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Serological biomarker profile in an experimental model of *Leishmania* infection in non-human primates and in new world leishmaniasis.

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Abstract

Leishmaniasis is are a group of vector borne tropical diseases caused by a protozoan parasites of the genus *Leishmania* with species dependent clinical manifestations. Visceral leishmaniasis is the most severe form. This pathology causes more than 20,000 deaths per year and although it presents these alarming numbers, it has been neglected for a long time. Accurate diagnosis is essential not only for effective treatment but also to trace asymptomatic infections that might pose a risk to the community. The detection of these asymptomatic infections is a currently unmet challenge. This study aims to evaluate the performance of 4 distinct antigens (SPLA, SALA, rK28 and rK39) in a longitudinal study in non-human primate model of *Leishmania* infection. Moreover the effect of treatment in the reactivity to these antigens will be evaluated. Finally, the performance of these markers in detecting active visceral leishmaniasis, in humans, in the context of patients from the new world. Two of these markers are based on extracts of parasite (amastigotes and promastigotes) and two are based on recombinant parasite proteins. In a longitudinal study, sera from 17 experimentally infected rhesus monkeys were evaluated to define an immune response profile considering the 4 antigens tested. The day 0 (before infection) was used to normalize the individual reactivity. It was observed that there is a tendency to have an early response to parasite extracts in the acute phase of the disease with progressive decrease with time. The profile for the recombinant antigens was distinct with a latter overtime increase. Among the recombinant antigens, rK28 was more promising than rK39. In fact, 10 animals showed a tendency to react to rK28. No major changes were found in the immune response profile in treated animals. Considering the study in humans, two groups were evaluated, the first referring to patients with confirmed active infection and the second to a negative control of patients from the same origin, but without known infection. These groups were used to generate the receiver operating characteristic (ROC) curve to define cut-off values. Using these cut-offs all antigens were able to differentiate infected samples from healthy samples. Among the positive samples, a very strong reactivity to antigens was observed. Overall, this data supports the possibility of distinct antigen specific reactivity during infection. rK28 seems to be the best marker for early detection of infection. The data in humans corroborates well with other studies carried out with animal and human models demonstrating that the available markers are adequate for the diagnosis of active visceral leishmaniasis.

Keywords: visceral leishmaniasis; *Leishmania*; non-human primates; new world; promastigotes; amastigotes; rK28; rK39; ELISA

Resumo

A leishmaniose é um grupo de doenças tropicais transmitidas por vetores causadas por parasitas protozoários do gênero *Leishmania* com manifestações clínicas dependentes da espécie envolvida na infeção. A leishmaniose visceral é a forma mais grave. Essa patologia causa mais de 20.000 mortes por ano e, embora apresente esses números alarmantes, é negligenciada por um longo tempo. O diagnóstico preciso é essencial não apenas para um tratamento eficaz, mas também para rastrear infeções assintomáticas que possam representar um risco para a comunidade. A deteção dessas infeções assintomáticas é um desafio atualmente não atendido. Este estudo tem como objetivo avaliar o desempenho de 4 antígenos distintos (SPLA, SALA, rK28 e rK39) em um estudo longitudinal em modelo de primatas não humanos da infeção por *Leishmania*. Além disso, o efeito do tratamento na reatividade a esses antígenos foi avaliado. Finalmente, o desempenho desses marcadores na deteção de leishmaniose visceral ativa, em humanos, no contexto de pacientes do novo mundo. Dois desses marcadores são baseados em extratos de parasita (amastigotas e promastigotas) e dois são baseados em proteínas recombinantes do parasita. Em um estudo longitudinal, soros de 17 macacos rhesus experimentalmente infetados foram avaliados para definir um perfil de resposta imune considerando os 4 antígenos testados. O dia zero (antes da infeção) foi utilizado para normalizar a reatividade individual. Observou-se uma tendência a uma resposta precoce aos extratos de parasitas na fase aguda da doença com diminuição progressiva. O perfil para os antígenos recombinantes foi distinto com um aumento tardio. Entre os antígenos recombinantes, o rK28 foi mais promissor que o rK39. Dez animais mostraram uma tendência a reagir à rK28. Não foram encontradas alterações importantes no perfil da resposta imune nos animais tratados. Considerando o estudo em humanos, foram avaliados dois grupos, o primeiro referente a pacientes com infeção ativa confirmada e o segundo a um controlo negativo de pacientes da mesma origem, mas sem infeção conhecida. Esses grupos foram utilizados para gerar a curva ROC (Receiver Operating Characteristic) para definir os valores de cut-off. Usando esses pontos de corte, todos os antígenos foram capazes de diferenciar amostras infetadas de amostras saudáveis. Entre as amostras positivas, foi observada uma reatividade muito forte aos antígenos. No geral, esses dados suportam a possibilidade de reatividade específica a cada antígeno ser distinta durante a infeção. O rK28 parece ser o melhor marcador para a deteção precoce da infeção. Os dados em humanos corroboram com outros estudos realizados com modelos animais e humanos, demonstrando que os marcadores disponíveis são adequados para o diagnóstico de leishmaniose visceral ativa.

Palavras chave: leishmaniose visceral; humanos; primatas não humanos; novo mundo; SPLA; promastigotas; SALA; amastigotas; rK28; rK39; ELISA

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Abbreviations

AIDS – Acquired immune deficiency syndrome

AUC – Area under the curve

BSA – Bovine serum albumin

CL – Cutaneous leishmaniasis

DAT – Direct aglutination test

DCL – Diffuse cutaneous leishmaniasis

DNA - Deoxyribonucleic acid

ELISA – Enzyme linked immunosorbent assay

HIV – Human immunodeficiency virus

I3S – Instituto de Investigação e Inovação em Saúde

IFAT – Indirect fluorescence antibody

KATEX – Kala Azar latex agglutination test

L. – *Leishmania*

L. t. – *Leishmania tropica*

LAMP – Loop mediated isothermal amplification

LPG – Lipophosphoglycan complex

MAC – Membrane attack complex

MCL – Mucocutaneous leishmaniasis

OD – Optical Density

OPD – o-phenylenediamine dihydrochloride

PBS – Phosphate-buffered saline

PBS-T – Phosphate-buffered saline with 0.05% Tween

PCR – Polymerase chain reaction

PKDL – Post-Kala Azar dermal leishmaniasis

qPCR – Quantitative real-time PCR

RDT – Rapid diagnostic test

ROS – Reactive oxygen species

SALA – Soluble *L. infantum* amastigote antigens

Sn – Sensitivity

Sp – Specificity

SPLA – Soluble *L. infantum* promastigote antigens

Th1 – T helper profile 1

Th2 – T helper profile 2

VL – Visceral leishmaniasis

WHO – World Health Organization

Leishmania

Leishmania is a protozoan and, due to its microscopic size, its recognition was not possible until the invention of the microscope and its use by Antonie van Leeuwenhoek towards the end of the 17th century. However, the study of protozoa only started after the discovery of bacteria and the germ theory carried out by Pasteur, in the late 19th century (4). Around 1900 a Scottish pathologist of the British Army, Sir William Boog Leishman (1865–1926), while in India, was examining spleen smears collected from a soldier stationed in the town of Dum Dum, that died from splenomegaly and emaciation. Leishman observed ovoid bodies in the samples collected which he believed were degenerated forms of trypanosomes and therefore concluded the soldier suffered from a form of trypanosomiasis. He coined the name of this illness "Dum-dum fever". Not much later, an Irish doctor, Charles Donovan (1863–1951), had similar findings of ovoid bodies in spleen smears from deceased native Indians from fevers and enlarged spleens. However, Charles Donovan wasn´t convinced these findings were in fact trypanosomes, but perhaps bodies from a different genus (5). The first to recognize the bodies on lesions of patients in the oriental sore was doctor Piotr Fokich Borovsky (Петр Фокич Боровский) (1863– 1932) (6). Although his findings remained unnoticed because he published his observations in a not popular russian journal in 1898. The British medical doctor Ronald Ross (1857–1932) published an article in 1903, in which he concluded that the observed ovoid bodies were not degenerate trypanosomes, but a new protozoan organism and that the clinical picture of the cases was similar to that of kala-azar. Ross then proposed these newly discovered ovoid bodies to be named, *Leishmania donovani (5)*. The nature of the Leishman's bodies continued to be discussed for another year but by the end of 1904 the term *Leishmania donovani* was generally adopted (7). The French bacteriologist Charles Jules Henry Nicolle (1866–1936) was the first to describe related visceral leishmaniasis (VL) causing species *Leishmania infantum* in children in Tunisia suffering from splenic anemia in 1908 (8). Later, in the same year, in collaboration with his colleague Charles Comte (1869–1943), he found the same parasite in dogs in Tunis as well(9) and since then, dogs have been implicated as important reservoir hosts for VL (10). The pathologist James Homer Wright (1869–1928) was credited to the discover of *Leishmania tropica*. In 1903, he published a detailed description of the organism from a specimen of a wound from an Armenian girl and named this organism as *Helcosoma tropicum* but, in 1906, the German zoologist and physician Max Lühe (1870–1916) changed the name of this parasite into *Leishmania tropica* (5). Later, in 1914, the Russian physicians Wassily Larionovich Yakimoff (Василий Ларионович Якимов) (1870–1940) and Nathan Isaakovich Schokhor (Натан Исаакович Шохор) (1887–1941) suggested a change in the classification of the parasite and, although they did not imagine, that change became the standard for the next 60 years. The Russians suggested that *L. tropica* should

be categorized into the two subspecies *L. tropica minor* and *L. tropica major* based on the size of the parasites found in skin lesions (*L. t. minor*, smaller amastigotes; *L. t. major*, larger amastigotes) (11).

After the discovery of this new parasite, studies were started to try to identify how it is transmitted. These studies started as early as 1904, however, it was only in 1921 that the presence of a vector was implicated in the parasite's life cycle. New World Leishmanial parasites were first described separately by the Brazilian doctor Adolpho Carlos Lindenberg in skin lesions of patients with 'Baurú ulcers' from the State of São Paulo, Brazil, in 1909 (12) and the Italian physician Antonio Carini (1872–1950) together with his Brazilian colleague Ulysses de Freitas Paranhos (1880–1954) . It was originally thought that New World leishmaniasis and Old World leishmaniasis were the same, but in 1911 Gaspar Vianna found that the parasites in South America differed from those in Africa and India and created a new species, *Leishmania braziliensis (4)*.

In the last decades there was an increase in cases of leishmaniasis in endemic and non-endemic areas (figure 1). It is possible to consider several factors that influence these

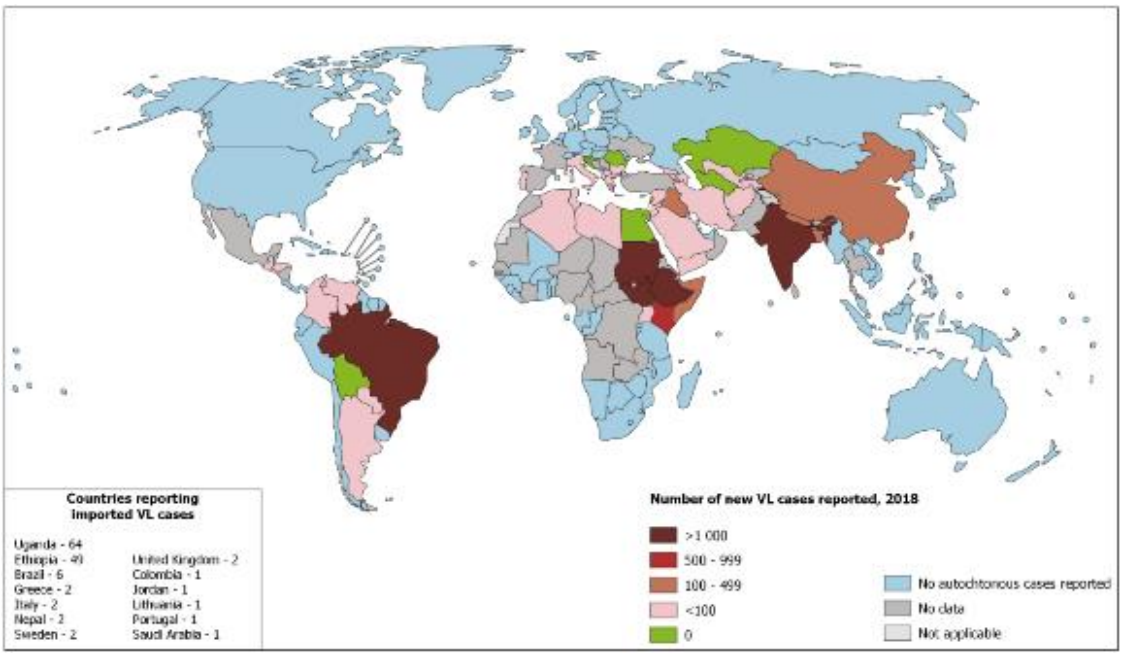

Figure 1 - Status of endemicity of visceral leishmaniasis worldwide, 2018. Adapted from (1)

numbers. Climate change and globalization are two factors that helps the spread of leishmaniasis to non-endemic areas (13). For example, over the years, the number of cases of leishmaniasis in international travelers has increased (14). There is also evidence that global warming will lead to an extension of the distribution of sand flies more northwards which could result in the transmission of leishmaniasis, in the future, to non-endemic regions (13). Another factor to be considered is the spread of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) and the immunodepression

associated with these conditions has resulted in the establishment of a number of new opportunistic parasitic infections throughout the world (15). There is a greater challenge in the diagnosis of these patients, since, as they are immunosuppressed, they do not have a reliable immune response against the parasite and, consequently, they have low titers of specific antibodies, making it difficult to use immunological methods of diagnosis. Asymptomatic patients also play an important role in the process of perpetuation of the disease because although they do not show symptoms, they act as a reservoir for the parasite and contribute to the transmission of the disease. The current diagnostic methods have a low performance in the diagnosis of these patients and the development of a way to identify and treat these asymptomatic patients is extremely important for the control and prevention of this pathology. In order to better understand the pathophysiology of the disease and the associated immune response, studies have been carried out for years in order to elucidate fundamental questions. Studies with animal models allow the longitudinal evaluation of the disease evolution and the immunological impact generated by the infection caused by the parasite. This type of approach makes it possible to assess very important issues and is very difficult to apply to humans. Among these animal models, the use of non-human primates helps to elucidate these issues. Because they share a genetic homology with humans, physiology and behavioral characteristics, this type of animal model offers a unique opportunity to assess issues in a species so close to humans but can still be maintained under controlled laboratory conditions (16). Leishmaniasis has been a major health problem in endemic areas for many years. Recent data from the World Health Organization (WHO) show that there are more than 1 billion people living in endemic areas at risk of infection. It is estimated that there are about 90,000 new cases of VL per year and the frightening number of deaths caused by this disease reaches 20,000 per year. More than 1 million cases of CL have been reported in the past 5 years (1). Although alarming numbers, leishmaniasis remains a neglected tropical disease.

The parasite

The leishmaniasis is caused by a protozoan of the genus *Leishmania* (order *Kinetoplastida* and family *Trypanosomatidae*). This parasite has a digenetic life cycle and infects vertebrate and invertebrate hosts. Thus, this protozoan can be found in the forms: flagellated paramastigote and promastigote, free or adhered to the digestive tract of invertebrate; amastigote, without flagella, mandatory intracellular in the cells of the mononuclear phagocytic system of vertebrate hosts. Amastigotes have spherical, spindle-shaped or oval morphology. The micrometric limits of their diameters are approximately 1.5 to 3 x 3 to 6.5 μm. The promastigote forms of the parasite are elongated, with a long free

flagellum emerging from the parasite's body in its anterior portion. They present a great variability in the measurements of the parasite body, whose diameters can be observed between 10-40 x 1.5-3 μm. Finally, paramastigotes are small and rounded or oval. The flagellum is short, externalizes in the anterior region of the body and the diameters vary from 5 to 10 x 4 to 6 μm (2). There are several species of *Leishmania* capable of causing disease in humans. These species are of epidemiological importance worldwide. In the Old World, the species that have the greatest expression are: *L. infantum*, *L. donovani*, L. *major*, *L. tropica* and *L. aethiopica*. In the New World, the main species that stands out are: *L. braziliensis*, *L. amazonensis*, *L. mexicana* among others (1). The parasite, in all its evolutionary forms, presents a series of surface molecules that are important in the host / parasite relationship. These molecules interfere in the process of virulence, pathogenesis and survival of the parasite. Among the flagellated forms of the parasite, a lipophosphoglycan complex (LPG) stands out, considered the most abundant molecule in this form of the parasite. Among proteins, the protease gp63 is very important and is found in both flagellated and fluted forms. The infective form for vertebrate hosts is the metacyclic promastigote, which is free in the anterior portion of the invertebrate host digestive tract and has intense motility as it has a very long flagellum (2).

The Vector

The vectors responsible for the transmission of the parasite are *diptera* known as “sand flies”. They are small insects (~ 3mm adults), golden-brown in color and with hair distributed throughout the body. Although they are commonly compared to mosquitoes, they are much smaller and do not produce audible sounds when flying.

The search for the form of transmission of leishmaniasis began shortly after the discovery of the parasite. It is known that *Leishmania* parasites are transmitted by the bite of infected phlebotomine sand flies, but studies have been carried out to reach this conclusion. These studies started as early as 1904, however, it was only in 1921 that the presence of a vector was implicated in the parasite's life cycle. In this year the Sergeant brothers, Edouard and Etienne had the first experimental proof of transmission to humans by sandflies belonging to the genus *Phlebotomus*, where it was demonstrated that the scarification of a suspension of sand flies on the skin of volunteers resulted in the appearance of lesions typical of Oriental sore(4). However, the results of this research were not accepted by the entire scientific community as proof that sand flies would be involved in the transmission of the disease acting as vectors. Finally, the currently accepted model has been demonstrated by the British-Israeli parasitologist Saul Adler (1895–1966) in 1941 when he successfully infected five volunteers with sand flies experimentally infected with *L. tropica* in the laboratory (5). Later it was found that the sand flies transmitting leishmaniasis in the New World belong

to the genus *Lutzomyia*. Meanwhile, recent studies shows that, at least, 56 *Lutzomyia* species and 42 *Phlebotomus* species have been involved in the transmission of leishmaniasis in the New and Old World, respectively (17).

Only females of the genus *Phlebotomus* (figure 2) perform blood meal because they need blood for maturation of their eggs (1). These *diptera* are present throughout the world in tropical and subtropical, arid and semi-arid areas and temperate zones (18). However, there is evidence that global warming will take this vector to the most northern areas of the globe and this may result in the transmission of leishmaniasis and other diseases to regions that are not currently endemic for these pathologies (13). Well implemented vector control through integrated vector management can play an important role in the elimination and control of leishmaniasis (1).

Figure 2 - Female phlebotomine sandflie - Adapted from (1)

Life Cycle

The vertebrate hosts are infected when metacyclic promastigotes are inoculated by females of the vectors when performing the blood meal. The insect's saliva has biological components that act as a vasodilator, anticoagulant and antiplatelet agent. These actions are important in the infection process of the vertebrate host and benefit the insect by ensuring the blood flow. *Lutzomyia Longipalpis* saliva, for example, contains the most potent vasodilator known, the maxadilan. In addition to these actions, the saliva of invertebrate vectors also acts in modulating the vertebrate host's immune system. Before its internalization by host cells, the metacyclic promastigote needs to survive the attack

mediated by the complement system, avoiding the formation of the membrane attack complex (MAC). One of the mechanisms responsible for this resistance is due, in part, to LPG that prevents the MAC from binding to the parasite. Gp63 also participates in the system evasion process by cleaving fractions of the complement system to prevent the formation of the MAC. Promastigotes are then endocitized by phagocytic cells (namely macrophages) using receptors on the surface of these cells. During the phagocytosis process, the cell intensifies its respiratory burst and naturally produces reactive oxygen species (ROS), for example, hydrogen peroxide, known to be very harmful to cell membranes. Again, gp63 plays its role because by cleaving fractions of the complement, it can use the fragments obtained as opsonizers to bind to macrophages without stimulating the increase in the respiratory process and consequent production of ROS. After internalization, the parasite is found inside the phagocytic vacuole, the phagosome. Then, there is the phenomenon of fusion between the phagosome and the lysosomes, which allows the passage of enzymes to its interior and the formation of the phagolysosome. Once again, the parasite needs strategies to evade this new attack and the LPG helps again as it can delay this fusion. At this point, differentiation into the amastigote form is essential for parasite survival. In this form, gp63 acts by cleaving lysosomal enzymes and ensuring the parasite's survival. By controlling the phagolysosome environment, amastigotes begin their multiplication process by simple binary division, and, without parasitic control, cell death is determined. Cell disruption releases the new amastigote forms that will infect new phagocytic cells using a mechanism similar to that mentioned when they are endocitated by new cells (2).

Another important factor for the establishment of the infection occurs during the blood meal. Next to the metacyclic promastigotes, a gelatinous substance is also deposited at the site of the bite, secreted by the promastigotes themselves (Promastigote Secretory Gel - PSG) (19). The active component of PSG is the glycan part of the Proteophosphoglycan filaments, the main constituent of this gel. The blockage caused by the PSG sand fly proboscite alters its form of feeding, increasing the number of bites and the time of meal (20). These changes in the insect's behavior favor the deposition of more parasites, PSG and saliva before the sandfly can perform the meal (21). Infection of the invertebrate host also occurs during blood meal. When ingesting the blood of the vertebrate host, the insect ingests macrophages infected with *Leishmania* amastigotes. 24 hours after ingesting the parasite and taking into account the change in temperature and pH of the medium, the amastigote forms differ in small ovoid, flagellated and with little mobility forms called procyclical promastigotes. After 48-96 hours of intense multiplication, differentiation occurs in long and thin promastigotes. These forms are attached to the middle and anterior portions of the insect's intestine using flagella and thus guarantee its development in this location. Between the end of the 4th or 5th day there are already viable metacyclic promastigotes for migrating

to the proboscis and contaminating new vertebrate hosts during the next blood meal (2). The figure 3 shows graphically the life cycle of this parasite.

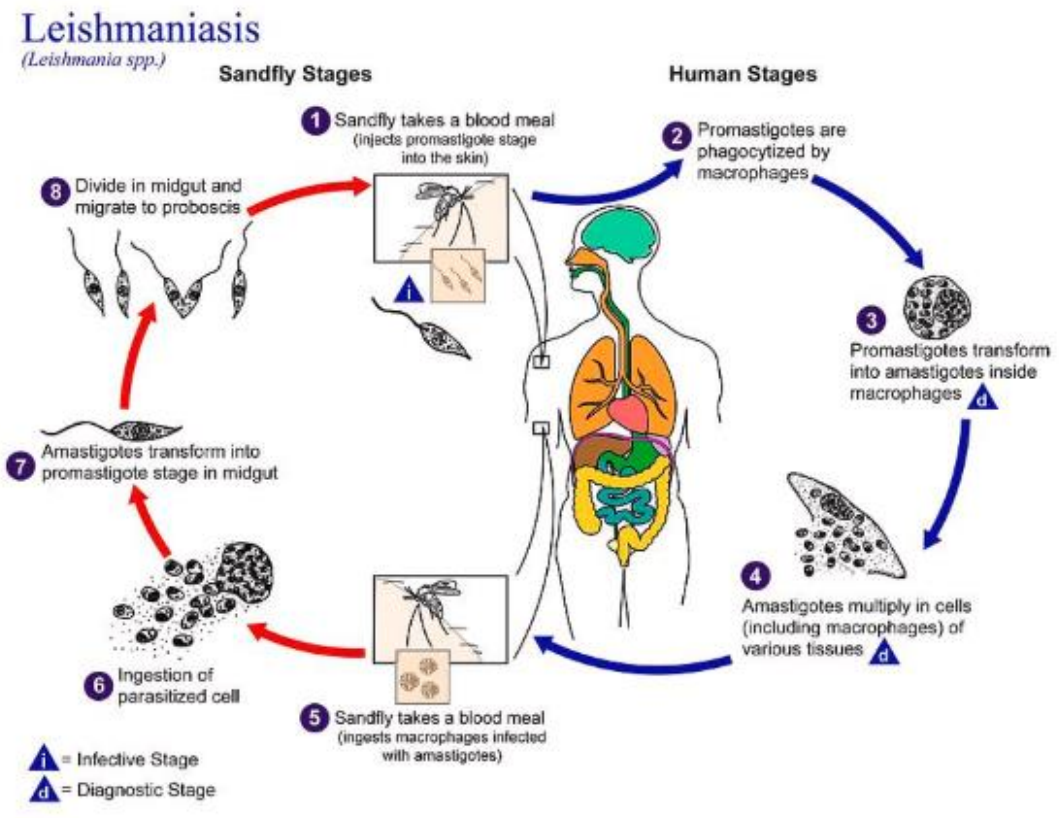

Figure 3 - Leishmania life cycle - Adapted from CDC (https://www.cdc.gov/parasites/leishmaniasis/biology.html) - Accessed in 14/04/2020.

The Disease

Leishmaniases are vector-borne parasitic diseases caused by at least 20 species of the genus *Leishmania* and are transmitted between vertebrate hosts by female sandflies. Leishmaniasis is primarily zoonotic disease where the parasite's animal reservoir plays a very important role in the propagation of these pathologies. An estimated 0·7–1 million new cases of leishmaniasis per year are reported from nearly 100 endemic countries. WHO lists leishmaniasis as one of the neglected tropical diseases for which the development of new treatments is a priority. It is important to remember that leishmaniasis cannot be characterized as just a single disease, but as a set of diseases that can occur depending on the species of parasite involved, the characteristic of the vector involved, or even on the immune response of the vertebrate host. These differences lead to diseases that vary in severity between skin lesions that heal spontaneously to a visceral form that can lead to the death of the host (22). With three main manifestations of the pathology, ineffective treatment and misdiagnosis leads to difficult management and control of the disease.

Transmission

The forms of transmission of leishmaniasis vary between zoonotic - animal-vector-human, and anthroponotic - human-vector-human. These forms vary according to the geographic region or according to the species of the parasite. Canines (dogs), rodents, and lagomorphs (rabbits, hares) are reservoir hosts that perpetuate zoonotic leishmaniasis. The classic form of transmission involves the mechanism mentioned in the parasite's life cycle; however, there are other possible forms of transmission although these are rarer. The world has changed a lot, especially in the last few centuries, and the parasite has accompanied this change. Nowadays it is known that social events, such as business, tourism or wars, can directly influence the increase in disease transmission.

During the last years it was noticed an increase in the number of cases of all forms of leishmaniasis throughout the world and this fact led many experts to consider leishmaniasis as an emergent disease in some areas and in others as a re-emergent one (23). Some factors influenced this increase in cases, from exposing people to endemic areas to procedures used to try to save lives. For example, Brazilian soldiers are sent on the jungle war training course near Manaus, Amazonas and over the years many have become infected (24), the same case is reported to American soldiers who carry out their jungle training in Panama (13). The number of cases also increases among people who travel to endemic areas for business or tourism. The globalization associated with the ease of movement between countries that we have today exposes many people from non-endemic areas to the parasite (25). An aggravating factor in this situation is the transport of animals during these trips. It is common for a family to take their pet dog on a trip to an endemic area. The disease incubation period in dogs is variable and symptoms may appear only after returning to the country of origin (13). This means that infectious dogs may go unnoticed for long periods and could serve the sources of new infections back home.

Other factors to be considered in the transmission of leishmaniasis are medical procedures. Although it is more difficult than classical transmission, blood and organ transfusions can be a form of contamination. Screening for leishmaniasis is not carried out in blood banks and the risk of contamination exists in endemic and non-endemic areas. One study showed that of 506 healthy French blood donors 76 had a positive leishmanial serology in a specific Western blot test and parasites were isolated from two (26). Even in non-endemic areas the risk exists. A few years ago, a lady contracted VL in Scotland when she received a blood transfusion during an operation. It was later discovered that the blood was of an Asian sailor who was in transit in the country (13). Organ transfusion is another form of transmission and has an aggravating factor, the immunosuppressive medication that must be administered to the patient after the transplant. This type of medication directly interferes

with the immune response to the parasite. The same phenomenon occurs in patients with HIV, the deficiency of the immune response increases the transmission of the disease among these patients. Lastly, there is also the possibility of transmitting the parasite horizontally or vertically. There are reports of disease transmission through semen (27), shared needle among drug users (28) and congenital transmission (29).

Reservoir Host

There seems to be no doubt that all *Leishmania* species have a zoonotic origin (13). Dogs are considered the largest animal reservoirs of this parasite (30). A fact aggravated by more than half of these animals showing no symptoms (31) and, thus, perpetuating the disease and putting the nearby human population at risk of infection. Some species of *Leishmania* (*L. donovani* and *L. tropica*) have adapted and humans have become the largest reservoir of these parasites. In addition to these, other mammals also play a role in reserving the parasite: rodents, hyracoides, hares, sloths, marsupials and even horses are possible animal reservoirs (22).

Human beings can play the role of reservoir for *Leishmania* species other than cited adapted ones. Many infections can run asymptomatically or be reactivated many years after the supposed cure if the patient has some depression of the immune system. There are reported evidences that patients that achieve apparent cure with elimination of the parasite can have apparent relapses after 1 to 30 years (32). In addition, sick patients also function as a reservoir for the parasite, especially if they are not treated. In all those cases, human beings work as the reservoir host for the parasite.

Pathological Forms

The leishmaniasis can be presented in different forms and can be considered symptomatic or asymptomatic. Different species of *Leishmania* can cause the same clinical manifestations. The most common forms of the disease are cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis. In addition, it exists another manifestation called post-Kala Azar dermal leishmaniasis (PKDL) and this is only happening in patients who have had VL previously. The clinical manifestations of leishmaniasis in humans is determined by the complex interactions between the virulence characteristics of *Leishmania* species, treatment and the immune response of the host.

Cutaneous leishmaniasis

In a clinical and epidemiological context, this is the most frequent pathological presentation among all possible ones. Characterized by the formation of a papule that develops from weeks to months and, subsequently, slowly ulceration of the site occurs. The lesions usually occur at the location of the vector's bite, and several lesions usually suggest several bites.

Therefore, injuries usually occur in normally exposed places on the body, mostly arms, legs and face. In general, patients are systemically well, and the lesions, although sometimes itchy, do not generate the pain that might be expected from their appearance (22). Cutaneous leishmaniasis is not life-threatening and these lesions are often self-healing or can be contained with proper treatment, but it can still lead to substantial cosmetic morbidity, social stigmatization and psychological effects. When the ulcers heal, they invariably leave permanent scars, which are often the cause of serious social prejudice (1).

About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia. In 2018 over 85% of new CL cases occurred in 10 countries: Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran (Islamic Republic of), Iraq, Pakistan, the Syrian Arab Republic and Tunisia. It is estimated that between 600.000 to 1 million new cases occur worldwide annually (1). It is important to note that lesions are commonly mistaken for boils, therefore, we can think that these estimated values may be even higher. Rare variant forms of CL, such as diffuse cutaneous leishmaniasis (DCL), can occur and depend on the patient's immune response. DCL develops as multiple widespread non-ulcerating papules and nodules resembling lepromatous leprosy. The condition reflects the absence of a cellular immune response, and the dermis is typically and heavily infiltrated with parasites.

The immune response related to CL is mediated by cells, mainly lymphocytes. After infection, the macrophages present *Leishmania* antigens to $CD4^+$ T lymphocytes (which can be divided into at least two populations: $CD4^+$ T lymphocytes with subtype T helper profile 1 (Th1) and $CD4^+$ T lymphocytes with subtype T profile 2 (Th2). Th1 plays an important role in host resistance, while Th2 is associated with susceptibility to infection. Depending on the active profile, the disease will follow a different course. While the activation of Th1 leads to resolution and resistance to the disease, the activation of Th2 confers susceptibility and progression of the pathology. Therefore, depending on the balance of the Th1 / Th2 response, the disease can solve itself or progress (33). Although the humoral response is present, the level of antibodies is generally low. After curing, the level of antibodies decreases and may even become negative in months after that. However, there is a variation in the pattern of the humoral antibody response of the IgG subclass, with an increase in IgG1 and IgG3 in the CL (2).

Mucocutaneous leishmaniasis

It is a pathology that starts its course with CL, however the disease causes secondary lesions and affects the mucous membranes causing infiltrated ulcers that can lead to partial or total destruction of the affected tissue. The most affected areas are the mucous membranes of the nose, mouth and throat (1). This destruction process is a slow process with a chronic course,

which has, in most cases, as the first sign of mucosal involvement the manifestation of an erythema and a slight inflammatory infiltrate of the nasal septum. This generates constant coryza and, subsequently, an ulcerative process that begins in the septum, then reaches the vestibule, the wings of the nose, the floor of the nasal fossa, the soft palate and the uvula, from there down to the pharynx, which can compromise the larynx and the trachea. In many cases it even destroys the entire cartilaginous structure of the nose (2). This process can take decades to happen and usually results from the lack of effective treatment for a CL. Although some patients may have subclinical infections and have no previous lesions (34). These serious injuries can cause difficulty in breathing, eating and speaking. At this stage, secondary respiratory infections are frequent and can lead the patient to death (2).

In general, the species that cause this type of disease are limited to the New World. Studies show that more than 90% of cases occur in Bolivia (especially), Brazil, Ethiopia and Peru (1).

The immune response to MCL is similar to that of CL, however, an exacerbated response is observed. Although there is a need for a Th1-mediated response to cure the disease, an exacerbated Th1 response and a high number of $CD8^+$ cytotoxic T lymphocytes are associated with the severity of the disease. The consequence of this response is development of MCL (35). Studies show that when disease progresses from early (non-ulcerated) lesions to late (ulcerated) lesions the ratio between $CD4^+$ and $CD8^+$ T cells changes, and more $CD8^+$ T cells are found in patients with ulcerated lesions (36). As in CL, the humoral response exists with low antibody values, although in this case they may be slightly increased, and, also as presented in the cutaneous form of the disease, there is a predominance of the IgG1 and IgG3 subclasses in this pathology (2).

Visceral leishmaniasis

Visceral leishmaniasis, also knew as Kala-Azar, is the most severe form of the clinical manifestations caused by *Leishmania* infection. Caused by parasites of the *donovani* complex - *L. donovani* in the Indian subcontinent and East Africa and *L. infantum* in the Americas, Europe and North Africa (37). Table 1 compares both species. Unlike the other pathologies presented so far where the parasite is in the patient's dermis and mucous membranes, VL is characterized by the migration of the parasite to the host's viscera. The clinical manifestations of this pathology range from asymptomatic to severe presentation with worrying visceral involvement. The disease has a systemic infection, of chronic development characterized by the presentation of irregular fever, splenomegaly and hepatomegaly, anemia, thrombocytopenia and hypergammaglobulenemia. If the patient is not treated, the state of weakness leads to cachexia and death. However, there are reports of people who contract the infection and never develop the disease; people who recover

spontaneously or, even, people that control the infection and remain asymptomatic (2). Actually, only a minority of infected humans develop this disease: most are infected at sub-clinical level (38). Although, asymptomatic patients may develop symptoms if they have an episode of severe immunosuppression. The onset of visceral leishmaniasis can be acute or insidious, and the incubation period is between 2 weeks and 8 months. Without treatment, the disease is typically fatal within 2 years as a result of secondary bacterial infection or severe anemia (22).

In this disease, the cells of the mononuclear phagocytic system affected are mainly those of the spleen, liver, lymph nodes and bone marrow. However, in more advanced stages of the disease, organs where the parasite is not found are rare. Some more specific changes occur in the splenic, hepatic and hemocytopoietic tissues. Splenomegaly is the most characteristic finding of VL. Although in the initial phase of the disease it may not occur or be slightly accentuated in the established and chronic disease, it is an invariable characteristic. Hepatomegaly is another important finding in this disease and is due to the fact that hyperplasia and hypertrophy of Kupffer cells occur, usually these cells are densely parasitized. Bone marrow is, in general, hyperplastic and densely parasitized. This leads to a change in the overall production of cells and, following the severity of the disease, to aplasia (2).

It is estimated that between 50,000 and 90,000 new cases of VL occur annually. This characterizes this disease as one of the parasitic diseases with the highest mortality potential in the world. In 2018, more than 95% of new cases reported to WHO occurred in 10 countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan.

The immune response again involves stimulation of $CD4^+$ T lymphocytes. And, likewise, it is related to the mediation of Th1 and Th2 response leading to disease control or severity, respectively. VL is characterized by the inability of macrophages to destroy amastigotes, leading to the maintenance of the disease. In this disease, however, the humoral response is quite marked by a very high IgG production. However, as the activation of B lymphocytes is polyclonal, most of the immunoglobulins produced are nonspecific (2).

Table 1 - Leishmania donovani complex species and their characteristics - Adapted from (2)

L. DONOVANI	Anthroponosis - responsible for VL at all ages and for PKDL in adults.	India, Nepal and East Africa.
L. INFANTUM	Zoonosis - Occasions VL mainly in children. Canids are the main reservoirs of the parasite.	Americas, Mediterranean and North Africa.

Post Kala-Azar Dermal leishmaniasis

Post Kala-Azar Dermal leishmaniasis is a late complication that affects patients who had VL caused by *L. donovani* and were treated and cured. The clinical presentations of this pathology are the appearance of hypopigmented papules, macules and nodules that usually start near the mouth and spread gradually throughout the body (22). Although it presents a cutaneous manifestation, it is not considered CL. PKDL usually manifests between 6 months and 5 years after the cure of VL and, although cutaneous parasitism is intense, there is usually no sign of parasitism in the viscera and bone marrow. These injuries can take months or years to disappear (2). Although the mortality caused by PKDL is not high, it is still a stigmatizing disease that carries a significant socioeconomic burden, further amplified by a reluctance to obtain treatment or due to noncompliance (39).

About the immune response, in post-kala-azar dermal leishmaniasis, parasites seem to persist in the skin after treatment, and, whereas systemically the Th1 response dominates, locally in the skin a Th2 profile dominate (22).

Epidemiology

Current data show that there are more than 1 billion people living in endemic areas and it is estimated that about 20,000 deaths occur each year, caused by leishmaniasis (1). In 2012 there were already cases of leishmaniasis reported in 98 countries on 5 different continents (40), however, if we consider the socioeconomic, climatic and health changes between 2012 and 2020, we can imagine that these numbers may be higher. Some factors actively contribute to the increase in leishmaniasis cases in the world and Dujardin (41) believes that the most important are the environmental changes, the immune status of the hosts and the ineffective treatment. In addition to these we can consider the role of other events, such as uncontrolled migrations caused by economic pressures or wars. In addition, plane tickets are more accessible than they used to be and many people, in search of sun and exotic places, end up in endemic areas and are infected with *Leishmania*. This also occurs with professionals who go to work in endemic areas and return home infected (13). Figure 4 shows the cases of CL reported to WHO in 2018.

Risk Factors

The progression of the disease is influenced by several factors. Immunosuppression, genetic factors, co-infection with HIV and / or helminths, malnutrition, poverty and exposure to the parasite are very important risk factors for the development of the pathology and its severity. Immunosuppression in leishmaniasis can be caused by the disease itself, but it can be exacerbated if there is co-infection with other pathogens such as the HIV or helminths

or, still, malnutrition **(42)**. In addition, the use of immunomodulatory drugs or immunosuppressive drugs after transplantation also play an important role in depressing the host's immune response. In all these cases, a fulminant activation or reactivation of the disease may occur if the host is immunosuppressed. In immunocompromised patients, *Leishmania* parasites can persist many years, even after treatment, since there appears to be no sterile immunity **(43)**. In addition, immunosuppressed patients have an atypical (more severe) presentation of the disease (2).

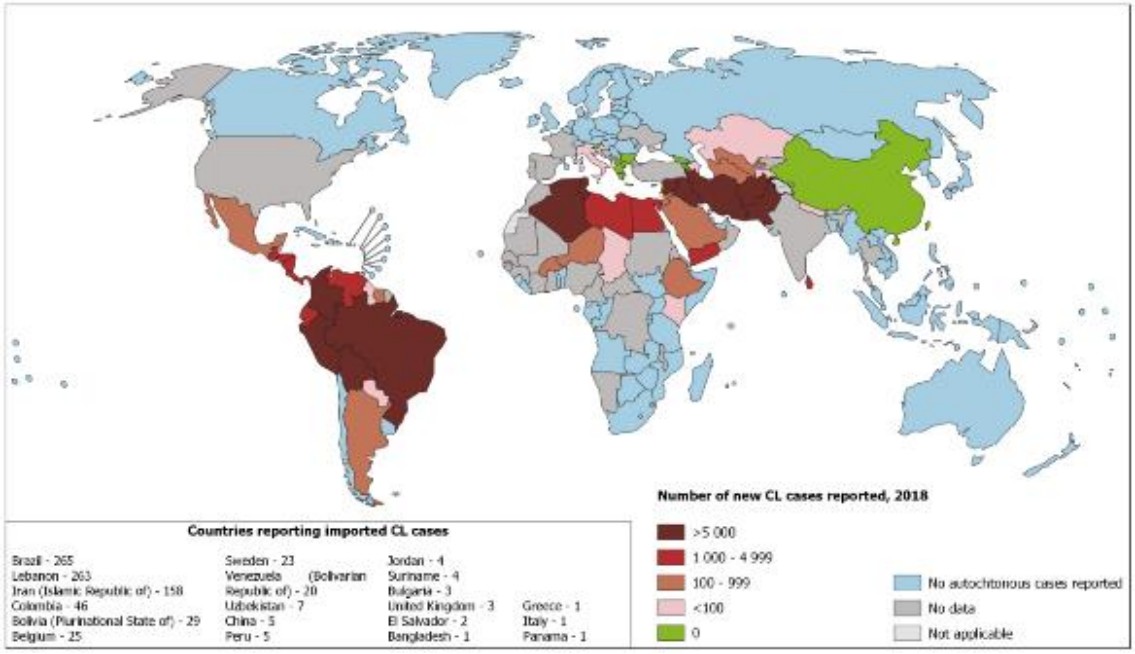

Figure 4 - Status of endemicity of cutaneous leishmaniasis worldwide, 2018. Adapted from (1).

Another risk factor associated with leishmaniasis is exposure to the parasite. The rates of sand fly biting may vary from almost constant to extremely sporadic depending on the region and the habits of the individual (13). It is has been described, experimentally, in mices that the bites of uninfected sand flies could provide some protection (44) against subsequent infection. This suggests that high levels of sand fly bites in endemic areas could be protective and result in asymptomatic infections in man. Poverty increases the risk for leishmaniasis in many ways. Poor housing and peridomestic sanitary conditions may increase sandfly breeding and resting sites, as well as their access to humans. Crowding of many people into a small space may attract peridomestic anthropophilic sandflies by providing a large biomass for blood-meals. Poor nutritional status resulting from a poor diet increases the likelihood that visceral leishmaniasis infection will progress to clinically manifest disease. Recent experiments in protein energy-, zinc- and iron-deficient mice suggest that this effect is mediated primarily through functional failure of the lymph node barrier and increased early visceralization of the parasite (45).

Treatment

Unfortunately, there are still no vaccines available for human leishmaniasis. There are, however, vaccines for dogs and vaccination of these animals on a large scale, especially in endemic regions, can help control the progress of the disease. This efficacy is still very controversial because the vaccinated dogs, although they do not have the disease, still have the infection, therefore, they still participate in the process of transmission of the pathology as reservoirs of the parasite. There are currently some recommended drugs for the treatment of leishmaniasis. The most used are: antimony, miltefosine, amphotericin B, pentamidine and paromomycin. Each of these drugs has unique advantages in the treatment, however, a common feature of all these drugs is the toxicity generated with prolonged use. The study of possible drug interactions, potential for parasite resistance, potential for drug failure and possible unexpected side effects is extremely important for us to be able to find the best way to treat the disease. A greater investment in the discovery and development of a vaccine would be a great help in combating this disease, but, as most cases occur in developing countries, there are not many investments directed at this issue.

Prevention and control

The prevention and control of leishmaniasis is a major public health challenge. As human vaccines do not yet exist, disease control is based on effective treatment of patients. Taking into account the fact that the drugs currently used for the treatment of leishmaniasis are very toxic, an effective and safe treatment can only be applied if there is an early and reliable diagnosis. This way the patient would not need to take the medication for a very long time. Disease prevention involves the most basic concepts. It is a parasite transmitted by a mosquito, so protection against the vector is the easiest way to prevent contagion. Avoiding exposure to the parasite is still the best way to prevent the disease. Effective treatment of infected patients and dogs also contributes to disease prevention by reducing the parasite's reservoir.

Diagnostic

There are several ways for the differential diagnosis of all clinical forms of leishmaniasis. In this work, the ways of diagnosing VL will be addressed.

Clinical Evaluation

The clinical evaluation is based on the signs and symptoms presented by the patient associated with the history of residing in an endemic area. The most common manifestations of VL are persistent fever, splenomegaly, weight loss and fatigue. These signs

are very common in other pathologies and, therefore, specific laboratory evaluation is necessary to confirm the diagnosis. The presence (isolated or combined) of anemia, thrombocytopenia, leukopenia or polyclonal hypergammaglobulinemia reinforces the suspicion of the disease (45).

Direct parasite detection

This form of diagnosis is based on direct research of the parasite in preparations of material obtained from aspirate of the spleen, lymph nodes or bone marrow for making a smear on a slide. The material is stained, and the reading is performed under an optical microscope. It is an inexpensive and quick technique although the material collection is invasive and needs to be performed by highly qualified professionals and this ends up limiting the use of this technique to laboratories that have a certain infrastructure. Correct diagnosis requires well-trained staff and that is an extra challenge in countries where leishmaniasis are rarely seen. Although the specificity of microscopy is high, among the materials used the sensitivity of the test varies, with the spleen aspirate being the material with the highest sensitivity (93-99%), bone marrow (53-86%) and lymph nodes have the lowest value of sensitivity (53-65%). One of the strategies to increase this sensitivity is the culture of the parasite through the material collected and the subsequent examination (45). Another limiting factor of this technique is the impossibility of carrying out tests on a large scale because the tests are carried out on a case-by-case basis. Parasite cultures can also be used as a form of diagnosis although they are time-consuming, expensive and laborious they are still used in some laboratories as a way of diagnosing VL. In addition, the biological materials used in this technique are difficult to obtain and the collection process involves many risks of post-collection complications.

Xenodiagnosis

Xenodiagnosis consists of exposing a patient to a specific vector and subsequently assessing whether the vector is contaminated with the disease investigated. This technique is rarely used in clinical practice but has an interesting feature to demonstrate the tested patient's ability to transmit the disease. An article published in 2020 (46) reports on the use of xenodiagnosis to assess leishmaniasis. In this article it is reported that, among sixty-two tests performed and more than five thousand dissected mosquitoes, 4 patients had a positive result. Among these, one patient was co-infected with HIV / *L. infantum*, one patient was immunosuppressed due to multiple myeloma and the others were immunocompetent with untreated active VL. This test shows great potential, including in the diagnosis of asymptomatic immunosuppressed patients. This technique brings as a disadvantage the issues of being a time-consuming technique, the need for trained

personnel to carry out the identification of parasites in sandflies and to have an almost exclusive use in research work.

Molecular Methods

Molecular methods aim to identify the parasite's DNA in patient samples and have a sensitivity far superior to direct parasite detection techniques. The use of molecular methods for diagnosing VL has been increasing and recent studies have demonstrated a high sensitivity (> 90%) in polymerase chain reaction (PCR) tests in spleen aspirate and bone marrow (47). Quantitative real-time PCR (qPCR) is a molecular method that makes quantitative analysis of the parasite's DNA possible. This technique presents faster, more effective, reproducible, and sensitive results regarding the DNA present in the analyzed sample. However, these are expensive techniques and need a larger laboratory infrastructure, which ends up restricting their use to some locations and excluding endemic areas that do not have funds to use these techniques.

Recent advances in the molecular area can help these areas that do not have enough infrastructure to perform PCR. The development of the loop mediated isothermal amplification (LAMP) of DNA technique aims to assist the use of molecular techniques in the field. LAMP is based on the use of only one enzyme (Bst DNA polymerase) that is capable of amplifying, in 40 minutes, a large amount of DNA and, because it does not need a thermocycler as it is based on just one temperature, it has great potential to be used in places with less infrastructure. Studies demonstrate that the use of this technique in the diagnosis of VL has a level of sensitivity / specificity similar to molecular methods based on PCR (47). However, there are disadvantages related to molecular methods. As these methods are based on the genetic material of the parasite present in the analyzed samples, the material with the easiest access (blood) has low sensitivity values because the parasitic load in the blood is not always sufficient to present positive results. In this way, falsely negative results are common. As the parasite presents tropism for certain tissues of the body, the other biological materials that have better sensitivity values and can be used in these techniques are very difficult and dangerous to collect. Collecting bone marrow and / or spleen material is always a complex procedure and involves too many risks of post-collection complications.

KATEX

Kala Azar latex agglutination test. This methodology proposes a non-invasive way to detect patients infected with *Leishmania*. Unlike other tests, this technique uses a fresh urine sample as material. This methodology is based on latex spheres coated with *Leishmania* antibodies which, upon contact with the patient's urine to be screened, generate agglutination (48). Comparative studies carried out demonstrate that this technique has sensitivity and specificity similar to other techniques, including in patients co-infected with

HIV (49). Actually, this technique is very interesting in the HIV coinfection context due the fact that these patients have a low antibody titer and the technique is based in the search of the antigens. Its use can be very interesting because it uses an easily accessible biological material with non-invasive collection. It is worth mentioning that the methods considered gold standard for the diagnosis of VL need noble biological material, difficult to obtain and with risks involved in the collection.

Serological methods

Serological methods are based on the detection of antibodies in the patient's blood or serum. Historically, several immunological techniques have been and are still used for the diagnosis of VL. In this work we will cover the following techniques: Indirect Fluorescence Antibody (IFAT); Direct Agglutination Test (DAT); Rapid Diagnostic Test (RDT) and Enzyme Linked Immunosorbent Assay (ELISA). It is important to remember that serological tests have a common disadvantage: low sensitivity in identifying positivity in coinfected-HIV and asymptomatic patients and the presentation of positive results for patients who have a history of cured VL (50). In contrast to these disadvantages, immunological methods are able to screen a large number of patients at the same time. This is very important in endemic areas that need a large number of tests to assess the local population context.

IFAT

The indirect immunofluorescence technique is based on the use of fluorescence to visualize the patient's antibodies. The patient's serum is added to the slides that have parasite antigens and, in a second step, a second antibody, marked with fluorescent molecules and directed to the constant region of the first antibody, is added to the slide. The reading is performed in an immunoflorescence microscope to visualize the presence of marked antibodies. A comparative study between techniques showed that IFAT has good sensitivity (87.5%) and specificity (95.8%) (51). However, the need for specific equipment and well-trained technicians is a limiting factor in the use of this technique.

DAT

As it is a simple, accurate and efficient test, the direct agglutination test is considered a serological test that can be performed in areas that do not have a very advanced laboratory infrastructure. This test is based on the direct agglutination between *Leishmania* promastigotes and specific anti-*Leishmania* antibodies present in the patient's serum, resulting in the agglutination of these promastigotes (52). As for the performance of the technique, a meta-analysis carried out recently demonstrated a sensitivity and specificity of

96% and 95%, respectively (53). It has limitations because it has a high incubation time (18 hours) which leads to long-term results.

RDT

The recent development of *Leishmania* recombinant proteins has made it possible to create a rapid diagnostic test. The rK39 protein is the most used in this type of tests and is composed of 39 amino acids originating from a parasite protein. This technique is based on immunochromatography and can be applied in any region without the need for laboratory infrastructure. Furthermore, it is a very quick and simple execution technique and has a very low cost. Studies carried out in the Indian subcontinent region have shown excellent sensitivity (97%) and specificity of 90%. Another recombinant protein has been studied and used in the diagnosis of VL, rK28 demonstrates sensitivity values like rK39 (50).

ELISA

The ELISA is a versatile serological technique that allows the detection and quantification of antibodies from any biological fluid (eg: serum, blood, urine). This technique can be used to search for parasitic diseases or other pathologies. In the context of leishmaniasis, ELISA tests are widely used for diagnosis and this technique consists of adding the material to be researched in a plate that has several wells containing *Leishmania* antigens. In a second step, secondary antibodies conjugated to an enzyme are added and they will bind to the patient's antibodies that remained bound to the plate antigens. After this stage, a substrate is added and promotes a change in color of each well and, after some time, the enzymatic reaction is stopped by the addition of an acid. The absorbance of each well is measured, and the result is obtained. This technique has limitations such as the fact that there is a need for a more complex laboratory infrastructure; the cross reactivity with other pathologies and the influence of the temperature because it is a technique that uses the enzymatic action. However, it allows the screening of many patients at the same time as each plate has 96 wells. The sensitivity and specificity of the tests varies according to the antigens used.

Immunological methods are very important, but they are still far from perfect. That is why the study and development of new forms of diagnosis and new markers is very important. In this work we seek to elucidate some things related to the use of markers for the diagnosis of leishmaniasis. Some of these markers are already used on a large scale, however the evaluation of the performance of these markers longitudinally in an animal model of non-human primates can provide interesting information about these markers.

Objectives

Considering all the known challenges in the diagnosis of visceral leishmaniasis, the objectives of this work are:

- Evaluate the performance of four different antigens (SPLA, SALA, rK28 and rK39) used in the diagnosis of VL in an animal model composed of asymptomatic non-human primates. In this way, the kinetics of the immune response in asymptomatic patients will be evaluated in order to try to identify a good marker for the diagnosis of these individuals. For this, the serum of rhesus monkeys, infected with a known quantity of parasites and later evaluated longitudinally, will be used. This type of study is very difficult to carry out with humans and it is expected that the results obtained can be correlated with humans due to the genetic similarity with the studied group. Understanding how these markers behave during the infection is imperative in the attempt to identify an ideal marker for the diagnosis of leishmaniasis, especially in the context of asymptomatic patients. It is expected to understand the immune profile of the response obtained and to correlate this profile with a natural infection.
- The objective of this work It is also to evaluate whether the profile of the immune response varies in treated animals. Among the animals studied, some were treated and it is sought to understand if there is any difference in the immune response of these animals. Some work with these antigens has already been carried out in the context of human VL in countries of the Indian subcontinent.
- This work also aims to evaluate the performance of these antigens in detecting active infection in humans in the context of new world leishmaniasis.

Materials and methods

This study was made at the Instituto de Investigação e Inovação em Saúde (I3S) and at the Faculdade de Farmácia, Universidade do Porto for the human sample's analysis. The monkey samples analysis was made at Faculté de Médecine, University Paris Descártes, Paris – France. Statistical analysis for this study was completed using Graphpad Prism 8 (Graphpad Software, San Diego, California USA).

Samples

This study was conducted in two groups of samples from monkeys infected with *L. infantum* and in two groups of samples from humans, one group of positive samples and one negative

control group. Even after the infection all the monkeys remained asymptomatic until the day of euthanasia.

Regarding the monkeys, two distinct groups of samples were evaluated. These groups were divided because these animals lived at different times and the studies were carried out in different institutions. In this way, the groups will be categorized into sample group I and sample group II.

The first group consisted of 7 rhesus monkeys, infected with *L. infantum*. Each monkey was infected with a load of 2 x 10^7 parasites per kg. Monkeys have not undergone any type of treatment from the time of infection to the time of euthanasia. During this period blood samples were taken at defined time intervals for each specimen. There is a day 0 sample for each specimen and these samples were used as the negative control for each monkey. The monkeys were sacrificed in three different time points to cover acute and chronic phases. One animal was euthanized with 11 days of infection, 2 animals were euthanized with 28 days of infection and 4 animals were euthanized on day 250. After infection, blood samples were taken on several moments and in this study 4 different time points to evaluate the antigens were selected and the table 2 shows the selected sample collection scheme carried out for this group of monkeys. All the samples were collected using EDTA tubes and were centrifuged and aliquoted in Fontenay-aux-Roses. After that they were sent, frozen, to the Faculté de Médecine, University Paris Descártes, Paris – France.

Table 2 - Selected sampling scheme - first monkey group. Animal identification on lines and days of collection on columns where D means the day after infection where the sample was collected.*

Animal	Do	D14	D21	EUTHA
28				D: 28
82				D: 250
114				D: 11
264				D: 28
268				D: 250
270				D: 250
274				D: 250

The second group consisted of 8 rhesus monkeys infected with *L. infantum*. Each monkey was infected with a load of 2 x 10^7 parasites per kg. Of these, 2 specimens did not receive any type of treatment (3106 and 3107), 3 received treatment with miltefosine (3100; 3101; 3102 - 5mg/Kg, administered orally, for 21 days) and another 3 received treatment with a test compound (3103; 3104; 3105 - 10mg/Kg, administered orally, for 21 days) after 1 day

of infection. During this period blood samples were taken at defined time intervals for each specimen. There is a day 0 sample for each specimen and these samples were used as the negative control for each monkey. After infection, samples were collected in a complementary manner to have the same scheme to both subgroup of treated monkeys with different drugs. One monkey of each subgroup was sacrificed at the same time points to evaluate the difference between the treatments. The monkey 3100 and 3103 were sacrificed 1 month post infection, 3101 and 3104 – 2 months post infection and 3103 and 3105 – 3 months post infection. The non-treated monkeys were sacrificed in two different time points: 3106 – 2 months post infection and 3107 – 3 months post infection. In this study 4 different time points were selected, considering the time of euthanasia for each subject, to evaluate the antigens and the table 3 shows the selected sample collection scheme carried out for this group of monkeys and the complementary time points between groups. All the samples were collected using EDTA tubes and were centrifuged and aliquoted in Lyon. After that they were sent, frozen, to the Faculté de Médecine, University Paris Descártes, Paris – France.

Table 3 - Selected sampling scheme – second monkey group. Animal identification on lines and days of collection on columns where D means the day after infection where the sample was collected.*

Animal	D0	D11	D15	D18	D25	D61	D62	D96	D97
3100									
3101									
3102									
3103									
3104									
3105									
3106									
3107									

1 month
2 months
3 months

***color matching - euthanasia points after infection**

To evaluate the selected antigens in the context of new world leishmaniasis two groups of human serum were selected, one group being collected from patients with diagnosed leishmaniasis (n = 17) and the other group from healthy volunteers without any history of leishmaniasis (n = 18).

Thus, the first group contained 17 samples from confirmed leishmaniasis patients (symptomatic and positive by real time PCR). This group came from the UFMG University, in the state of Minas Gerais, in the southeastern region of Brazil. No additional information on the geographical origin of the patients, nor the gender, age, how long since the diagnosis of leishmaniasis was provided.

A second group of samples were collected from Brazilian volunteers who, at the time of collection, lived in Portugal. The group consists of 18 adults with an average age of 29 years (min: 20 years; max: 41 years; median: 29 years) who had arrived in Portugal, on average, 1 year before blood collection (min: 0 year; max: 5 years; median: 0 year). Participants come from 13 different cities, which are distributed in 9 of the 26 states in the country and from the federal district covering all 5 regions of Brazil (North, Northeast, Midwest, Southeast and South). All volunteers answered a questionnaire and in this they all informed that they do not have any known disease, nor that they have had a previous diagnosis of leishmaniasis.

Ethical context

Monkey Samples

Subgroup I

The entire process from infection to euthanasia was conducted by Centre CEA Paris-Saclay/Site de Fontenay-aux-Roses, Fontenay-aux-Roses – France, following all the process previously approved by the competent ethics committee.

Subgroup II

The entire process from infection to euthanasia was conducted by Cynbiose - National Veterinary School of Lyon (VetAgro Sup - Campus Vétérinaire), Marcy-l'Étoile - France, following all the process previously approved by the competent ethics committee.

Human Samples

Leishmania human positive samples were approved by the UFMG Ethics Committee on Human Research (logged under protocol number CAAE – 2343114.9.0000.5149). Negative human samples were collected based on Volunteers' signed consent.

Antigens

The four antigens evaluated in this study for ELISA diagnosis were: soluble *L. infantum* promastigote antigens (SPLA), soluble *L. infantum* amastigote antigens (SALA) and two highly conserved recombinant *L. infantum* proteins rK39 and rK28. So far, rK39 RDT assay is used as a reference standard for the diagnosis of VL but its inability to discriminate between clinical and subclinical infection in endemic population drives the attention towards more specific and sensitive novel antigens (54). The rK39 antigen is widely used for diagnosis of African, American, Indian, Nepalese, Sudanese, and Brazilian VL with a sensitivity of rK39 has been reported as 98% with a specificity of 89% (55). Other studies show that rK39 and rK28 antigens have similar sensitivity and specificity and rK28 can also be used as a serodiagnostic tool in VL diagnostic (54).

Soluble Promastigote and Amastigote and *Leishmania* Antigens: SPLA, SALA

L. infantum promastigotes were cultured in MAA base solution. After five days in culture, the promastigotes were counted using a Neubauer chamber (dilution 1:40). The number was registered, and the washing steps began by pelleting the parasites at 1,800g for 10 minutes at 4°C. This step was repeated three times. The parasites were suspended in a theoretical volume of $2x10^9$/ml PBS and counted one more time (dilution 1:4000) to register the concentration and dilute to a final concentration of $1x10^9$/ml. 2 mM of PMSF—protease inhibitor, was added to the final concentration. Next, 10 full freeze-and-thaw (by hand) cycles at -80 °C for 10 minutes. Followed by a final centrifugation of the extract at 13,000g for 30 minutes at 4°C. The supernatant was recovered, and the pellet was discarded. Protein quantification of the antigen was performed, and single use aliquots of 50 µg were stored. Extraction protocol for the amastigote antigens were similar to the promastigote antigens.

Recombinant proteins-based antigens – rK28 and rK39

In 1993 Burns et al. (56) described an antigen, rK39, based on a *Leishmania* kinesin that showed excellent affinity. It is a repetitive sequence with 39 conserved epitopes related to *L. chagasi* and *L. donovani*. Although this antigen is based on these species, there is a cross reaction with *L. Infantum*. This sequence is part of a protein present mainly in amastigotes and, since its description, has shown very promising results. This antigen is currently used on a large scale in rapid diagnostic tests. Single use aliquots with 5 µg of recombinant protein were prepared and subsequently frozen for use in ELISAS.

The rK28 antigen is a synthetic gene based on the fusion between a kinesin region related to the rK39 antigen with repetitive sequences of the *L. donovani* haspb1 protein. The initial idea in producing this antigen was to try to diagnose patients who had low antibody titers against rK39. This antigen was described in 2010 by Pattabhi et al. (57) and in the article describing this antigen, a comparison was made between the new rK28 antigen and rK39, where it was concluded that this new antigen had great potential in helping to diagnose leishmaniasis. Single use aliquots with 5 µg were prepared and subsequently frozen for use in ELISAS.

Protein quantification

To determine the concentration of our antigens, Bradford protein quantification assay was done. Serial dilutions of BSA, used as a protein standard, with an initial concentration of 4mg/ml was used to generate a standard curve. Each dilution was added into a microtiter plate with 5µl per well in triplicates. The antigens were added as a pure sample and at a 1:10 and 1:100 dilution. 200µl of Reagent B (Folin reagent) and 25µl of Reagent A (copper

tartrate) were added to each well. After 15 minutes in the dark, the plate absorbance was read at 750nm.

Enzyme Linked Immunosorbent Assay – ELISA – Monkey Samples

To define the dilution used in the monkey samples, a first test was performed using the same samples in two different dilutions (1:50 and 1: 100). As the analytical signals generated were weak and the more concentrated dilution did not interfere with the blank values obtained, the use of 1:50 dilution was defined as the standard for all tests. The ELISA protocol, briefly, 96-well flat-bottomed microtiter plates (Greiner, microtiter immunoplate) were coated with 50µl coating buffer (0.05M carbonate/bicarbonate buffer pH 9.6) of solutions with either 1µg/ml of rk39 or rk28, 10µg/ml of SPLA, SALA. The plates are incubated overnight at 4°C. The coating contents of the plates were discarded, and the plates were blocked with 200µl of PBS-low-fat-milk 10% for 1 hour at 37°C. Next, the plates were washed with PBS-Tween 0.05% (PBS-T) and the sera were diluted 1:50 in PBS-T 1% milk, 100µl/well, were added to the plate in duplicates and incubated 1 hour at 37°C. A positive control human serum that consisted of a blend of confirmed positive samples diluted 1:500 in PBS-T 1% milk 100µl/well, one well per antigen, 4 wells/plate. This internal control acted as an indicator to assure if the assay had any issues. After washing with PBS-T the plates were incubated with an antihuman peroxidase IgG secondary antibody, diluted 1:5000 in PBS-T 1% milk, 100µl/well for 1 hour at 37°C. Following the incubation, the plates were washed with PBS-T and incubated with 0.5mg/ml of o-phenylenediamine dihydrochloride (OPD, Sigma), 100µl/well, for 10 minutes in dark. The reaction was stopped with 50µl of HCl. Absorbance values were read at 492 nm in an automatic ELISA reader (Synergy 2 Multi-Detection Microplate Reader - Biotek). Replicates were reviewed and repeated, at least 2 times, to increase reproducibility.

Enzyme Linked Immunosorbent Assay – ELISA – Human Samples

The ELISA protocol, briefly, 96-well flat-bottomed microtiter plates (Greiner, microtiter immunoplate) were coated with 50µl coating buffer (0.05M carbonate/bicarbonate buffer pH 9.6) of solutions with either 1µg/ml of rk39 or rk28, 10µg/ml of SPLA, SALA. The plates are incubated overnight at 4°C. The coating contents of the plates were discarded, and the plates were blocked with 200µl of PBS-low-fat-milk 10% for 1 hour at 37°C. Next, the plates were washed with PBS-Tween 0.05% (PBS-T) and the sera were diluted 1:400 in PBS-T 1% milk, 100µl/well, were added to the plate in duplicates and incubated 1 hour at 37°C. A positive control serum that consisted of a blend of confirmed positive samples diluted 1:500 in PBS-T 1% milk 100µl/well, one well per antigen, 4 wells/plate. This internal control acted

as an indicator to assure if the assay had any issues. After washing with PBS-T the plates were incubated with an antihuman peroxidase IgG secondary antibody, diluted 1:5000 in PBS-T 1% milk, 100µl/well for 1 hour at 37ºC. Following the incubation, the plates were washed with PBS-T and incubated with 0.5mg/ml of OPD, 100µl/well, for 10 minutes in dark. The reaction was stopped with 50µl of 3M HCl. Absorbance values were read at 492nm in an automatic ELISA reader (Power Wave XS, Bio-Tek). Replicates were reviewed and repeated, at least 2 times, to increase reproducibility. Samples with OD > 4.000 were diluted 1:6400 in PBS-T 1% milk to fit the OD on the reading calibration line. Samples with OD less than 4.00 were not diluted due to the low sample volume.

Statistical Analysis

Monkey Samples

To assess the reactivity of the antigens, it was considered the value obtained, for each antigen, in the collection performed one day before the infection as the individual and unique negative control for each of the monkeys tested. After analyzing the raw data generated by ELISA´s, the results obtained in all other time points, for each animal and in each antigen, were divided by the value of the negative control of each animal in each antigen and, thus, It is possible to evaluate the proportional increase of each collection point in relation to day zero (negative control). These results obtained after the divisions were passed to a logarithmic base of 10 for a better graphic dispersion. These results were inserted in Graphpad 8 to generate the individual graphs of each sample of both groups of monkeys tested.

Human Samples

The statistical evaluation started with the determination of sensitivity (true positives) and specificity (true negatives) for each antigen tested. To determine the sensitivity and specificity the optical densities (OD) obtained, in at least 2 ELISAS tests, the average of the ODs obtained in each sample was compiled and used to determine a ROC curve for each antigen. The area under the curve (AUC) and shape of the ROC curve support the estimations of the discrimination power of the tests. The AUC have values between 0 and 1 and indicate the strength of the tests, 1 being the strongest. The cut-off value for each antigen was defined as the OD value that had, at the same time, the highest sensitivity (Sn) and specificity (Sp). Sensitivity / specificity were calculated as Sn = true positive / (true positive + false negatives) x 100% and Sp = true negative / (true negative / false positives) x 100%. After this determination it was defined that samples with OD < cut-off for the respective antigen would be considered negative samples for the analyte. Likewise, samples with OD > cut-off for the respective antigen would be considered positive samples for the

analyte. For a better graphic visualization, some representations created in Graphpad 8 used a logarithmic base of 10. In addition, an analysis of the correlation between the antigens was carried out according to the results obtained for each one of them. This relationship was performed considering that all values followed a Gaussian distribution and Spearman's relationship coefficients were used.

Results

Monkey Samples

The tested antigens can be divided into two groups, SPLA and SALA in the group of parasite extracts and rK28 and rK39 in the group of recombinants. The first sample group presented, on day 0, the following average OD values for SPLA, SALA, rK28 and rK39, respectively: 0.017; 0.024; 0.038; 0.032 (table 5 – time point 0). The second sample group presented the following values for the same antigens, respectively: 0.024; 0.034; 0.021; 0.034 (table 7 – time point 0). If the medians for each of the antigens are evaluated on the same day 0, It is obtained similar values for some antigens and considerably different for other antigens, this is due to the fact that each monkey has a distinct immunological characteristic before infection. The medians obtained for the SPLA, SALA, rK28 and rK39 antigens are, respectively: Sample group I: 0.014; 0.023; 0.008; 0.017 (table 5 – time point 0) and Sample group II: 0.016; 0.023; 0.009; 0.019 (table 7 – time point 0). It is possible to evaluate the heterogeneity of the immunological responses of each animal in figure 5 where all the OD values obtained on day 0 are represented graphically for each antigen.

The minimum and maximum OD values obtained are described in table 4 for the first subgroup of samples and the minimum and maximum OD values obtained for the second subgroup of samples are described on table 6.

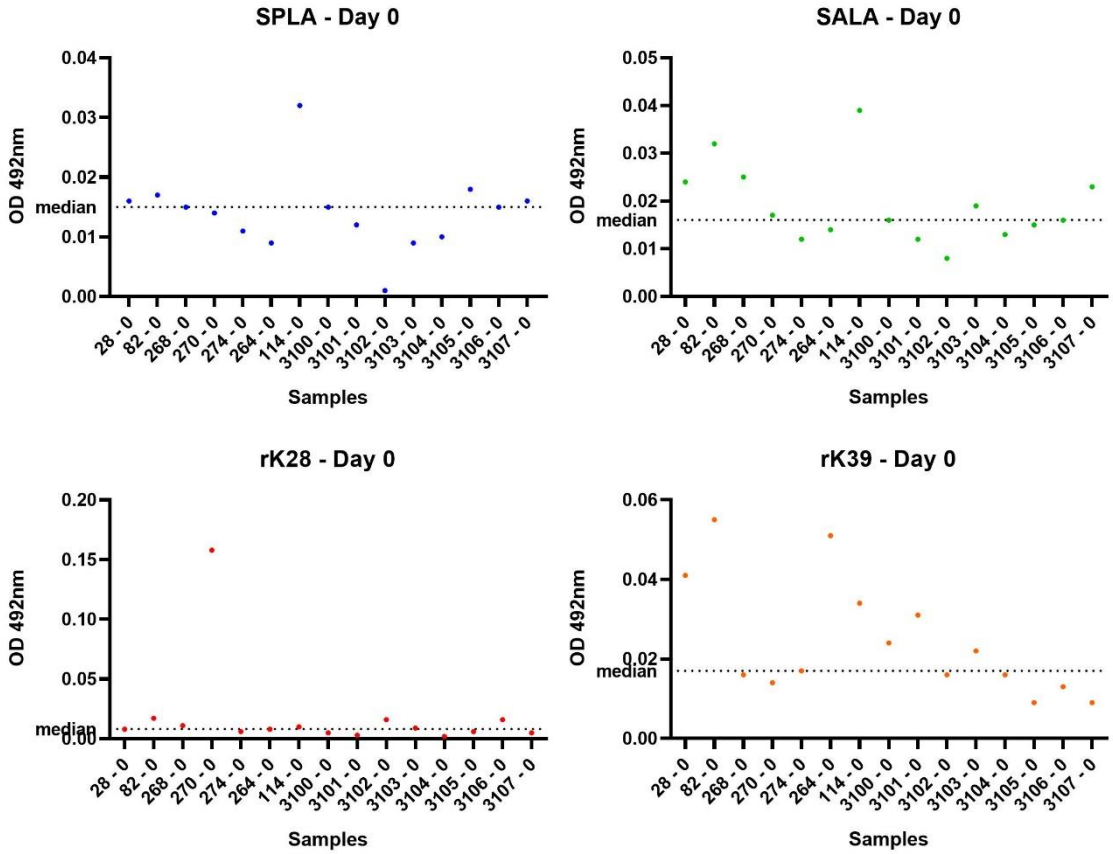

Figure 5 - OD values for each sample in each antigen on day 0 for all animals tested. The lines represents the median values for each sample.

Sample Group I

In this group some animals had high OD values (compared to the group average) for some antigens on day 0: 3 of the 7 animals had a high OD value for the rK39 antigen, 1 animal had a high for the rK28 antigen and 1 animal had high values for SPLA, SALA and rK39

Table 4 - Minimum and maximum OD values, for each antigen, for the first group of monkeys. The numbers on first column refers to each time point of blood collection

	SPLA		SALA		rK28		rK39	
	min	max	min	max	min	max	min	max
0	0.009	0.032	0.012	0.039	0.006	0.158	0.014	0.055
1	0.008	0.041	0.011	0.052	0.005	0.149	0.009	0.048
2	0.005	0.028	0.009	0.041	0.004	0.109	0.008	0.039
3	0.007	0.035	0.008	0.064	0.002	0.211	0.006	0.225

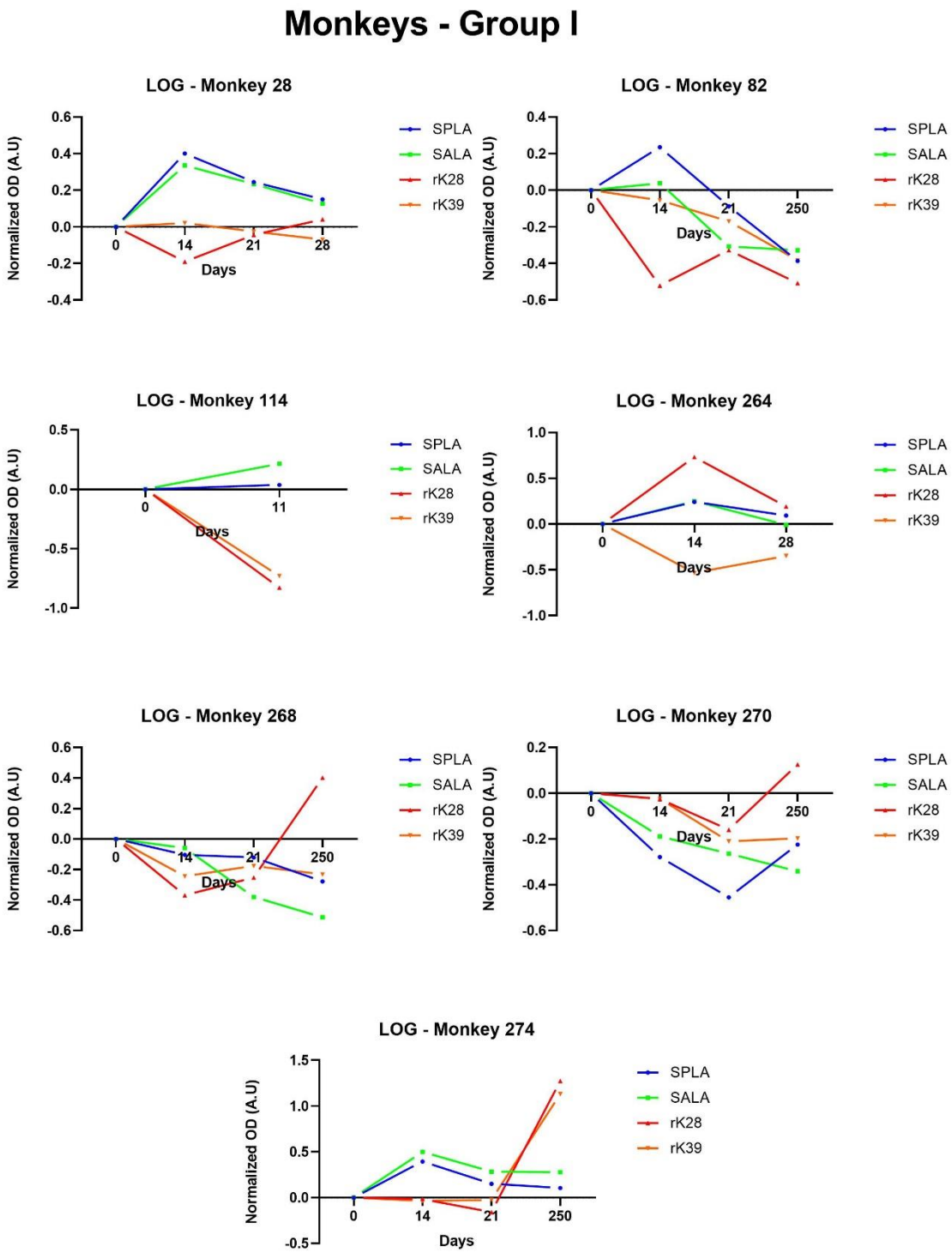

Figure 6 - Group I - Individual charts for all antigens for each monkey. These charts demonstrate the normalized OD (arbitrary unity) over time of each antigen in each monkey - color matched with the respective antigen.

antigens. This last cited animal was euthanized on day 11 and only two collections of material from this animal were performed. Figure 6 shows the behavior of the immune responses of each of the animals in this group of samples. There is a trend towards a peak response to antigens based on parasite extracts in the initial stage of infection and a

tendency towards reactivity to recombinant antigens over time. The minimum and maximum OD values obtained for each antigen, at each time, are reported in table 4.

The values of the means and medians of the optical densities obtained for each of the antigens, in each of the tested times were described in table 5.

Table 5 - Means and medians (med) OD values, for each antigen, for the first group of monkeys. The numbers on first column refers to each time point of blood collection

	SPLA		SALA		rK28		rK39	
	mean	med	mean	med	mean	med	mean	med
0	0.016	0.015	0.023	0.024	0.031	0.010	0.032	0.034
1	0.022	0.022	0.030	0.030	0.036	0.006	0.024	0.015
2	0.015	0.014	0.020	0.016	0.027	0.007	0.022	0.016
3	0.015	0.012	0.023	0.015	0.055	0.013	0.047	0.023

Sample Group II

This group of individuals has a different characteristic from the previous one regarding treatment. These individuals were separated in subgroups, so that 3 animals were treated with miltefosine, 3 were treated with test compound and 2 received no treatment at all. In this group, two of the eight individuals also had a high OD value for rK39 on day 0. Among the 8 monkeys tested, only 1 did not show reactivity to any of the antigens. The others showed reactivity to at least one of the 4 antigens tested. Figure 7 shows the behavior of the immune responses of each of the animals in this group of samples. There is also a trend towards a peak response to antigens based on parasite extracts in the initial stage of infection and a tendency towards reactivity to recombinant antigens over time. The

Table 6 - Minimum and maximum OD values, for each antigen, for the second group of monkeys. The numbers on first column refers to each time point of blood collection

	SPLA		SALA		rK28		rK39	
	min	max	min	max	min	max	min	max
0	0.001	0.018	0.008	0.023	0.002	0.016	0.009	0.031
1	0.013	0.094	0.015	0.138	0.002	0.066	0.010	0.057
2	0.007	0.053	0.014	0.091	0.003	0.076	0.012	0.044
3	0.008	0.045	0.010	0.059	0.003	0.209	0.006	0.429

minimum and maximum OD values obtained for each antigen, at each time, are described in table 6.

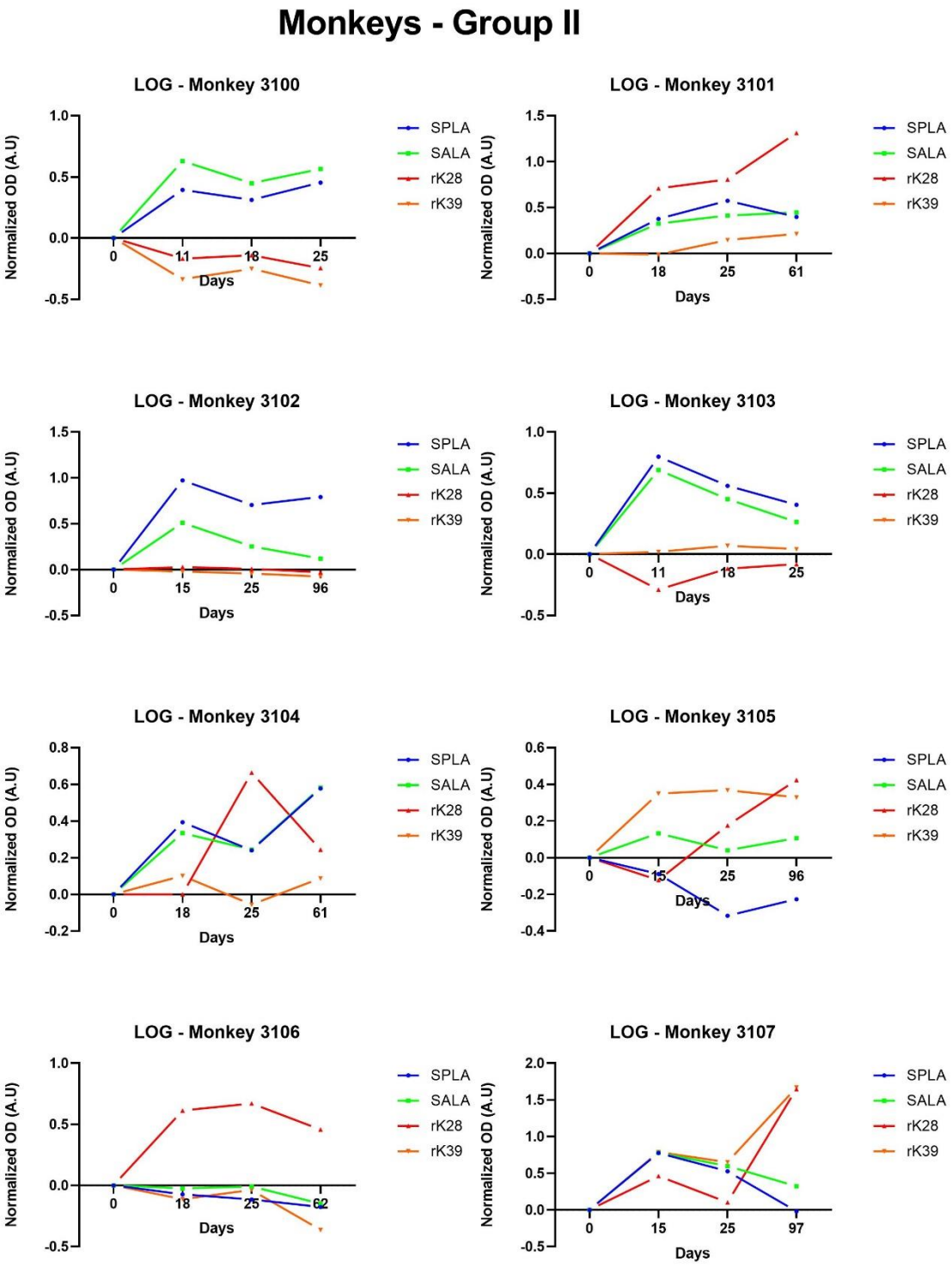

Figure 7 - Group II - Individual charts for all antigens for each monkey. These charts demonstrate the normalized OD (arbitrary unity) over time of each antigen in each monkey - color matched with the respective antigen.

The values of the means and medians of the optical densities obtained for each of the antigens, in each of the tested times were reported in table 7.

Table 7 - Means and medians (med) OD values, for each antigen, for the second group of monkeys. The numbers on first column refers to each time point of blood collection

	SPLA		SALA		rK28		rK39	
	mean	med	mean	med	mean	med	mean	med
0	0.012	0.014	0.015	0.015	0.008	0.005	0.017	0.016
1	0.035	0.026	0.051	0.027	0.015	0.009	0.023	0.020
2	0.026	0.024	0.036	0.026	0.018	0.009	0.023	0.017
3	0.024	0.019	0.033	0.032	0.039	0.015	0.070	0.019

Figure 8 is the compilation of the seroreactivity for all non-treated subjects in each antigen. These charts demonstrate the tendency to the referred peak of immunological response against the parasite extracts on the acute phase for both parasite extract antigens. Also is it shown the tendency that immunological response against the recombinant antigens develop with elapsed time. Figures 9 and 10 shows the same compilation of seroreactivity for the treated animals. These charts refer to monkeys with miltefosine and the test compound.

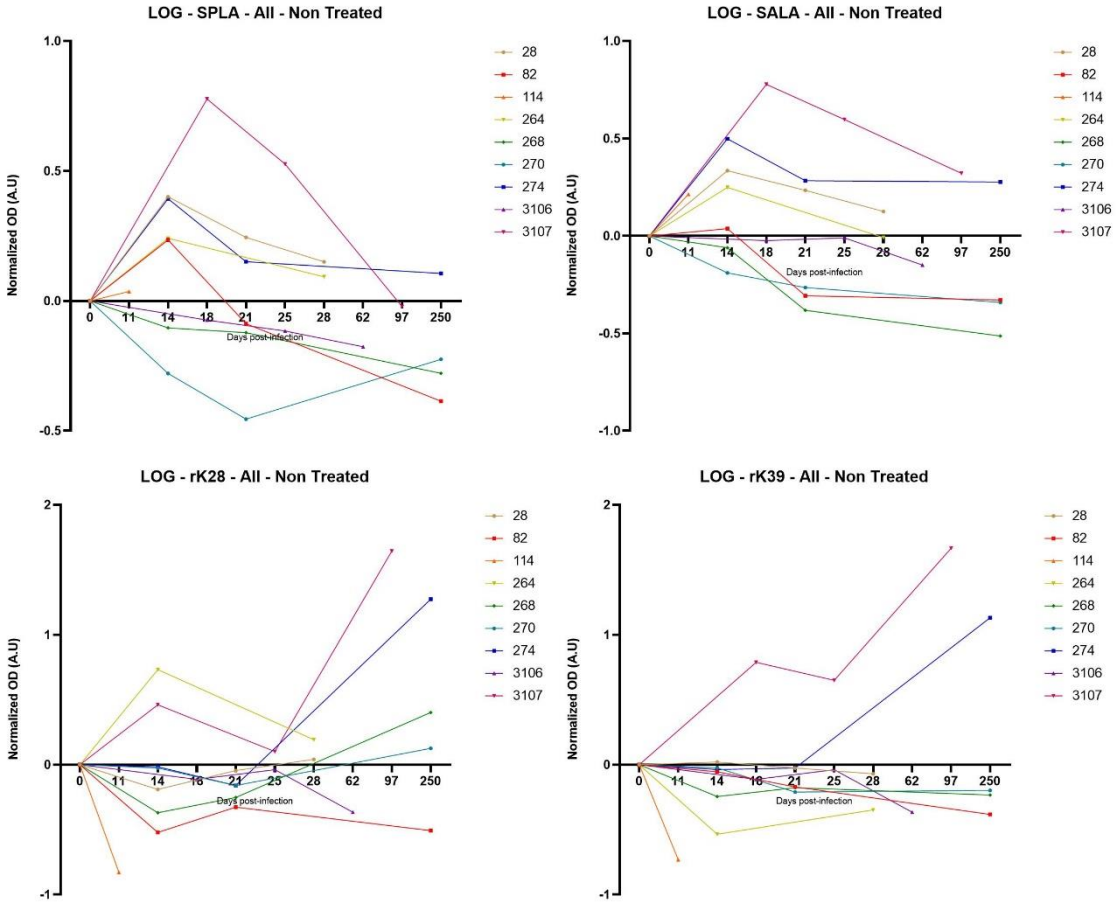

Figure 8 - Individual charts for each antigen with all non-treated monkeys. These charts demonstrate the normalized OD (arbitrary unity) over time of each animal in each antigen. Color matched with the respective animal. These charts shows the tendency to seroreactivity of all antigens.

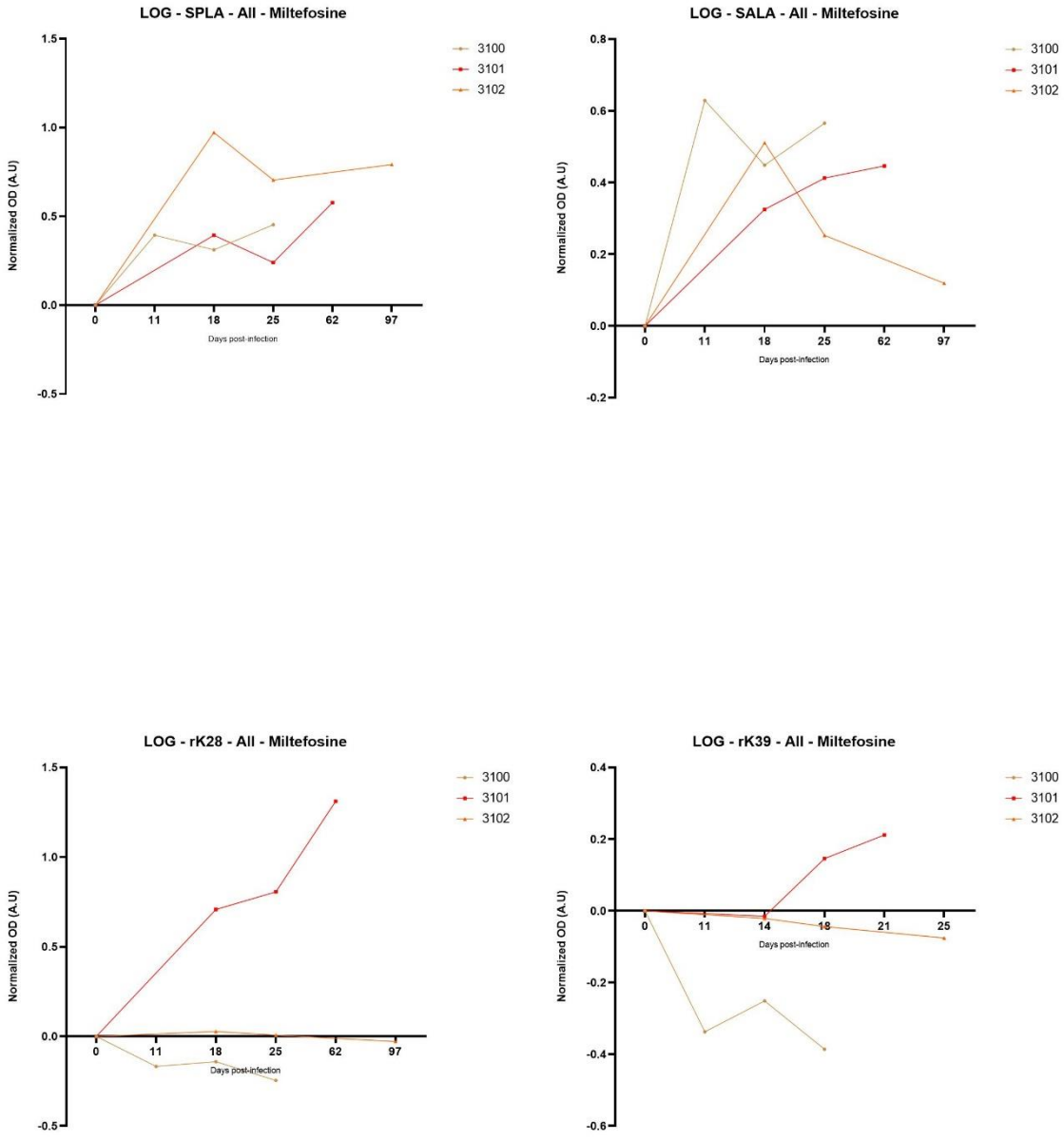

Figure 9 - Group II - Individual charts for all antigens contemplating all the monkeys treated with miltefosine. These charts demonstrate the normalized OD (arbitrary unity) over time of all the monkeys (separately) in each antigen.

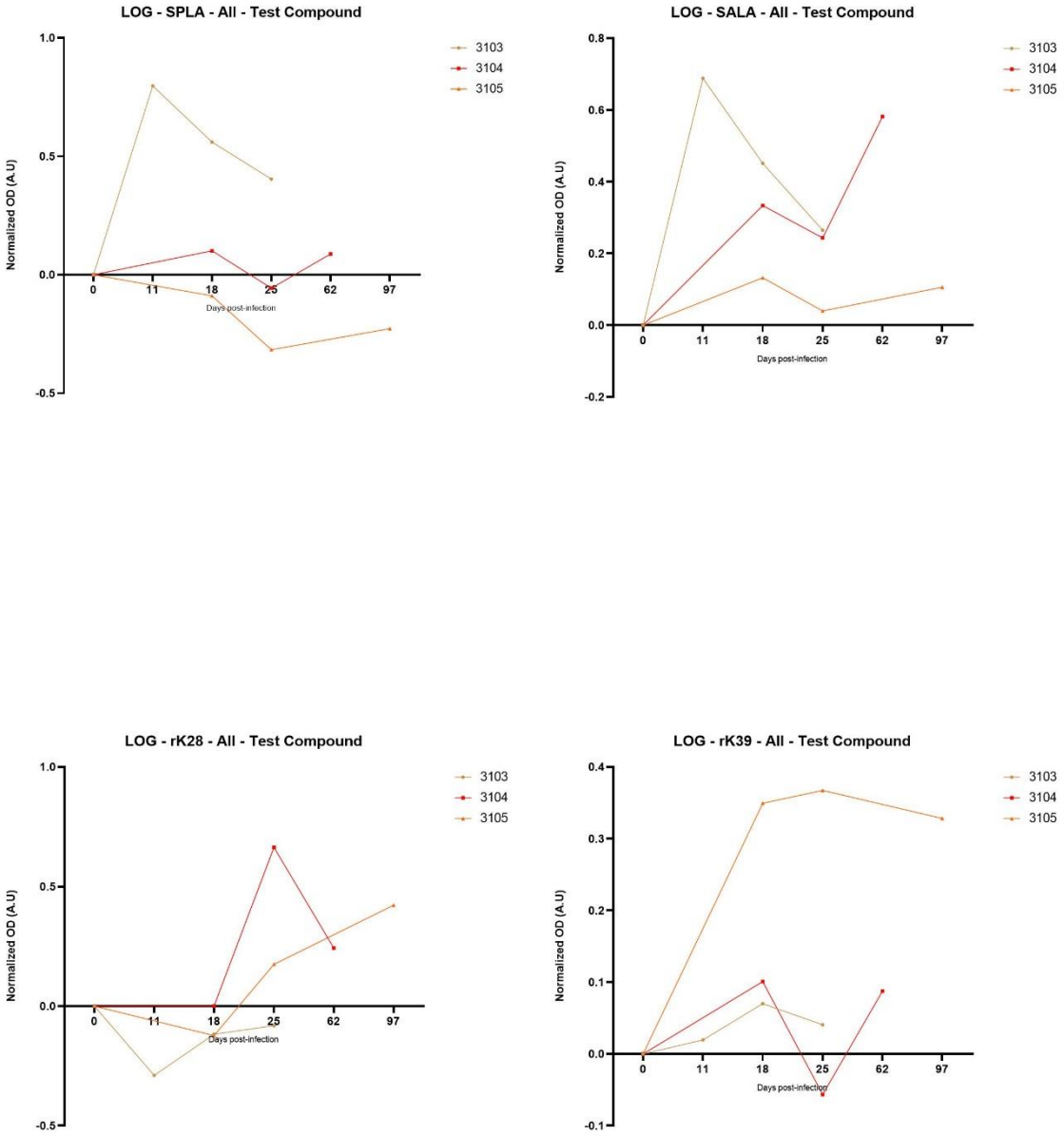

Figure 10 - Group II - Individual charts for all antigens contemplating all the monkeys treated with test compound. These charts demonstrate the normalized OD (arbitrary unity) time of all the monkeys (separately) in each antigen.

Human Samples

For human samples, the results obtained were used to determine the cut-off value for each of the antigens. The determination of a ROC curve was performed using the values of the negative samples compared to the positive samples. The ROC curve determination function of the Graphpad program was used and the results are shown in figure 11. Table 8 demonstrates the values obtained on the determination of the ROC curves.

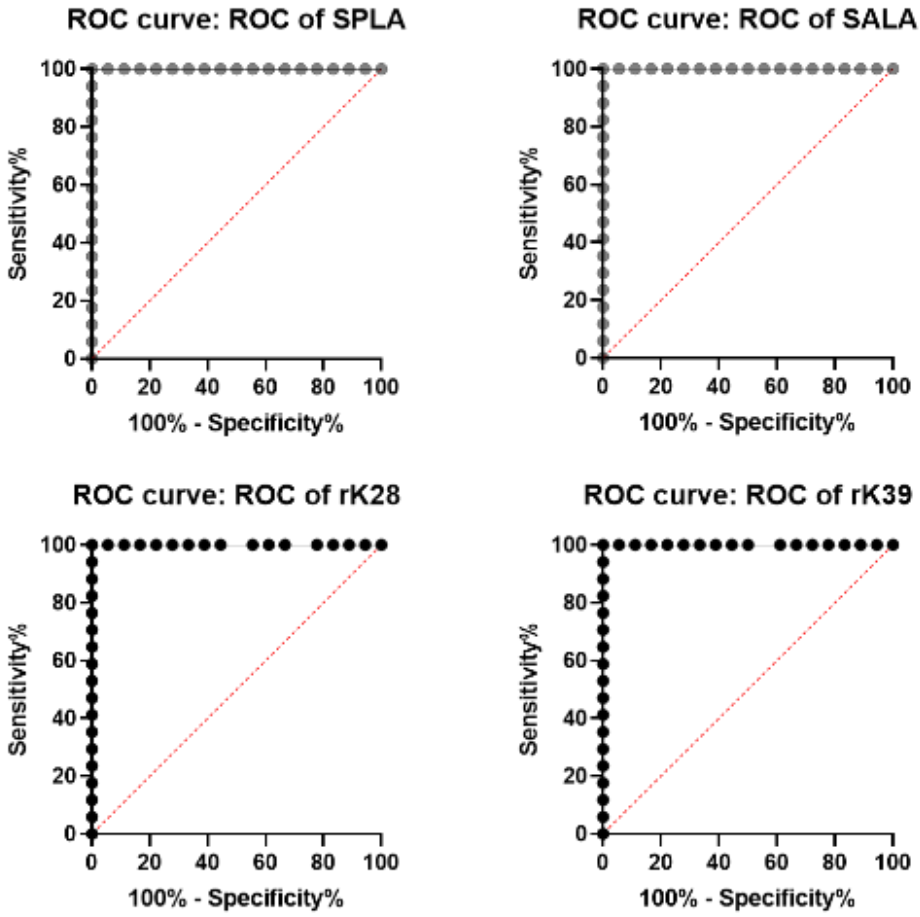

Figure 11 – Defined ROC curves of each antigen used to define the cut-off value for each antigen.

Table 8 - Described ROC information for each antigen. Defined cut-off value for each antigen

	SPLA	SALA	rK28	rK39
AUC	1.00	1.00	1.00	1.00
Std. Error	0.00	0.00	0.00	0.00
95% confidence	1.00 to 1.00	1.00 to 1.00	1.00 to 1.00	1.00 to 1.00
P value	<0.0001	<0.0001	<0.0001	<0.0001
Defined Cut-Off	0.133	0.055	0.133	0.118

According to the values obtained in the ROC curve, the cut-off values for each of the antigens were determined. The cut-off values for each antigen are shown on table 8. The OD values were compared with the cut-off values and samples with a value lower than the cut-off value were considered negative and samples with OD values higher than the cut-off value were considered positive. In all four antigens it was possible to define a cut-off value that could differentiate positive samples to negative samples. Figure 12 shows graphically the disposition of OD for each sample in all four antigens. The graphs were made with a logarithmic base of 10 for better dispersion of the values and the lines represent the cut-off values for each antigen. The medians of the positive samples varied for each antigen and the values obtained were 0.533; 0.213; 8.736; 8.480 (SPLA, SALA, rK28 and rK39, respectively). It is possible to see a large difference in comparison to the medians obtained in the negative samples. These were 0.038; 0.029; 0.018; 0.029 (SPLA, SALA, rK28 and rK39, respectively).

Table 9 - Descriptive statistics for each antigen - Human Samples – "POS" representing the positive samples group and "NEG" representing the negative samples group.

	SPLA		SALA		rK28		rK39	
	POS	NEG	POS	NEG	POS	NEG	POS	NEG
Minimum	0.193	0.014	0.059	0.010	0.235	0.007	0.139	0.010
Median	0.533	0.038	0.213	0.029	8.736	0.018	8.480	0.029
Maximum	1.538	0.074	0.958	0.052	55.856	0.032	59.920	0.098
Mean	0.667	0.039	0.317	0.029	16.598	0.018	15.501	0.035
Std. Deviation	0.409	0.015	0.283	0.011	17.526	0.009	17.332	0.024

As two well-defined groups were tested, the AUC values were all 1. Although the human sample group did not have any samples that were in a gray area for analysis, it can be considered that the cut-off values obtained are a good parameter in the case of extrapolation of these values for a population with unknown results.

The rK28 was the antigen that had the lowest results compared to the others, with a minimum OD value of 0.007 for a sample and an average OD 0.018. The same antigen had the highest mean (OD 16.598) although the highest value was found in a sample for the rK39 antigen (OD 59.920). Considering the averages obtained, for positive samples, the decreasing order of antigens are rK28, rK39, SPLA and SALA (16.598; 15.501; 0.667; 0.317, respectively). For negative samples, the order changes. Presenting, in descending order, the following antigens: SPLA, rK39, SALA and rK28 (0.039, 0.035, 0.029, 0.018, respectively). Table 9 shows the minimum, maximum, median, mean and standard deviation values for

each of the antigens. An evaluation of the correlation between the results obtained in each antigen was carried out to verify a possible relationship between them. As expected, in positive samples, the SPLA and SALA antigens have a relationship with a Spearman ρ of 0.80, demonstrating a strong correlation. The antigens rK28 and rK39 have a Spearman ρ relation of 0.98, also demonstrating a very strong relation. The relationship between recombinant antigens and parasitic extracts shows Spearman´s ρ values around 0.75, indicating a moderate to strong correlation. Among the negative samples wasn´t observed, as expected, one strong correlation between the antigens. Figure 13 shows the correlation between antigens in positive sample group and negative sample group. Figure 14 shows the results obtained for each sample in each antigen among the samples in the negative group. There is no response profile as seen in samples from the positive group. Also figure 15 shows the dispersion of the obtained OD´s of each sample for all 4 antigens for the positive group of samples. In general, the results of the recombinant antigens were similar to each other and higher than the results of the extracts of the parasites. Only two samples from the group of positive patients had a different profile from the others.

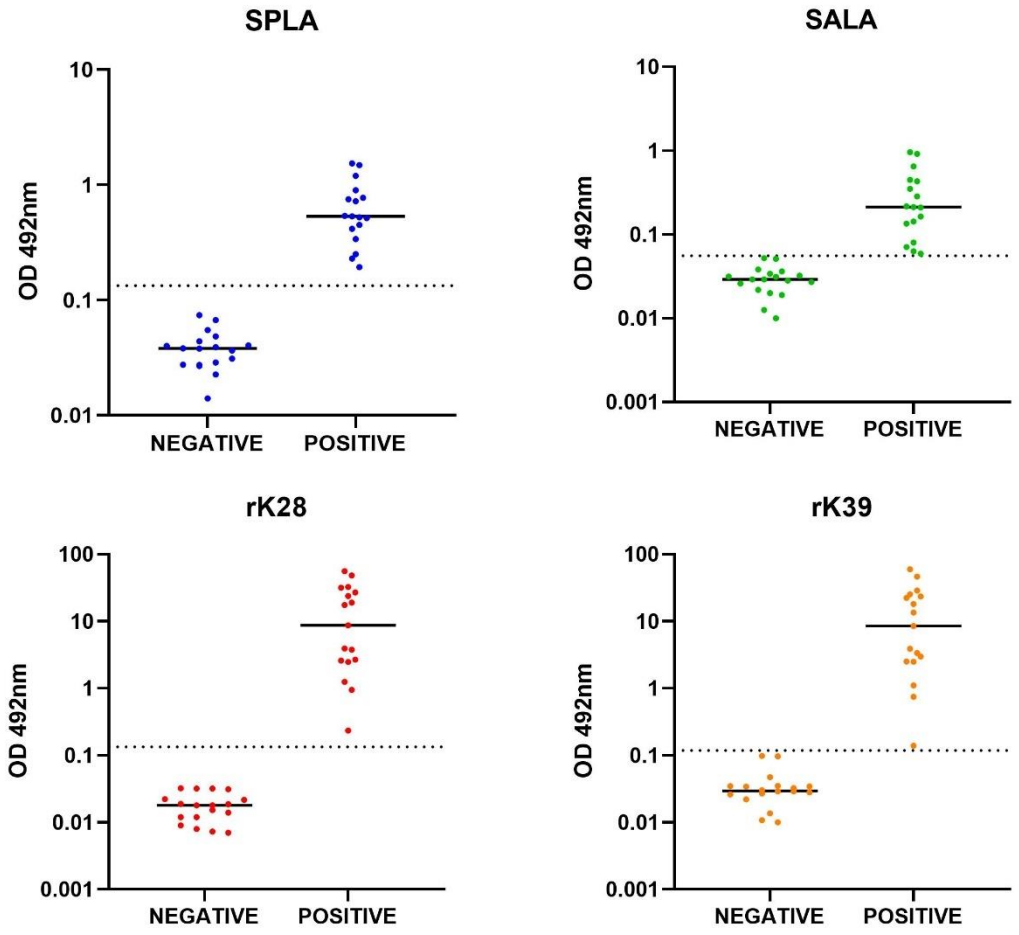

Figure 12 - Graphical representation of the ODs obtained for each human sample tested in the negative sample group and in the positive sample group in each antigen. Each dot represents a sample. The dotted lines represents the defined cut-off values.

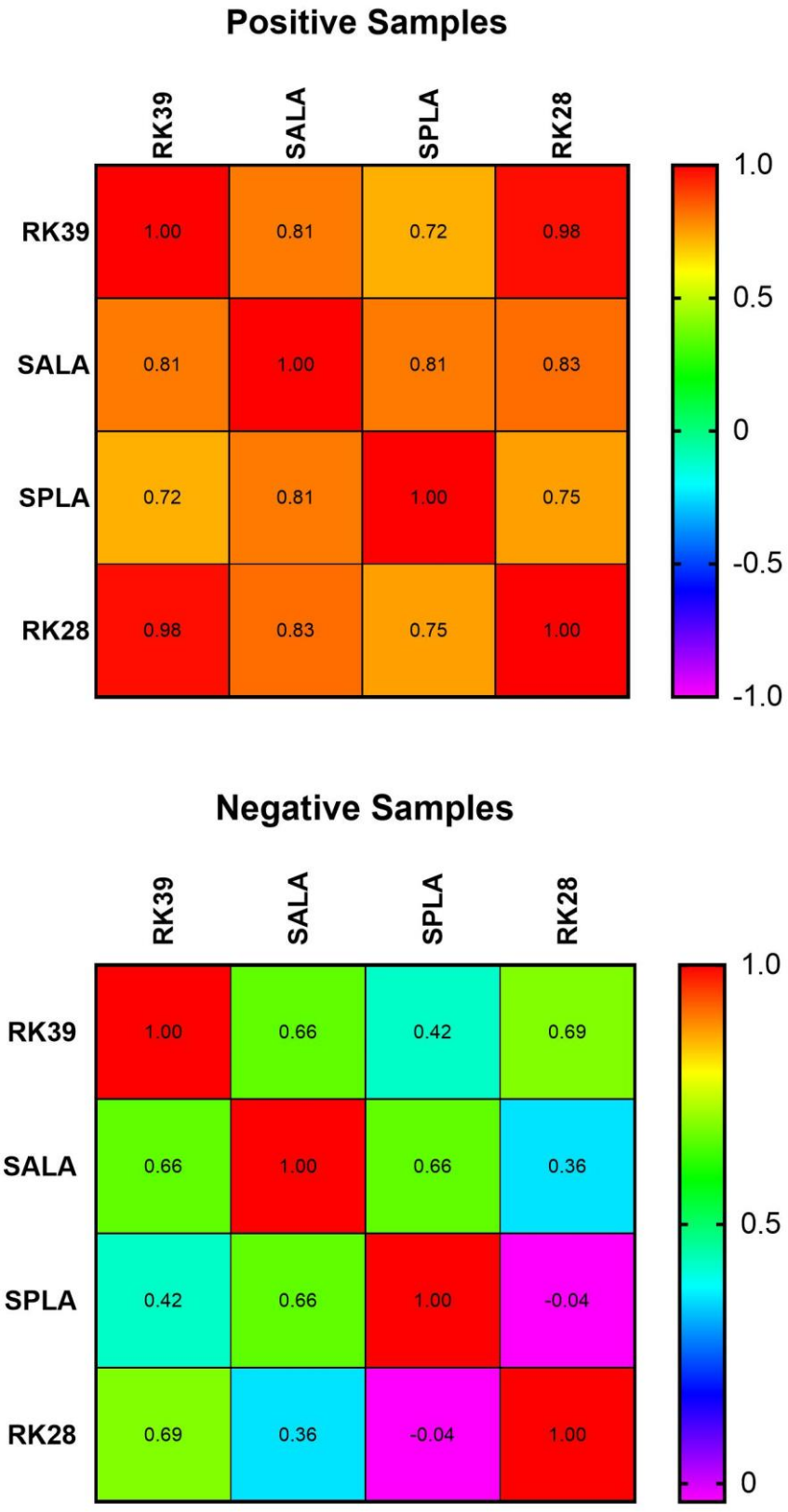

Figure 13 - Spearman correlation among the obtained OD´s in all antigens for both human sample groups. Positive sample group on top and negative sample group on bottom

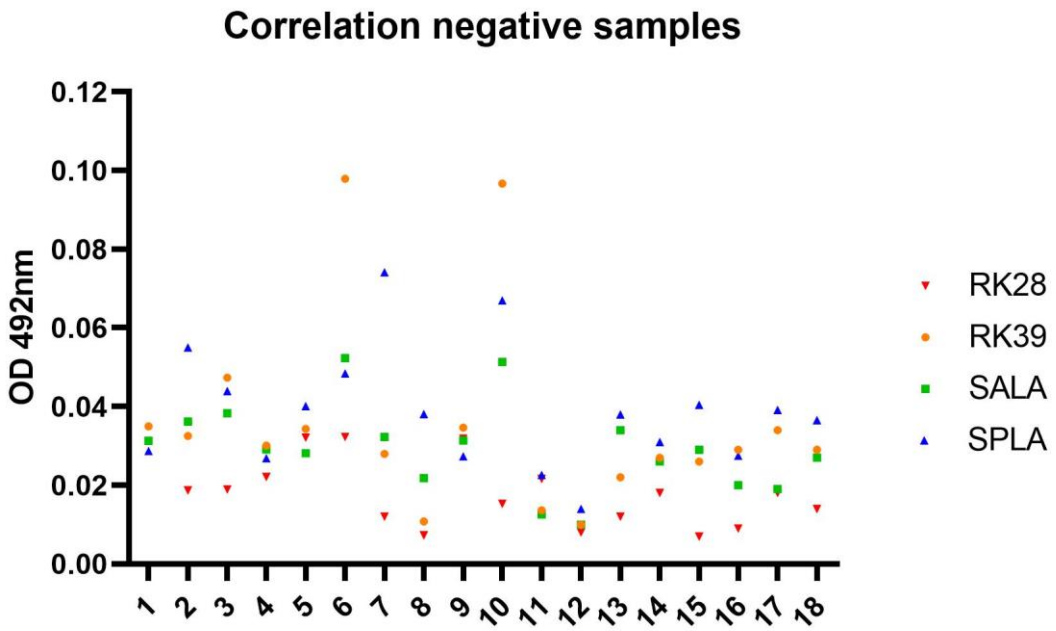

Figure 14 - Graphical representation of the ODs obtained for each antigen in each sample of the negative group. Each point corresponds to an antigen in each sample. The dots have a color correlation with the tested antigens.

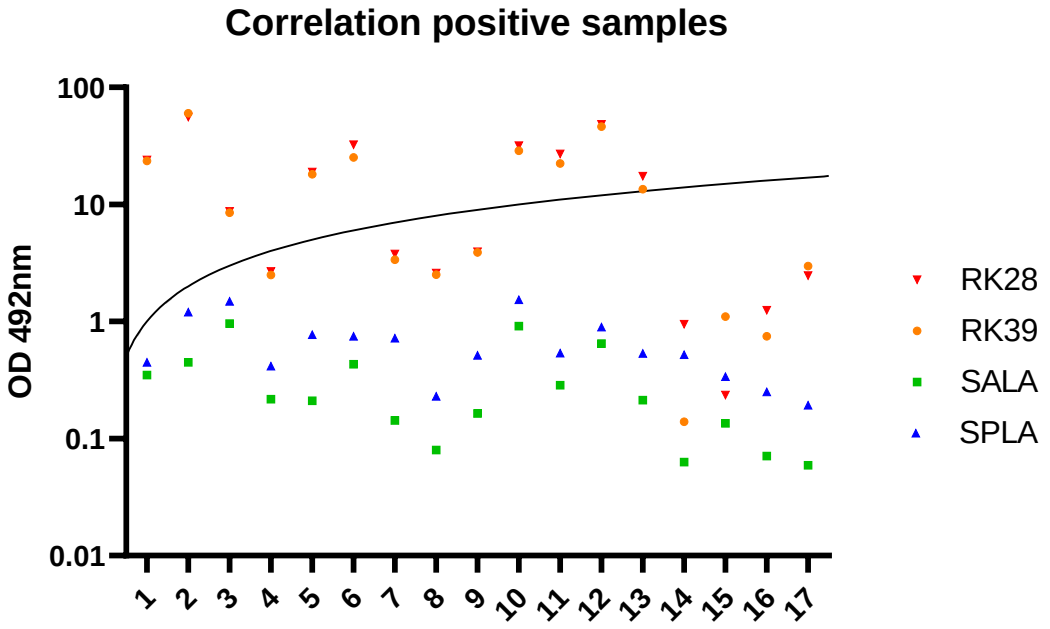

Figure 15 - Graphical representation of the ODs obtained for each antigen in each sample of the positive group. Each point corresponds to an antigen in each sample. The dots have a color correlation with the tested antigens.

Discussion

Monkey Groups

In this study it was possible to evaluate the performance of antigens in two different species. It was believed that because monkeys are phylogenetically closer to humans, the immune responses would be very similar. Considering the fact that the number of individuals used in this study was limited and that in both species it was identified reactivity in all antigens, the results were elucidative.

While in the group of humans were obtained a super strong analytical signals (OD> 50.00) even with a dilution of 1: 400, in the group of monkeys the greatest signal obtained did not reach 1.00 even with a much higher concentration (1 : 50).

For both groups of non-human primates, cut-off values were not defined to assess the positivity of antigens. Instead the values obtained for each antigen, in each individual, were used as the individual negative control for each animal. The cut-off wasn´t defined because the OD values on day 0 was very heterogeneous as shown on figure 5. That's why for the animal group the tendency of immunological response instead of absolute values was evaluated. Assessing the groups of monkeys studied It is seen that although they were distinct groups, both presented similar mean and median values in each of the antigens on day 0. This shows that even considering the individual characteristics of each individual studied, the difference about the time and place where they were kept and the immunological background, the groups have common characteristics. Table 10 shows the means and medians of the OD values obtained for each animal group.

Table 10 - Means and medians (med) of both tested animal groups for each antigen before the infection.

	SPLA		SALA		rK28		rK39	
	mean	med	mean	med	mean	med	mean	med
Group I	0.017	0.014	0.024	0.023	0.038	0.008	0.032	0.017
Group II	0.024	0.016	0.034	0.023	0.021	0.009	0.034	0.019

The first objective of this work was to evaluate the performance of the antigens tested in a group of non-human primates infected with *L. infantum*. The results demonstrated that the tested antigens can be used as markers to predict infection in monkeys. All antigens showed reactivity. Effectively, the longitudinal monitoring of these animals allowed the identification of a non-linear response profile among all animals (treated and non-treated). There is a trend towards a peak response to antigens based on the parasite extracts in the acute phase with gradual decline, as can be seen in almost all animals tested. With the

exception of animals 268 and 270 in group I and animal 3106 in group II, all other animals tested showed reactivity against parasite extracts as shown in figures 6 and 7. This type of immune response profile had already been described in another animal model (dogs) by Santarem et. al. (58). Parasite extracts are richer in the number of epitopes available compared to recombinant antigens and, therefore, may have a greater capacity to stimulate the animals' immune system, in addition to the immune response generated by *Leishmania* infection tends to promote production polyclonal antibody which may also explain the peak of extracts richer in binding sites. With a large amount of non-specific antibodies, the tendency of these immunoglobulins is to bind to the antigens present. Rodrigues demonstrated that although there is a hypergammaglobulinemia characteristic of VL, the number of specific antibodies against *Leishmania* is very low and, although they peak in the acute phase, they end up returning to baseline levels in the chronic phase of the disease (3). Another possibility that could justify this peak is also based on the plurality of epitopes of the extracts. This fact can be correlated with the reactivity of other antibody clones to other microorganisms that can cause an immunological recall and a cross reaction with other antigens is obtained. The response to recombinant antigens, on the other hand, tends to appear after some time of infection. The rK28 performed better compared to the rK39. Ten of the seventeen animals tested showed reactivity to this antigen (figures 6 and 7). The issue of reaction to recombinants happening late can be explained by taking into account that these markers are very specific for *Leishmania*. Thus, the animals' immune system needed more time to produce specific clones against *Leishmania*. This first group of primates is the same group of animals that was used in the work of Rodrigues et al. (3) and, at the time of euthanasia, the organs were collected for further analysis. The same collection was made for the second group of animals. The organs were also collected for further analysis. Figure 16 demonstrate the parasitemia values that was carried out by the Cell death in host-pathogen interactions research group at Université Paris Descartes and these values were used to check if a correlation between the individual immune response of each animal with the parasitic load constant in the spleen at the time of euthanasia exists. The parasitemia values in the spleen of each animal of the first group are shown in figure 17. These values were used with the same purpose as the values of the second group of animals.

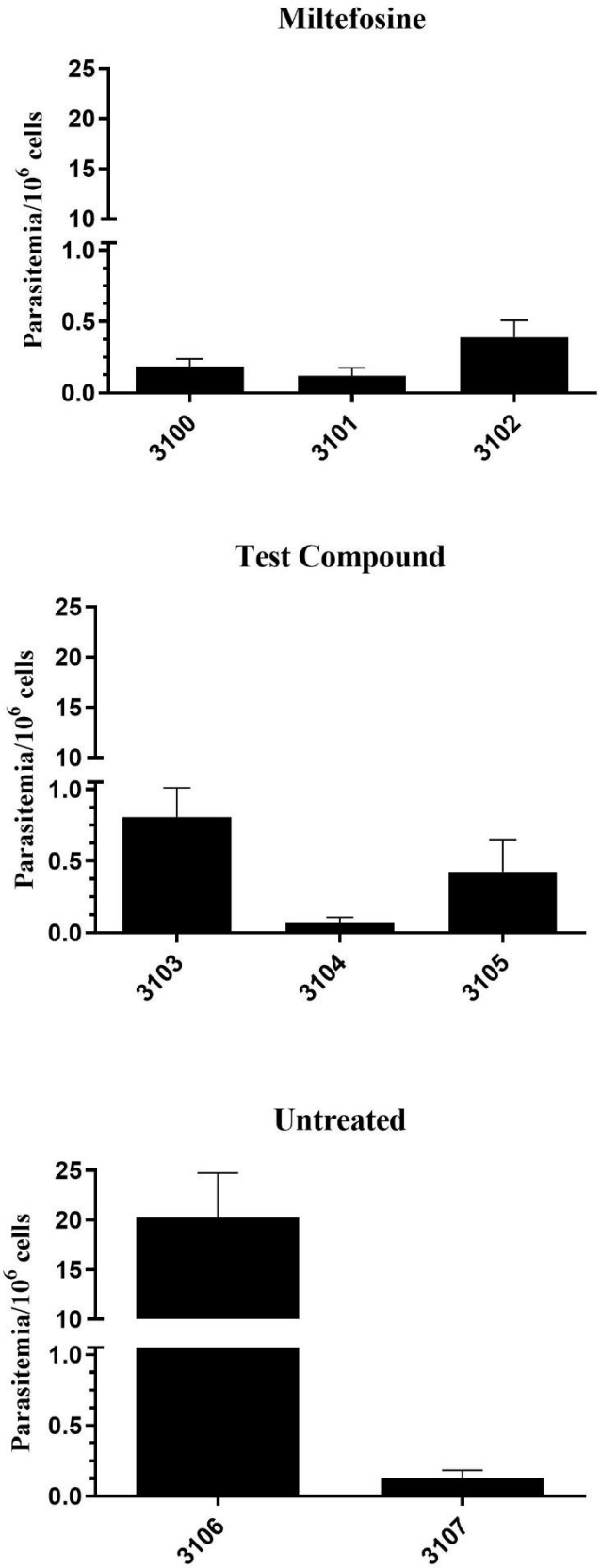

Figure 16 - Quantification of parasitemia in the spleen of each monkey at the time ofeuthanasia – group II – divided in subgroups among the treated and non-treated animals. Unpublished data from the Cell death in host-pathogen interactions research group

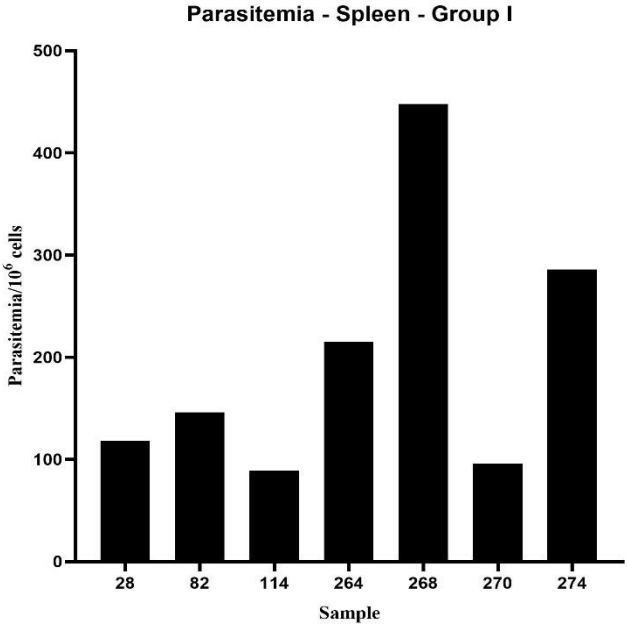

Figure 17 – Parasitemia in spleen at euthanasia - Group I – Monkeys. Adapted from (3)

Paradoxically, no linear relationship was observed between parasitemia and the immune response. Suggesting that there are other factors, in addition to the parasitic burden, that influence the immune response. Rodrigues had already proposed that a high parasitemia could cause changes in the organization of the spleen leading to a greater difficulty in the process of maturation of antibodies (3). Among the animals tested in each group, specimens 268 (in group I) and 3106 (in group II) showed the highest parasitemia value compared to the other animals in the respective groups. Coincidentally, it is the animals that showed the lowest reactivity rate to most antigens (figures 6 and 7). There was only response to rK28 in both animals. Although it is impossible to state because it is just two animals, these findings corroborate the statement by Rodrigues et. al.

Another objective of this work was to observe if there is a difference in the immune response between treated and untreated specimens. As can be seen in Figures 8 (untreated) and 9 and 10 (treated with both drugs) the tendency to peak reactivity to parasite extracts followed by an increase in antibody titers against recombinant antigens over time is common among treated and untreated animals. This demonstrated that even while the treatment was being administered, it did not prevent the immune reaction against both parasite extracts and recombinant antigens. However, while all animals treated with test compound showed an immune response against rK39, among animals treated with miltefosine there was only reactivity against rK28 among recombinant antigens, suggesting an inhibition of the production of specific antibodies against the marker most used in rapid tests today (rK39). As only three animals treated with miltefosine were tested, subsequent studies with a larger

number of specimens can elucidate whether treatment with this drug may interfere with the production of specific antibodies against that kinesin.

Comparing the antigens to each other, it is concluded that there is no evident advantage when the extracts of parasite are evaluated among them. The results presented show that the performance of these antigens is very similar. This fact was to be expected because both markers are based on soluble antigens and have similar epitopes. Recombinant antigens differ.

Although rK39 is the most used in clinical practice, for the studied cohort, this antigen showed reactivity in less than half of the animals tested. RK28, on the other hand, demonstrated a great potential for use. First, it was the marker with the lowest median value on day 0 (OD: 0.008), demonstrating that it would be the marker with the least possibility of cross-reaction with other pathogens. Two thirds of the animals tested showed reactivity against this marker, even asymptomatic, and these data suggest that this may be a good diagnostic marker for asymptomatic patients. The development of such a marker is a very important issue, especially in endemic areas. A study carried out with dogs (59), comparing two different techniques, demonstrated that rK28 had a better sensitivity in the detection of asymptomatic individuals in relation to the other markers. Our data corroborate these statements in a distinct species and with genetic homology in relation to humans.

Human Group

Finally, the last objective of this work was to evaluate the performance of these antigens in a cohort of infected and healthy patients in the context of leishmaniasis in the new world. All the tested markers demonstrated an excellent performance. Considering the negative control group, it was noticed that none of the samples reacted against any of the tested antigens. All were truly negative. As for the samples in the positive group, all reacted against all antigens. Therefore, the ROC curve of all antigens showed a value of 1.00. It is realized that this sensitivity and specificity obtained could have been lower if a larger group of samples were analysed, where there were individuals with gray-zone results.

The samples of the human group had shown a very strong reactivity to antigens in general. Especially recombinant antigens. It is known that rK39 is used on a large scale, mainly in the field context in endemic regions. It is a marker that has great sensitivity and specificity and is widely used in quick tests that are easy to perform. Among the samples from the positive group of humans all reacted against this antigen. Another antigen that demonstrated very considerable values was rK28, which has also been described in the literature. It is an antigen with performance comparable to rK39. At the time of submitting this work, no studies using rK28 were found in human samples from the new world. It was

observed that, in general, reactions to parasite extracts were weaker than reactions to recombinant antigens. Considering the immunological profile seen in the non-human primates group of peak response to extracts in the acute phase with inversion of response to recombinants during the chronicity of the disease, and how the information about the subjects were not provided, it was inferred that these patients were already in the chronic phase of infection. However, two individuals had a different profile from the others. Serums 14 and 15 showed higher SPLA results than recombinants (figure 15), suggesting that they could be at an earlier stage of the disease where the reversal of the immune response had not yet occurred. Considering the correlation between the results obtained in each antigen, it was noticed that, for the group of positive sera, as experienced, the soluble antigens based on parasite extracts are very correlated with each other while the recombinant antigens, because they are very specific, have a strong relationship with each other. Figure 13 demonstrates this correlation both for the group of positive sera and for the group of negative sera.

When the group of negative sera is evaluated, it is noticed that there is not such a strong correlation between the results obtained. The immunological background of each patient generates different ODs among uninfected patients. A better way to visualize this dispersion is through figure 14, which shows a point for each OD obtained. When comparing with figure 15, it can be seen that in the first there is no correlation while in the second, a correlation between the results obtained is perceived.

Conclusion

One of the objectives of this work was to evaluate the performance of the four antigens in populations of two different species. All antigens have demonstrated effectiveness in helping to diagnose leishmaniasis and have the potential to be used on a large scale in the diagnosis of this pathology. In general, all the results were very elucidative and important to this work.

Evaluating all the results obtained in this work it was possible to elucidate some questions. The good performance of recombinant antigens was confirmed regarding the diagnosis of leishmaniasis. Both tested antigens (rK28 and rK39) showed good reproducibility, sensitivity, and specificity. There is a characteristic kinetics in the response to antigens that begins with a first response to the parasite extracts and, with the course of the pathology, develops specific antibodies against the recombinant antigens. The rK28 antigen proved to be a good marker for asymptomatic individuals in groups of non-human primates because it is the first recombinant antigen to show reactivity. The possibility of further studies with

this antigen may be interesting to try to improve the diagnosis of asymptomatic patients in endemic regions. The results about treatment suggests that the drugs could interfere in some immunological response but, in general, does not stop the production of antibodies nor the parasitemia. It was concluded that these markers were also effectives in the new world leishmaniasis context. All of them were capable of distinguish positive samples from negative samples.

Although studies and WHO data show many deaths related to this disease, unfortunately leishmaniasis remains a neglected tropical disease because it is endemic mainly in populations with low socioeconomic development. A better understanding of the disease is necessary so that one day it will be possible to control the transmission of this parasite. There are several people studying this parasite and there is an effort to try to better understand the mechanisms related to the disease, however they believe that only a joint effort of the countries affected and not affected by this pathology can lead to the point where it will be possible to reduce the problems related to this parasitic infection remains strong. The major hope is that, as soon as possible, good news will be part of a new reality.

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