard curve of the assay.

CXCL13, also known as B-Lymphocyte Chemoattractant (BLC) or B Cell-Attracting chemokine 1 (BCA-1), belongs to the CXC subtype of the chemokine superfamily. It is secreted by dendritic cells, which are immune cells. CXCL13 and its receptor CXCR5 assist the migration and homing of B lymphocytes to lymphoid tissues follicles. CXCL13 concentration was measured in four monkeys and followed the same protocol as B2MG. It was possible to measure monkeys' CXCL13 with this kind of kit. The dilution 1:4 was chosen. It was less sample consuming, in the middle of standard curve and had more detectable results.

Neopterin, 2-amino-6-(1,2,3-trihydroxypropyl)-1H-pteridin-4-one, is a purine nucleotide and product of guanosine triphosphate (GTP) by macrophages upon stimulation with interferon-γ (IFN-γ). Neopterin concentration presents a pro-inflammatory immune status and the activation of cellular immune system under the control of Th1. Furthermore, it estimates the extent of oxidative stress evoked by the immune system. In the most results, Neopterin levels were higher than the limit of detection in four monkeys. The non-diluted was the best because
of recognition with antibodies against human Neopterin. The mean concentration of time points after treatment (91/98 dpi) diminished compared to after infection. It seemed that after DA treatment in 28 dpi, its level decreased in CSF.

Matrix metallopeptidase 9 (MMP-9) is an enzyme, which breaks down proteins in the extracellular matrix (ECM) in normal physiological processes and course of diseases. It is expressed by many cell types upon response to adhesion molecule, cytokine, growth factors and hormones. MMP-9 concentration was below the limit of detection except of two time points, therefore, the non-diluted concentration should be considered with care. In addition, the three detected values were above but close to the limit of detection.

Vascular cell adhesion molecule-1 (VCAM1), also known as CD106, has a significant role in the immune system. It is expressed by cytokines-stimulated endothelial cells. VCAM1 interacts with the beta-1 integrin VLA4 on leukocytes and mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium and also performs signal transduction. VCAM1 was clearly detected and its concentration increased at 91/98 dpi in monkey 561 and decreased in monkeys 553 and 563. It was safe to consider non-diluted as the best because all the results were above the limit of detection and middle of the standard curve.

Inter-Cellular Adhesion Molecule 1 (ICAM1), also known as CD54, is expressed on endothelial and immune cells. Its level increases upon cytokine stimulation. By means of ICAM-1/LFA-1, leukocytes (lymphocyte) bind to endothelial cells and migrate into tissues. IL10 (Interleukin-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an counterinflammatory cytokine, which produces by a variety of cells such as T-cells, macrophages, mast cells, etc. It down-regulates a number of cytokines including IFN-γ, IL-2, IL-3, TNF and GM-CSF. CXCL10, also known as IP-10, belongs to the CXC chemokine family. It is secreted by several cell types (monocytes, endothelial cells and fibroblasts) in response to IFN-γ. CXCL10 by binding to chemokine receptor CXCR3 has been attributed to several roles such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, T-cell activation and migration and antitumor activity. CXCL8, also known as Interleukin-8 (IL-8), is a chemokine produced by macrophage and epithelial cells. CXCR1 and CXCR2 are its most frequent receptors. It is produced upon proinflammatory stimuli by many cell types including monocyte/macrophages, T cells, neutrophils, fibroblasts, endothelial cells, etc. IL-8 is an important mediator of the inflammatory response and serves as a chemical signal in order to attract neutrophils, basophils, T cells and eosinophils. ICAM1, IL10, CXCL10 and CXCL8 were detected in a very low concentration and below the limit of detection in four monkeys. Therefore, it is better to measure these proteins in monkeys with other kits or antibodies.

H-FABP, heart-type fatty acid-binding proteins are present in different cell types. It is rapidly released after acute myocardial infarction. It could be a diagnostic marker for chronic and acute brain damage (54). H-FABP levels in both serum and CSF were mostly below the limit of detection. Although there were few results, it was not enough satisfactory to measure its concentration with this kind of kit.
GSTP1, glutathione-S-transferase-π, is a protein expressed in normal tissues at various levels by different cell types to protect them against cytotoxic and carcinogen agents. Interestingly, variations of GSTP1 concentration were investigated in both serum and CSF samples during the disease progression. After invasion of parasites into the blood, GSTP1 concentration in serum varied according to the disease process and administrated treatments. However, the protein level was lower than controls before 63 dpi which was not expected. Up and down movements were shown. They could be due to the fact that several time points were not available for all monkeys and/or physiological changes during the disease progression. They were different animals and different levels of proteins could be measured. Therefore, in BI which corresponded to non-infected, GSTP1 level was lower than controls. After diminazine aceturate treatment, after some time (84 dpi), although the parasites were supposed to be removed from serum, the protein level was increased, it might be due to the cytotoxic action of the drug on the body cells, delay for recovering the body in order to achieve normal status and penetration of some GSTP1 from CSF to serum. In addition, After MelB treatment in 152 dpi, GSTP1 level increased, it could be explained by some hypothesis. For GSTP1, it takes some time to reach to the normal value and MelB which is normally used for second stage treatment is highly toxic. As a matter of fact, protein concentration diminished in 273 dpi as expected. It showed lower expression, which could be the sign of the restoration of the health.

When parasites have invaded the cerebrospinal fluid at an unknown time point, GSTP1 expression changed based on the infection course and different treatments. The changes were low and most of the time non-significant.

There were nine infected monkeys. Only three monkeys survived until 300 dpi. Therefore, for these monkeys, the available time points were from -14 to 273 dpi in both serum and CSF samples. Whereas the rest six monkeys did not remain alive more than 98 dpi, so, the available time points for them were from -14 to 91 dpi. In addition, some time points were not available in some monkeys for both serum and CSF samples. These facts could be the limitation of this research to interpret the results and to present the significant values.

As a conclusion, GSTP1 presented different concentrations in diverse situations including disease, sub-treatment and treatment. These variations were measured in both serum and CSF from -14 to 273 dpi. By GSTP1 expression, it was possible to monitor the disease progression in experimentally infected Vervet Monkeys by T. b. rhodesiense. In fact, it is feasible to measure some potential biomarkers of monkeys' sample with human kits and follow the changes during the disease progression, as a test of cure.
V. Acknowledgements

I would like to thank so much Dr. Jean-Charles Sanchez, my supervisor, for giving me the opportunity to perform my internship and participate in such an interesting project in his research group (BPRG), trusting in my capability as well as helping me to write this report. I am particularly grateful to Natalia Tiberti & Alexandre Hainard for guiding me during this training and taking the time to answer all my questions, in addition, because of their availability, advice and sympathy. I would also like to thank Dr. Patricia Palagi, coordinator of master's, for her support. Thank to the members of BPRG for their help, encouragement and kindness. And finally, I would like to thank my family and friends for their valuable support.
VI. Appendix

The pink color corresponds to the concentrations below the limit of detection.

Figure 21: B2MG results of CSF samples.

Figure 22: CXCL13 results of CSF samples.
Figure 23: MMP-9 results of CSF samples

Figure 24: VCAM1 results of CSF samples.
Figure 25: Neopterin results of CSF samples.

Figure 26: H-FABP concentration in serum of monkey 553 and CSF of monkeys 559, 569 & 553.

Figure 26: H-FABP concentration in serum of monkey 553 and CSF of monkeys 559, 569 & 553.
VII. References


69. www.lucerna-chem.ch

70. www.bio-rad.com
