Development of an innovative immunosensor for Leishmaniasis screening

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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Resumo

Leishmaniose é uma doença tropical negligenciada causada pelo parasita *Leishmania* e é transmitida a humanos e cães pela mosca da areia fêmea Phlebotominae. A forma mais severa da doença é a leishmaniose visceral e é fatal se não tratada, estimando-se até 30 000 de mortes por ano. Devido a vários fatores como alterações climáticas e migração, o número de casos de leishmaniose tem vindo a aumentar. Outro problema é a falta de especificidade dos métodos de diagnóstico atuais, que resultam em falsos resultados. Indivíduos infetados, mas sem sintomas, são potenciais propagadores desta doença. Neste contexto, existe uma necessidade emergente de diagnóstico simples e eficiente capaz de fazer triagem da doença e consequentemente controlo da sua disseminação.

Neste trabalho, pretende-se desenvolver um imunossensor imobilizando biomarcadores de soros caninos em elétrodos impressos, já que estes são descartáveis, baratos, fáceis de usar e de resposta rápida.

Técnicas eletroanalíticas, como voltametria cíclica e potenciometria, são usadas para a caracterização dos imunossensores. O protocolo usado na imobilização da proteína baseia-se na imobilização do antigénio pela cauda de histidina (atualmente a proteína recombinante CPX2) a micropartículas magnéticas funcionalizadas com ácido nitrilotriacético, sendo o mesmo protocolo usado pelo grupo para citometria de fluxo. Um dispositivo de microfluídica que contém o elétrodo impresso foi especialmente desenvolvido para este trabalho, onde um campo magnético é gerado no elétrodo de trabalho para reter as partículas magnéticas, que foram previamente imobilizadas com o respetivo antigénio e anticorpos. Para obter volumes reprodutíveis, a suspensão que continha as partículas magnéticas foi propulsionada até ao elétrodo de trabalho através de uma válvula FIA, com a ajuda de uma bomba peristáltica. Os resultados obtidos são promissores, no entanto verifica-se alteração da superfície do elétrodo devido à adsorção inespecífica de proteína. Atualmente, está-se a trabalhar no sentido de bloquear a superfície do elétrodo de forma a evitar adsorções inespecíficas e a avaliar formas de remover proteínas que possivelmente adsorvam.

Em paralelo, exossomas foram isolados de plasmas de pacientes infetados com leishmaniose (sintomáticos, assintomáticos e curados). Estas vesículas são constituídas por proteínas antigénicas envolvidas na resposta imune contra o parasita e têm potencial para serem utilizados como novos biomarcadores da doença. Esta hipótese

foi verificada pela resposta das várias frações de material extracelular contra soros de cães e humanos infetados com leishmaniose.

Palavras-chave: *Leishmania infantum*, Leishmaniose visceral, Reservatórios de doença, Cães, Casos assintomáticos, Imunossensores, Partículas micromagnéticas, Elétrodos impressos, Resposta potenciométrica, Vesículas extracelulares.

Abstract

Leishmaniasis is a tropical neglected disease caused by the parasite *Leishmania* and it is transmitted to humans and dogs by the female Phlebotominae sandflies. The most severe form of the disease is visceral leishmaniasis that is fatal if left untreated. It is estimated that every year up to 30 000 people die. Due to several issues such as climatic changes and migration, the number of leishmaniasis cases has been rising. Another concerning issue is the lack of specificity in the current diagnosis methods, that lead to false results and unidentified infected dogs (asymptomatic cases) that can also contribute to the spreading of the disease. There is an urge to have an efficient and simple device to diagnose and screen dog serums, as they are natural disease reservoirs and therefore we want to control the silent spreading.

In this project, we intend to create an immunosensor based in a well-known canine leishmaniasis biomarker by using screen-printed electrodes, as they are disposable, cheap, they respond quickly and they are easy to use.

Electroanalytical techniques, as cyclic voltammetry and potentiometry, are currently in use to characterize the immunosensor. The protocol used for the protein immobilization is based in the immobilization of the antigen through its histidine tail (currently the recombinant protein - CPX2) to magnetic microbeads functionalized with nitrilotriacetic acid, the same strategy used by the group in flow cytometry protocol. A flow device containing the screen-printed electrode was specifically developed for this work, where a magnetic field is generated in the working electrode for fixing the magnetic beads with the respective immobilization of the antigen and antibody. To allow reproducible volumes, the dispersion containing the magnetic beads is propelled to the working electrode by using flow injection analysis, with the help of a peristaltic bomb. When the samples go through the flow system, a magnet is activated which assures the magnetic microbeads fixation to the screen-printed electrode. The obtained results are promising; however, an alteration of the electrode surface is verified due to unspecific protein adsorption. We are currently working to block the electrode surface to avoid unspecific adsorption and to evaluate the best approach to remove proteins that might adsorb.

In parallel, exosomes (EXos) were isolated from plasma of infected patients leishmaniasis (symptomatic, asymptomatic and cured). These vesicles are constituted by antigenic proteins involved in the immune response against the parasite and might have potential as new disease diagnostic markers. This hypothesis was verified by the

response of several extracellular material fractions against leishmaniasis infected human and canine serums.

Key words: *Leishmania infantum*; Visceral Leishmaniasis; Reservoirs; Dogs; Asymptomatic cases; Immunosensors; Micromagnetic beads; Screen-printed Electrodes; Potentiometric response; Extracellular vesicles.

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Abbreviations

CL Cutaneous Leishmaniasis

CSA Crude Soluble Antigens

CV Cyclic Voltammetry

CVL Canine Visceral Leishmaniasis

DAT Direct Agglutination Test

EIS Electrochemical Impedance Spectroscopy

ELISA Enzyme-Linked Immunosorbent Assay

EVs Extracellular Vesicles

EXO Total Exoproteome

EXos Exosomes

FC Flow Cytometry

FIA Flow Injection Analysis

HIV Human Immunodeficiency Virus

IC Immunochromatografic

IFAT Indirect Fluorescent Antibody Technique

IgG Immunoglobulin G

IMAC Immobilized Metal-Ion Affinity Chromatography

MCL Mucocutaneous Leishmaniasis

NTA Nitrilotriacetic Acid

PCR Polymerase Chain Reaction

PTFE Polytetrafluoroethylene

PVC Polyvinyl chloride

qrtPCR Real-time quantitative PCR

Rct Charge transfer resistance

SAM Self-Assembled Monolayer

SLA Soluble *Leishmania* Antigen

SPLA Soluble Promastigote *Leishmania* Antigens

SPE Screen-Printed Electrodes

spp. Species

SWV Square Wave Voltammetry

VDE Vesicle Depleted Exoproteome

VL Visceral Leishmaniasis

WHO World Health Organization

Introduction

1. *Leishmania*sis - a neglected tropical disease

Infectious diseases are the second cause of mortality around the world, according to the World Health Organization (WHO)[1]. Among the parasitic diseases, Leishmaniasis is the second cause of death after Malaria. This neglected tropical disease exhibits high morbidity and mortality putting at risk people from 98 different countries worldwide[2]. It is endemic in 88 countries and 1.8 million of new cases are estimated to occur each year[3].

Leishmaniasis is a vector-borne disease caused by the parasites *Leishmania* species and transmitted by the bite of infected female phlebotomine sandflies. Combining 30 species of phlebotomine sandflies to more than 20 species of *Leishmania* species capable of causing disease, makes this opportunistic infection more complex to understand.[4]

Several factors such as climate changes and changes in animal migratory movements have been contributing to the spread of this infectious disease[5].

Organism prevalence differs by geographical distribution, being an endemic disease in the tropics, subtropics and the Mediterranean basin[6]. The protozoan parasites of the genus *Leishmania* take advantage of the mammalian host to survive and affect predominantly least developed and emerging countries with limited resources and individuals with deficient immune system (for example, individuals infected with HIV – Human Immunodeficiency Virus)[5,7].

1.1 Pathological forms of the disease

More than 20 *Leishmania* species are known to cause disease and the outcome of the infection will depend not only on the parasite species but also on the immune response of the host and the environment[8]. Different parasite species can cause identical pathology[9].

Leishmania species (spp.) lead to specific clinic-pathological categories: cutaneous Leishmaniasis, mucocutaneous Leishmaniasis and visceral Leishmaniasis, also known as kala-azar (Figure 1). Symptoms range from skin lesions to affecting cutaneous and mucosal tissues or even to vital visceral organs damage[4,8].

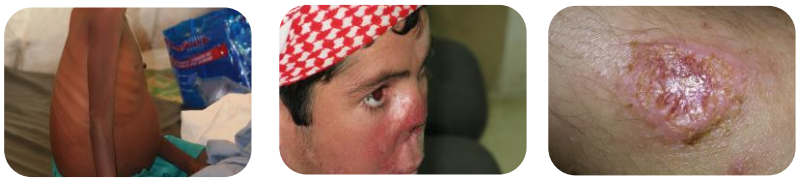

Figure 1 - Pathological forms of leishmaniasis – visceral, mucocutaneous and cutaneous[109].

1.1.1 Cutaneous Leishmaniasis

Cutaneous Leishmaniasis (CL) is the most studied form of the disease. It is caused by *L. tropica* and develops as a nodule in the site of inoculation, consequence of the infection of macrophages in the dermis that may develop to a dermal granuloma, an ulcer that heals spontaneously or several inflamed ulcers that take longer to heal (a few months to years). Histologically it is characterized by a lymphoid and monocytic infiltrate with granuloma formation. *L. mexicana* and *L. aethiopica* are responsible for a particular kind of CL in which the nodules resemble to lepromatous leprosy[8,9].

1.1.2 Mucocutaneous Leishmaniasis

This clinical presentation of Leishmaniasis is caused by *L. braziliensis.* Mucocutaneous Leishmaniasis (MCL) on the contrary of CL, which is confined to small areas of the skin, spreads to the oral and nasal mucosa and is characterized by disfigurative ulcers that take long time to heal. These progressively destructive ulcers, unlike cutaneous Leishmaniasis, are not self-healing and appear months or years after the first episode of CL[4,10].

1.1.3 Visceral Leishmaniasis

Visceral Leishmaniasis (VL), also known as kala-azar, is the most severe form of Leishmaniasis and is fatal if left untreated[6]. Each year there are 500 000 new cases of VL, mainly in endemic areas (90% of these in India, Bangladesh, Brazil, Nepal and Sudan)[11] (Figure 2). This disease strikes the most in poorest countries and can have a disastrous impact when it strikes a non-immune population.

VL is caused by the *Leishmania donovani* complex, which includes *L. donovani, L chagasi* similar to *L. infantum*[12].

An incubation period (from 1 month to 2 years) is followed by clinical manifestations such as fever, splenomegaly, hepatomegaly, weight loss, progressive anemia, pancytopenia. These typical symptoms of this systemic infection are characteristics of the dissemination

of parasites throughout the blood and reticuloendothelial system which lead to enlarged lymph nodes, spleen and liver[8].

Post kala-azar dermal Leishmaniasis is considered a sequel of VL in which skin is the focus of infection and appears like a nodular rash. This condition is frequent in Sudan and in the Indian subcontinent[13].

As an opportunistic infection, the parasite prevails in people whose immune system has been compromised. Thus, coinfection is common among HIV patients in Mediterranean countries, Brazil, east Africa and the Indian subcontinent[14].

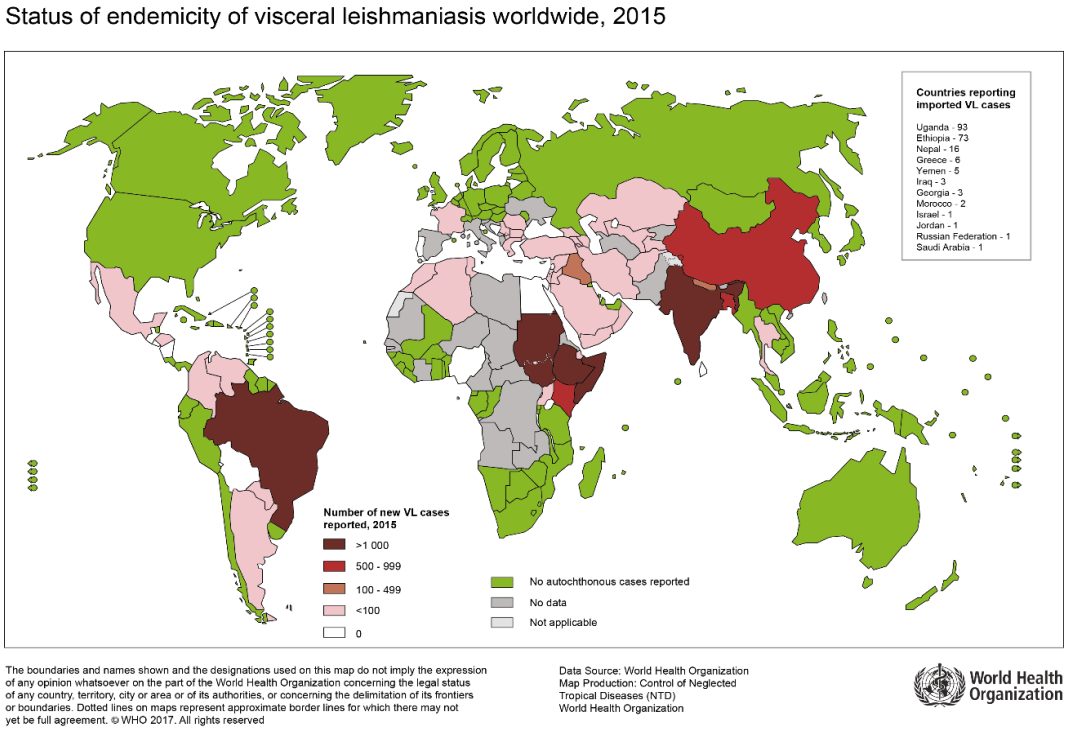

Figure 2 - Distribution of Endemic Visceral Leishmaniasis worldwide 2015. World Health Organization[15].

1.2 Epidemiology

Leishmania spp. capable of causing disease are divided in two main groups: Old world species (*L. major, L. tropica, L. aethiopica, L. donovani, L. infantum*) and New World species (*L. mexicana*, *L. amazonensis*, *L. brasiliensis*, *L. guyanensis*, *L. chagasi*).[8]

Visceral Leishmaniasis (VL) is caused by *L. donovani* (East Africa and the Indian subcontinent) and *L. infantum* (Europe, North Africa) in the Old World and *L. chagasi* in the New World (Brazil). While *L. donovani* infects all age groups, *L. infantum* infects mostly children and immunosuppressed individuals. Among other regions, *L. infantum* is responsible for VL in children in the Mediterranean basin. However, due to increasing prevalence of human immunodeficiency virus (HIV) infection in this region, HIV-VL coinfection in the adult population is being reported frequently. *L. chagasi* similar to *L.*

infantum causes VL in children in Latin America, where lymphadenopathy is a dominant clinical feature[16].

Due to urbanization and HIV pandemic, the incidence of VL is rising. More than 350 million people live in active parasite transmission areas, putting their health at risk[17]. Furthermore, each year there are up to 30 000 deaths due to this severe form of the disease[9].

This disease affects mostly least developed countries and poorest regions, where malnutrition, displacement, poor housing, weak immune system and lack of resources are common issues. Although not fully recognized, socioeconomics subjects are tightly connected to poverty and endemicity of this disease[9].

1.3 Transmission

1.3.1 *Leishmania* life cycle

Sandflies used to be limited to their natural distribution areas but, as previously mentioned, several causes (migration, climate changes, economic development, etc.) lead to the spread of sandflies as well as *Leishmania* reservoirs and consequently lead to the disease incidence.

There are more than 800 known species of phlebotomine sandflies but only certain species of sandfly of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) transmit the *Leishmania* spp. parasites[11].

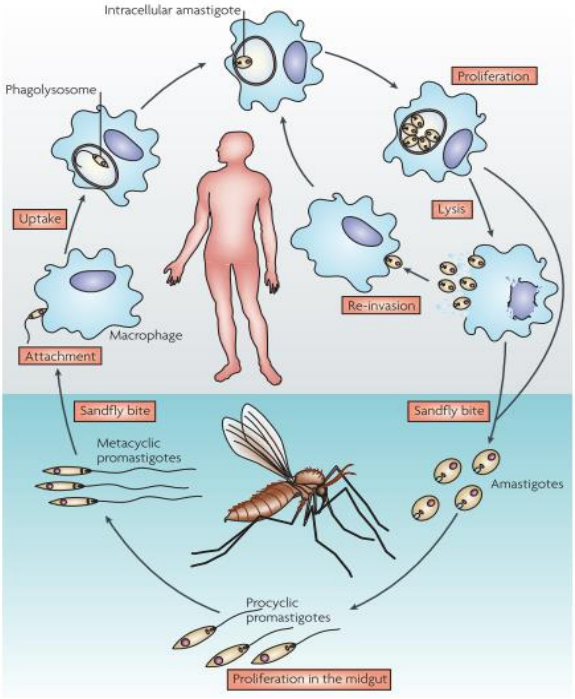

Figure 3 - *Leishmania* spp. life cycle. Ref 168 © (2001) American Society for Microbiology [4].

The parasites have a digenetic life cycle that includes an extracellular developmental stage in the female phlebotomine sandfly and another one in the vertebrate host, mostly intracellular. The parasite development in sandflies occurs in the alimentary tract, where promastigotes (the motile, flagellated, extracellular form of the parasites) are formed. Maturation of the parasite occurs in the midgut, where it develops to the infectious development form of the parasite: metacyclic promastigotes.

The transmission of the parasites to the mammalian host occurs during insect blood feeding. Usually, an inoculum contains around 100–1000 metacyclic promastigotes that are rapidly engulfed by mono and polymorphonuclear cells. Within these cells, the parasites go through other morphological changes, differentiating to an ovoid shape with short flagellum known as amastigote. Once they take this form, they multiply and the cycle becomes complete when the sandfly takes another blood meal with the amastigotes[18,19] (Figure 3).

1.3.2 Transmission Pattern

There are two types of VL transmission: zoonotic, when the disease can be transmitted from animal to vector to human, and anthroponotic is transmitted from human to vector to human. Transmission characteristics also differ according to geographical regions, being *L. donovani* responsible for the anthroponotic transmission and *L. infantum* for the zoonotic transmission. Anthroponotic transmission occurs in the Indian subcontinent and the zoonotic type is mainly transmitted in the Mediterranean basin and South America regions.

1.3.2.1 Zoonotic disease

Zoonotic visceral Leishmaniasis is a serious public health problem that affects some mammals and results in significant mortality and morbidity where it is endemic[20]. Animals such as wild canid, marsupials and rodents have been described as reservoirs of human VL[21]. However, zoonotic VL epidemics have been associated only in areas where canine Leishmaniasis is endemic.

Canine visceral Leishmaniasis (CVL) is caused by *Leishmania infantum* in the Mediterranean area, Middle East and Asian countries and is caused by *Leishmania chagasi* in Latin America. Due to their genotypic relationships, these two species are considered identical[22].

1.3.2.1.1 Dogs as main reservoirs

Although dogs are recognized as the main source of infection, the current control diagnosis methods and treatment are directed to humans[23].

In fact, from an epidemiological point of view, canine visceral Leishmaniasis is more important than Leishmaniasis affecting humans since its incidence is higher and both symptomatic and asymptomatic dogs are infectious to sandflies[24]. Besides, CVL is a veterinary issue because in endemic areas such as the Mediterranean Basin and Brazil, domestic dogs are the primary source of infection by the vector, allowing its life cycle perpetuation.

After an incubation period that can range from three months to several years, dogs might start presenting some clinical signs. Nevertheless, some dogs remain asymptomatic and never develop clinical signs[11].

Thus, dogs can present different forms of the disease: symptomatic, oligosymptomatic and asymptomatic. It is of great relevance mentioning that all forms of the disease enable the transmission of the parasite.

Symptomatic CVL typically results in death and it is based on the presence of at least two clinical manifestations. Infected dogs present cutaneous alterations such as alopecia, onychogryposis, dermatitis, skin ulceration and visceral manifestations with splenic, renal, hepatic, and neurological disorders. Other symptoms as anorexia and weight loss are also usual[25]. Infected dogs can remain asymptomatic, not presenting detectable clinical signs of disease. This is a grave problem, since these animals can contribute to the maintenance of *Leishmania* cycle[24]. Dogs that develop a few mild symptoms are classified as oligosymptomatic.

Despite evidences showing that killing seropositive animals leads to a decrease incidence in CVL and VL in children, this control method is not well accepted[26].

However, due to lack of accuracy in diagnosis methods the number of detected infected dogs cannot be elucidative of the real prevalence of infection. The number of infected dogs are estimated in few millions but it is believed that the real burden of disease is much higher[23].

We would expect that seroprevalence of CVL was higher in rural areas, where the sandflies are mainly found but the fact that there are more hosts infected in urban areas leads to the increase of CVL and higher prevalence when compared to rural areas. Unlike expected, urban areas favor the transmission of parasite due to high number of dogs and gardens. It provides a good environment to sandflies proliferation[27].

A sensitive and specific method to detect canine Leishmaniasis in early stages is needed to avoid false positives and false negatives that lead to unnecessary euthanasia and

disease transmission, respectively[11]. Detection of asymptomatic dogs is critical for controlling the spread of the disease among dogs and between dogs and humans.

The foundation to an effective control is prevention, screening and detection of the disease, since treating infected dogs is not that effective (relapses occur frequently and they can regain infectivity weeks after treatment)[28]. Besides, VL drugs might lead to parasite resistance.

2. Prevention and treatment

One approach to control the infection is doing vector control. This can be achieved with insecticides, such as pyrethroids and DDT. However, sandflies may develop resistance to DDT but remain sensitive to other insecticides. Bed nets and spraying are also alternative options to minimize the contact of some species with the host. Nevertheless, consequences concerning sandflies populations resistance are still under study[29,30].

Regarding dog protection, collars impregnate with deltamethrine reduce the risk of infection in dogs.

Few drugs are available for the treatment of Leishmaniasis and besides the risk of developing drug resistance, treatment efficacy depends on strains and species[9]. Furthermore, there is no Leishmaniasis human vaccine available. Vaccination would be a good option to prevent infection[31].

Vaccination of dogs would still be the best strategy if an efficient vaccine was developed. Being lauched in Portugal in 2011, CaniLeish® was the first vaccine for canine Leishmaniasis in the European Union. In Brazil, LeishTec® vaccine was also registered but only about 40% of protection against infection was found. Despite the efforts, *Leishmania* vaccinology still has a lot to improve till an effective and universal vaccine is developed[32].

3. Screening, detection and surveillance

Monitoring and controlling the spread of emerging diseases is a topic that should concern all individuals.

Disease surveillance is a crucial component to eliminate or at least to minimize the spreading of this infectious disease before the burden is devastating[33].

The first step to treat a condition or to take preventive measures is to diagnose. Taking appropriate actions will lead to improved health outcome. However, to achieve proper and accurate diagnosis a robust, rapid diagnosis test is needed[5]. Once we have a

diagnosis test with these characteristics efficient clinical and epidemiological management of infections can be made[34].

There is an urge to develop a tool both sensitive and specific that allows an easy screening in endemic regions, capable of identifying asymptomatic cases[35]. This diagnosis method should also be accessible, providing the possibility of doing periodic monitorization.

The process to diagnose should be automated, requiring inexpensive reagents and minimal operator intervention without diminishing the fidelity of the results. Considering that this disease affects mostly less developed countries the tests should be cheap and easy to perform in difficult field conditions[5,36].

4. Diagnosis methodologies

Accurate diagnosis still remains a problem to disease control since leishmaniasis presents a wide range of manifestations[37]. Clinical manifestations can also be confused with other illnesses often common in *Leishmania* endemic areas such as malaria, toxoplamosis and tuberculosis[38].

Many Leishmaniasis noninvasive diagnosis are available but none has both specificity and sensitivity to diagnose in endemic areas. We must have into account that age, medical history and host immune system response are crucial parameters in diagnosis.

According to WHO, diagnosis of Leishmaniasis can be confirmed with more conventional laboratorial techniques such as visualization of parasite in tissues by microscopic examination of the stained specimen or *in vitro* culture of the parasite from biopsies or aspirates from lesions, lymph nodes, spleen and bone marrow and other diagnosis methods that include molecular detection of parasite DNA in tissue samples and serological tests that detect anti-*Leishmania* antibodies[9].

4.1 Parasitological diagnosis

Parasitological diagnosis of Leishmaniasis is specific and remains the gold standard to diagnose this infectious disease[39].

One of the reference standards for diagnosis is the demonstration of parasites in tissue samples from bone marrow, skin lesions, liver, lymph nodes and spleen. Diagnostic is obtained by observation of the *Leishmania* amastigote forms in stained microscopic preparations with Giemsa[6].

The best results are obtained with spleen aspirates (93%-98% of sensitivity). When it comes to bone marrow aspirates, the sensitivity decreases to 60%-85% and worse results are obtained with lymph nodes aspirates (sensitivity ranges between 52% to 58%)[4].

Besides this method requiring trained personnel and its sensitivity being variable, it involves invasive sampling being a risky procedure that can lead to fatal hemorrhage so it must be carried out in settings with access to surgical facilities. Lymph node and bone marrow aspirates are safer but the material obtained is more diluted and therefore less sensitive, raising the risk of diagnosing false negatives[40].

The culture of parasite from infected tissues is another classical confirmatory test for VL. A major problem with this technique is that different species of *Leishmania* have different growth factors and contaminations are recurrent. Despite being more sensitive than microscopic examination, it is time consuming and expensive, so it is rarely used for clinical diagnosis[12].

Identification of amastigotes by direct examination of aspirates must be done by experts because the results are dependent on the observer[9]. It can also harvest false negative results because of the low number of parasites in some samples, particularly in asymptomatic cases, where the parasite charge is lower.

4.2 Serological diagnosis

Serological tests are based on the screening of antigens or antibodies.

The first approach is an excellent method to diagnose an infection since is more specific that antibody-based immunodiagnostic tests. Thus, antigen levels are expected to correlate with parasite charge, and this method might be useful when antibody prediction is deficient. Besides, this approach should avoid cross-reactivity and should distinguish active from past infections.[6] However, this technique is still unreliable at the moment (lack of specificity and variable sensitivity), so efforts are being made to improve this tool, as it stands as a promising approach.

Currently, most clinical and surveillance laboratories of the developing world use serological techniques to detect pathogen-specific antibodies, since direct methods are either invasive and potentially fatal or expensive.

VL infection is characterized by the presence of humoral response that leads to the production of antibodies specifically against to *Leishmania* spp.. Thus, serological methods to detect anti-*Leishmania* antibodies are useful as alternative diagnosis tests

for both human and canine *Leishmania* infections[27]. They have the advantage of being easily applied to a large amount of samples with specificity and sensitivity, mostly used in seroepidemiological studies[41].

The presence of anti-*Leishmania* antibodies in both asymptomatic and symptomatic infected dogs has allowed the development of agglutination tests, immunofluorescent serologic tests, such as Western blotting, immunochromatographic tests, and enzyme-linked immunosorbent assays (ELISAs).

The sensitivity depends on the methodology but the specificity will always depend on the antigen used. In most serological tests, the sensitivity and specificity data are compared against the standard methodology [16].

Serological tests are the elected to diagnose CVL. Nevertheless, when dogs present low antibody titers these tests lack sensitivity and specificity. Is not unusual to occur cross-reactivity too[42]. Moreover, after a successful treatment, it takes a while till antibody levels decrease so it can mask a relapse, making impossible to diagnose it.

4.2.1 Direct Agglutination Test

As an attempt to substitute the risky procedure of splenic aspirates, a noninvasive serological test was developed. Direct agglutination test (DAT) was the first antibody detection test used for VL diagnosis and has been used for more than 25 years.

DAT is a simple semi-quantitative diagnostic tool used in many developing countries with a high sensitivity (91-100%), specificity (72-100%), accuracy, reliability and inexpensiveness[4,43].

DAT detects parasite antibodies in the blood or serum of those infected through direct agglutination. Whole Coomassie-stained promastigotes, either in a suspension or in freeze-dried form are incubated with serial dilutions of serum. If the result is negative (absence of anti-*Leishmania* antibodies) DAT antigen accumulates at the bottom of the plate. If there are anti-*Leishmania* antibodies present, the antigens form a film over the well (positive result). In a positive reaction, agglutination can take till 18 hours of incubation to occur[9,41].

The disadvantages of DAT are requiring moderate technical expertise (the interpretation of the results depends on the person analyzing the results, creating inter-observer discrepancy)[44], serial dilutions must be done (requires a considerable volume of antigen) and relatively long incubation time[43,45].

DAT results remain positive long after the patient is cured (anti-*Leishmania* antibodies can persist for years as a result of a VL infection), so this test is not appropriate to detect relapses[6].

4.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an important serodiagnosis technique used in almost all infectious diseases, including VL.

These ELISA assays are based on the detection of antibodies present in the blood or serums. An antigen/recombinant protein is used to coat the plate and then the samples containing antibodies are incubated. To see the development of signal, a secondary antibody conjugated to an enzyme recognizes and binds to the primary antibody. After adding a substrate, a colorful product is developed and the results can be measured by optical density techniques.

This tool is frequently used to detect anti-*Leishmania* antibodies due to its high sensitive and specificity as well as good reproducibility and high throughput screening of large number of samples at affordable expenses. However, the specificity of this technique depends on the antigen used.

The sensitivity of crude soluble antigens (CSA) in an ELISA is high. Nevertheless, cross-reactions with serum from patients with trypanosomiasis, toxoplasmosis and tuberculosis can occur. VL diagnosed by ELISAs is based on crude soluble *Leishmania* antigens (SLAs). Though its high sensitivity, the specificity is low due to the antigens related with *Leishmania* and other pathogenic protozoa. It also has low competence to detect seropositivity in asymptomatic dogs[27,45,46].

ELISA's major disadvantage is its inadaptability for field conditions in resource-poor settings and its requirement for specialized operators.

4.2.3 Indirect Fluorescent Antibody Technique (IFAT)

Fluorescent antibody techniques are extremely valuable tools that allow evaluation of anti-*Leishmania* antibody titers produced by infected individuals. It is a quite useful method in epidemiological studies and in clinical practice[47].

IFAT is based on the use of fluorophores to detect antibodies present in the sample. It involves the use of a primary antibody to bind to the antigen and allow the formation of antigen-antibody complex and posteriorly the binding of a fluorophore-conjugated secondary antibody that results in an amplified signal that can be examined by fluorescence microscopy.

IFAT is the serological gold standard for the diagnosis of CVL in most countries around the Mediterranean basin. This methodology is different from ELISA and it resorts to the whole body parasite as antigen[47]. Although high specificity and sensitivity (100% and 90%, respectively), IFAT sensitivity is lower for asymptomatic infections when compared to ELISA. Also, the result interpretation depends on operator's expertise[46,48].

4.2.4 Immunochromatographic tests

Immunochromatografic (IC) tests are a great option to be used in large-scale surveys, in which antibody titers are not required. The results are always evaluated considering the epidemiological context of the area and the aim of the investigation[49].

IC test principal is similar to the one used in ELISA but the main difference is that the reaction occurs in chromatographic paper by capillary action. Immunochromatographic tests are based in the formation of an antigen-antibody complex. Then, the sample migrates in the membrane and the labelled antibody gets in contact with the immobilized antibody in the membrane and it results in the formation of color. The appearance of color is indicator of the antigen presence in the sample.

IC tests are inexpensive, practical, rapid and suitable for field use. However, in resemblance to DAT, a significant portion of healthy individuals is diagnosed as positives in endemic regions after long cure period (false positive cases). So, patients with suspect of relapse are not candidates for diagnosis[49].

4.2.5 Flow cytometry

Flow cytometry (FC) is considered an emerging technology for the diagnostic of several infectious diseases. It usually refers to the measurement of cells but the approach of making optical measurements in a flowing sample stream is a general analytical approach[50].

Flow cytometry is a technique based on the analysis and sorting of cells or particle suspensions in a controlled fluid stream through the measurement of fluorescence and scatter induced illumination. Using specific fluorescent markers, flow cytometry can be used to the quantification of structural and functional parameters on a single-particle basis.

Flow cytometry technique can quantify specifically the antibodies against *Leishmania* surface antigens, restraining potential cross-reactivity against more conserved intracellular structures. All the approaches to date used live or fixed promastigotes as targets to detect specific antibodies[51].

Among others, flow cytometry has high throughput capacity, possibility of quantification, high reproducibility and sensitivity and potential for multiplexing[52].

Recently, alternative methodologies were proposed to improve the serological approaches. One of them is immunoglobulin G (IgG) flow cytometry to detect anti-*Leishmania braziliensis* antibodies in sera of active cutaneous Leishmaniasis patients [38]. This method has shown to be reliable, achieving high levels of sensitivity and specificity[51]. Studies have shown the FC potential to monitor postchemotherapy cure of VL, in order to evaluate the success of treatment. The antibodies which are detected in FC are the anti-membrane-specific antibodies and are only present during active disease which makes it a perfect technique to evaluate the effectiveness of the treatment[53].

4.3 Detection of parasite DNA

Polymerase chain reaction (PCR) technology has become an indispensable tool for the diagnosis of many parasitic diseases, including Leishmaniasis. Amplification-based methods include the conventional PCR (polymerase chain reaction) and qPCR (quantitative polymerase chain reaction)[54].

PCR is based on the amplification of specific parasitic DNA sequences. The sensitivity of a PCR assay depends upon three factors: the physicochemical conditions of the reaction, the concentration of the DNA target and the selected PCR primers[20,55,56]. It has higher sensitivity for asymptomatic animals and early-stage infections when compared to serological methods. In patients with compromised immune system, PCR is also more sensitive than the classical parasitological methods.

Some PCR drawbacks are collection of samples, storage conditions and difficult access to sophisticated equipment[57]. Lack of standardization in the selection of target *Leishmania* DNA sequences and experimental protocols used worldwide also makes it harder to compare the sensitivity and specificity of these tests.

Real-time quantitative PCR (qrtPCR) is an innovative technology that has revolutionized molecular diagnostics by adding sensitivity, speed, broad dynamic range of target DNA quantification and reduced contamination. When compared with conventional PCR, it has the major disadvantage of costing three times more[55,58].

As we have seen above, every technique has its downsides.

Parasite demonstration in tissue smears and culture provide definitive diagnosis of VL, but generally has lower sensitivity than serologic methods. Particularly, splenic aspirate has the highest sensitivity of available tissue sampling techniques, but it has a risk

associated. Moreover, microscopy techniques lack sensitivity, whereas culture requires long time to obtain a result and is vulnerable to contamination[59].

Molecular diagnostic tools like PCR and real-time PCR are quite sensitive and specific but are difficult to perform and have a high cost.[6] So, its use remains largely restricted to some hospitals and research centers[37].

Serological tests are often not sensitive enough to detect asymptomatic individuals so they have to be combined with classical methods of diagnosis to confirm. ELISA, IFAT, DAT and rK39 immunochromatographic strip test (ICT) are highly sensitive and specific when analyzing active VL in immunocompetent individuals. This doesn't happen when titers decline and parasite charge is lower. Due to these reasons, tests give false-negative results frequently in immunocompromised patients and for asymptomatic *Leishmania* infections[60].

For those reasons, it is crucial to identify and produce new proteins capable of detecting asymptomatic Leishmaniasis and individuals in early stages of infection in VL endemic regions.

5. Recombinant proteins and novel biomarkers

Every diagnosis platform has its detection limits and sensitivity but these factors are also dependent on the specificity of proteins for producing high confidence results with lower detection limits. There is an urge to develop a marker for the active diseases[6].

Antigens related to the disease are discovered and validated through genomics and proteomics research. The development of new technology to diagnose can enable rapid introduction of these new antigens into clinical practice[36]. This tool must provide a signal of presence or absence of a particular antibody but also provide quantitative information[36].

The main problem with Leishmaniasis diagnosis is identification of markers that can detect the presence of disease since early stages until recovery after treatment. Seroconversion does not happen right after infection so serological tests may lead to false-negative results. New recombinant antigens are needed to contribute for a more accurate Leishmaniasis diagnosis and hard work has to be put in the development of a more sensitive and specific recombinant protein-based immunoassay capable of detecting asymptomatic cases in large screening studies[61,56].

Recently, several *Leishmania spp.* proteins were cloned, purified, and characterized to improve the diagnosis of Leishmaniasis[62,63]. The major advantages of using recombinant

proteins for the diagnosis of Leishmaniasis infections are: knowing the precise antigenic composition applied to the serological tests; good reproducibility and easy method standardization[23,64,51,63].

Using recombinant polypeptides containing specific *L. donovani* / *L. chagasi* epitopes that elicits an immune response in the majority of dogs and humans with VL can overcome some cross-reactivity problems[11]. Moreover, the variability in the humoral response concerning different parasite antigens observed in infected dogs suggests that a combination of recombinant proteins can improve the diagnosis efficiency[65].

Different families of proteins like kinesin-related proteins, heat shock proteins, nuclear proteins, ribosomal proteins, enzymes and other antigens were evaluated regarding their performance (sensitivity and specificity) of immunological diagnosis[40].

Recombinant protein rK39 (39-amino acid repeat of a kinesin-related protein), highly conserved in *Leishmania* spp., is a great tool to the diagnosis of VL in HIV patients and a prognostic indicator for monitoring patients undergoing drug treatment[64,59,61]. An important aspect of anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potential for use in predicting response to therapy[16]. Among the recombinant antigens, this one showed promising diagnosis and has been extensively tested in the last 5 years with IC tests in several Leishmaniasis endemic areas[4,6], with several commercial applications (Appendix 1). rk39 has also been tested for ELISA and FC assays showing high sensitivity and specificity in detecting clinical forms of CVL[52].

However, rK39 doesn't have the same accuracy in asymptomatic cases with proven infection. Several proteins such as *Leishmania infantum* cytosolic tryparedoxin peroxidase (LicTXNPx, also known as CPX), a member of enzymatic *Leishmania* cascades for detoxification of peroxides expressed in all stages of parasite development, and rK28 (a synthetic gene generated by fusing multiple tandem repeats of haspb1 and k39 genes) were already described as valuable tools for the detection of infection[6,23]. LicTXNPx antigen has a highly immunogenic probe during both human and canine infections. Previous studies indicated that anti-LicTXNPx antibodies were present in both symptomatic and asymptomatic experimental canine infections, making this antigen a good candidate marker and a prognostic indicator for monitoring the response to VL treatment[64].

When testing rk39 and LicTXNPx in ELISA, rK39 demonstrated a better performance in the symptomatic group[23] whereas LicTXNPx showed a better performance in the detection of infections in asymptomatic infected dogs. An ELISA with both LicTXNPx and rk39 antigens (LAM-ELISA) was performed and both symptomatic and

asymptomatic dogs had higher specificity (96.3%). LAM-ELISA is a simple and sensitive (87.1% sensitivity) test that associated with DAT may be a valuable tool for screening CVL[23,27].

Attention to extracellular vesicles (EVs) has been rising in the last years as they are believed to be potential biomarkers to use in the diagnosis of diseases[66]. Our group has been working with EVs as we are interested in the proteic material contained in exosomes and its great clinical impact potential in nanomedicine[67]. This type of vesicles is naturally released from cells and its context of infection is not still very clear[68]. Particularly, the exoproteome of *L. infantum* is composed by two main fractions: the vesicle fraction and the free protein fraction. It is believed that extracellular proteins derived from the parasite have an active role in host-parasite interaction[69]. This is an indicator that EVs can be used as future markers for infectious diseases.

6. Biosensors

A major obstacle in defining prevalence and incidence of infection is the lack of an easy and accurate tool for the detection of infected individuals[35].

Since many infectious diseases spread quickly before any symptoms are identified, the diagnosis device must be a sensitive analytical tool that can easily go down to very low detection levels of antibodies without losing selectivity. Biosensors meet these field requirements as they are simple, cheap, robust, accurate, rapid and provide high-throughput[33]. Even more, they offer the possibility of real-time monitoring, and the distribution of these diagnosis tools in the field provide a rapid infection detection[33] of the disease that has a significant effect on the success of disease spreading, control, or eradication[5].

A biosensor is a sensing device containing a bio-recognition element that interacts with a biomarker and a transducer that converts the changes in its physicochemical properties (optical, thermal, electrical, and thermodynamic properties) into an electrical signal (Figure 4). The biosensors can be classified based on the type of bio-recognition element or the transducing method used[5]. Based on the bio-recognition element, the biosensors can be classified as enzyme sensors, immunosensors, nucleic acid probe (DNA and RNA) sensors, or cell-, tissue-, or organelle-based sensors[70]. Based on the transducing method, biosensors can be classified as piezoelectric, optical, or electrochemical biosensors[5].

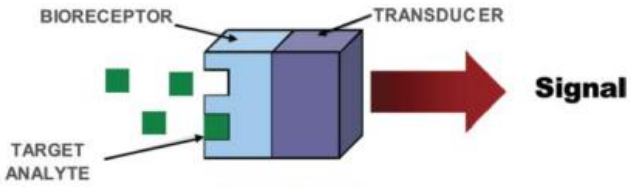

Figure 4 - Schematic diagram of biosensor elements: target analyte; bioreceptor; transducer. Image adapted from "The role of biosensors in the detection of emerging infectious diseases", 2006 [33]

There are several factors that affect the efficacy of the biosensor. Thus, all characteristics that influence the accuracy of these biosensors (biomarker/bio-recognition element ligation, surface preparation/immobilization conditions, incubation time, temperature, etc.) have to be optimized during the development process.

One of the major problems with biosensor are matrix interference and adsorption of blood components onto the sensor surface. Furthermore, biosensors have to be robust and work outside laboratories to be applied to real-time detection of infectious diseases in biological samples (i.e., blood)[33].

As we intend to develop a biosensor to detect an infectious disease and the host immune response to the parasite involves a humoral response and the development of antibodies against it, we decided to focus our attention on immunosensors in this revision.

6.1 Immunosensors

Antibody-based sensors, also known as immunosensors, are well accepted due to the specificity of antibody-antigen reaction to produce a change in the transducer signal[5]. They are considered more versatile than enzyme-based biosensors, because the antigens can be produced specifically to any biomolecule of interest[71]. These biosensors are ideal as they rely on the high affinity between the antibodies and the antigens (recognition element), resulting in very low detection limits[72,73], with less level of interferences, non-destructive approach to sample, stability, good precision and high sensitivity[74]. These devices should be compact and allow simple operation[75].

The disadvantage of these immunosensors is that the antigen is not easily released from the antibody after the measurement has been made. Thus, several strategies have been applied to design inexpensive biosensors[71]. Another option involves the use of a flow cell configuration in which the immunochemicals can be partially removed from the sensor before the next measurement[71].

Independently of the biosensor's type, immobilization of the bioreceptor onto a solid support is a crucial aspect in determining the overall performance of the device[33].

To obtain a diagnosis device with the characteristics mentioned above, we have to overcome some drawbacks regarding sensitivity, stability and longevity that mostly depend on the amount of the immobilized immune molecules on the surface, their conformational stability, remaining activity after the immobilization procedure and their orientation on the sensor surface[74]. The last is particularly important since immunoglobulins are asymmetrical molecules and wrong immobilization can lead to hindered interaction with the biomarker. Highly controlled orientation immobilizations are preferable to maximize their antigen-binding efficiency and achieve the best immunoassay sensitivity and selectivity. Oriented binding may require many chemical modifications that decrease the control over the nanostructures[76].

There are three main methods of immobilization: adsorption, entrapment and covalent binding.

Adsorption is a rapid and simple procedure, especially for disposable biosensors. This physical type of immobilization is based on Van der Waals attraction between biomolecule and the solid support surface. The most relevant drawbacks of this technique are that bonding forces between biomolecule and support are weak and an even monolayer cannot be guaranteed. As a consequence, the biological component can be leached during the assay, depending on experimental conditions such as pH, ionic strength, temperature and solvent[71].

Electropolymerization is an easy and attractive example of the entrapment technique. A biological molecule is homogenized in a monomer matrix and this is then deposited by electropolymerization[71].

Immobilization cross-linking, or covalent immobilization, is used to coat electrode surfaces with specific biotransducer molecules. Covalent immobilization of the biorecognition component (enzyme, antibody, cell, etc.) can be achieved by use of the self-assembled monolayer (SAM) procedure[71]. The method is based on the formation of three-dimensional links between the biological material and multifunctional reagents. Antibodies can be directly immobilized on the surface of the transducer by covalent bonding through amino, carboxyl, or aldehyde groups[5]. This technique has several advantages, such as stability and reusability of immunosensors due to covalent bonds[74].

Several immunosensor technologies advances have been made in the last years, with distinct notability to transducer approaches and their clinical potential such as for the detection of *Salmonella pullorum*[77], hepatitis B[77,78] and dengue[79].

Specifically for *Leishmania* spp., some developments have been made. A biosensor for Leishmaniasis had already been developed, in which proteolipossomes from mice were immobilized in interdigitated electrodes[80]. The concentration of antibodies anti-*L. amazonensis* was detected by EIS down to a concentration of 10^{-5} mg/mL. However, this specie of *Leishmania* has its clinical manifestations as cutaneous and mucocutaneous Leishmaniasis[80]. A piezoelectric immunosensor for detecting *L. chagasi* antibodies from canine serums was developed[81]. This immunosensor was capable of detecting antigen concentrations up to 3 µg/mL and to recognize antibodies anti-*L. chagasi* in canine positive serums up to 1:3200 dilution resorting to a quartz crystal microbalance[81]. Also capable of detecting canine serums antibodies of *L. infantum*, a surface plasmon resonance based immunosensor was successfully constructed detecting antibodies up to 1:6400 dilution[82].

Even though the two previous techniques had good results they were only tested for a limited number of positive samples of infected Leishmaniasis canine from endemic areas and for non-infected dogs. Nevertheless, asymptomatic canine serums remained to be studied. The lack of further study in this type of samples and the fact that electroanalytical immunosensors are taking an important place in the detection of infectious diseases gave us the opportunity to do experiments and to construct an immunosensor that meets the needs of diagnosis. Due their potential and their progress in the last few years it was decided to use electroanalytical techniques as the preferential approaches to construct the canine leishmaniasis immunosensor.

6.2 Electro analytical biosensors

More than half of the biosensors used for the detection of pathogens are based on electro analytical methods. They can be classified as amperometric, impedimetric, potentiometric and conductometric methods based on the electrical parameters they measure.

They constitute, possibly, the most practical and quantifiable of all low-cost diagnostic assessments of protein presence. Electroanalytical methods provide accurate and rapid response times, they are easy to use, efficient, portable, and low cost. Electrochemical detection also offers selectivity, as different electroactive molecules can be oxidized/reduced at different potentials. Additionally, this technique is compatible with miniaturization methods, has minimal power requirements and is independent of sample turbidity and color[83]. Thus, it allows a rapid, permanent control and a direct transduction of the biomolecular recognition event into electronic signals[71,84].

6.2.1 Amperometric biosensors

Amperometric biosensors transduce biological recognition events caused by oxidation/reduction of the electroactive species into an electrical current (typically, in the nanoampere to microampere range) to quantify an analyte within a sample matrix[5].

Amperometric transducers are used to study the charge transfer between the interfaces of phases. One of the half-cell reactions within the electrochemical cell is carefully controlled to study the changes in charge transfer at the interface of the other half-cell reaction, known as the working electrode. On formation of the overpotential, electron transfers occur and oxidative or reductive reactions follow. These processes are known as Faradaic processes. Other processes as the development of a layer, change the interfacial surface but do not cause charge transfer across the interfacial boundary, are termed non-Faradaic processes[85].

The aspects that influence the reaction rate are: concentration of the analyte and other species within the matrix at the interface; mass transport of species from bulk solution to the interfacial boundary; electron transfer across the interfacial boundary; electrode interactions such as adsorption and electrodeposition; and external factor like temperature.

In voltammetric methods, the potential applied to the electrode-solution interface changes as a function of time, together with the measurement of the current. Cyclic sweep voltammetry is often the chosen method for information acquisition such as oxidation/reduction potential, kinetics, and reaction mechanisms (Figure 5). Among all the electrochemical characterization methods, pulse strategies such as Square Wave Voltammetry (SWV) are possibly the most sensitive and the most used in electro analysis. The main advantage of pulse techniques resides in the different decay rates of the charging and faradaic currents. Charging current decays more rapidly compared to the faradaic current, during each pulse, so the capacitive current is negligible, allowing lower detection limits. Numerous studies relate detection of pathogens and the use of SWV[86].

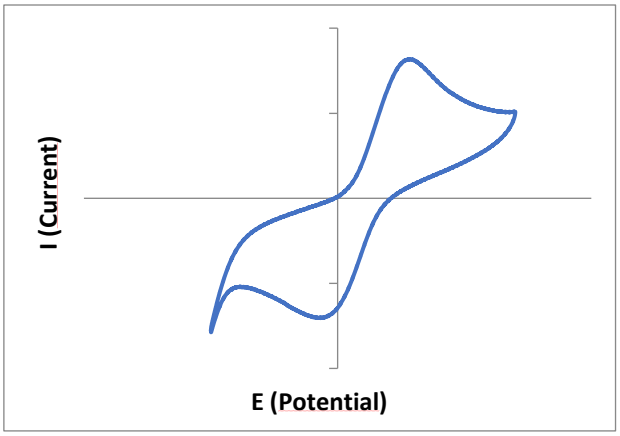

Figure 5 - Representative voltammogram resulting of a cyclic voltammetry

6.2.2 Impedance biosensors

Impedance spectroscopy is another electrochemical method that has also interest for the detection of infectious diseases.

To simplify, the real part of impedance is linked to resistive processes and the imaginary part to capacitive processes. In Nyquist plots, the charge transfer resistance (Rct) of impedimetric biosensors can be measured based on the calculation of the diameter of the semi-circle of the real part of impedance[87] (Figure 6). Rct represents the electrostatic and/or steric barrier presented to the redox probe at the surface as the films are being constructed[79]. As the sensing layer is being designed and layer-by-layer assembly processes on to a sensor surface occur, the Rct increases[79].

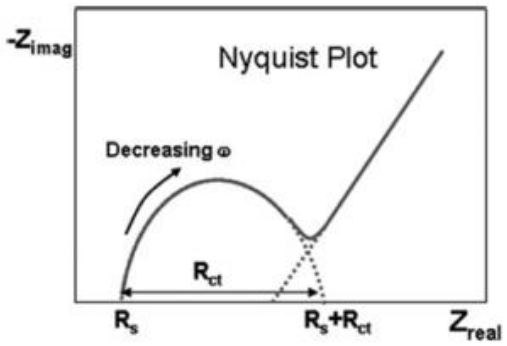

Figure 6 - EIS Nyquist plot (Zimag against Zreal). Image adapted from “Electrochemical biosensors and nanobiosensors”, 2016, Portland Press Limited [85]

Electrochemical impedance spectroscopy (EIS) is subdivided in two main categories: Faradaic and non-Faradaic. In the Faradaic measurements, redox probes are applied in the experiment and the analysis is based on charge transfer resistance changes generated by the blocking presence of the analyte when it binds to the surface. In non-

Faradaic EIS exploits charging currents; redox probes are not used and double layer capacitance changes upon target binding is the main method of analysis[85].

Impedance detection involves measuring the change in impedance (combined measurements of the charge transfer resistance and interfacial capacitance) caused by the binding of biomolecules to receptors (antibodies, DNA, proteins, etc.) immobilized on the surface of electrodes. Changes in the surface properties can be linked to the biochemical changes[33]. EIS is a direct method for probing classical antigen–antibody binding events with good sensitivity and reproducibility[79,88,89].

During the last decade, the number of EIS applications in biosensing (detection of cancer and other disease biomarkers, polluting agents, bacteria, toxins) has increased making EIS one of the most preferred electrochemical techniques.

6.2.3 Potentiometric biosensors

Potentiometric sensors for the detection of infectious diseases have received a great deal of attention. This type of sensors have the longest history and are distinguished for having the largest range of applications[33]. Potentiometry with ion selective electrodes is a well stablished technique, frequently used in pharmaceutical, food, ambient, biological and other type of analysis[90].

Great efforts are being made to improve immunoassays to fit the requirements in clinical diagnosis. Potentiometric sensors are those that meet these characteristics the most. Besides being portable and affordable devices, they are highly selective and sensitive, easy to use and have been already applied in some clinical immunoassays[91,92]. Sensors with potentiometric transduction have unique characteristics and valuable possibility of measuring ionic activity, the measurement of a specie in a certain oxidation state with high analytical sensitivity, in a wide range of concentrations. Besides, the rapid response highlights this analytical technique for analytical control processes as a replacement for of conventional methodologies that are more expensive and time consuming.

Potentiometric transduction is based on the measurement of potential differences occurring in the electrochemical cell composed by the reference and working electrode, both in contact with the sample, in electrical current absence. A change in electrode potential can be used as the quantifiable transducer response in potentiometric sensors. Potentiometric measurements provide a dependence between logarithmic concentration and potential. This detection method is sensitive, time-effective and economical.

The activity alteration of an ion in solution results in an alteration in the chemical system potential that is measured relative to the reference electrode. The relation between ionic

specie activity and potential differences can be translated by the Nernst-Nicolsky equation (Equation 1):

$$E = E_0 + \frac{RT}{z_A F}\log a_A \qquad \text{T=298,15K}$$

Equation 1: Nernst-Nicolsky equation. E corresponds to potential difference obtained by the working electrode in relation to the reference electrode; E_0 represents the sum of all contributions in the measurement cell, namely, selective electrode potential, potential of the reference electrode and liquid junction independent of sample that corresponds to the abscises interception of the linear response; R is the rare gases constant, 8,3144$JK^{-1}mol^{-1}$; T is the absolute thermodynamic temperature , K; F is the Faraday's constant, 9,64846x$10^4 Cmol^{-1}$; z_A is the primary ion charge; a_A represents the activity of the primary ion expressed in concentration units.

6.2.4 Conductometric biosensors

Conductometric biosensors rely on the fact that the majority of enzymatic reactions lead to the consumption or production of charged species, changing the ionic composition of the solution[93].

The conductivity of liquids results from the dissociation of the electrolyte into ions and the migration of the latter induced by an electrical field. When a potential difference is applied to the electrode, the electrical field regenerated induces the cations moves towards the cathode and the anions moves towards the anode. Thus, the current in the electrolyte is caused by the ion movement towards the electrodes and is determined by the sum of the ions present in the solution[93].

This type of biosensors does not require reference electrode, involve low voltage, involve inexpensive technology and permit the analysis of a wide spectrum of compounds[93].

7. Nanotechnology

During the last decade, many research in the field of nanotechnology has been made, especially in the nanoparticles and optical detection methods. This top edge research of study can be applied to drug delivery, *in vivo* imaging (mainly in cancer diagnosis) and *in vitro* diagnosis (molecular diagnostic, single-cell or molecule identification/signaling, biomarkers detection)[94,95]. Nanotechnology involves the use of materials and systems on the nanometer scale, and nanodiagnostics are defined as the use of nanotechnology for clinical diagnostic purposes. The use of nanotechnologies for diagnostic applications intends to meet the sensitivity and cost-effectiveness required by clinical laboratory[96].

Most nanotechnology-based assays are adaptable to automation which makes them very appealing diagnostic tools. The fact that most biological molecules and cell organelles and structures fall within the nanometer scale empowers the potential of

nanodiagnosis, since it offers a wide range of tools and applications[95,96]. Nanoscale materials offer excellent prospects for designing powerful bioanalytical protocols with remarkable sensitivity and multiplexing/coding capability[36,94]. Nanoparticles possess certain size-dependent properties, particularly regarding optical and magnetic parameters which can be manipulated to achieve detectable signals. The signal enhancement associated with the use of nanoparticle amplifying labels and with the formation of nanoparticle-biomolecule assemblies provides the basis for ultrasensitive electrochemical detection[36].

Nanoparticle-based assays are based on the binding of a nanoparticle label to the target biomolecule that will produce a measurable signal or using nanoparticles as transduction materials. Nanotechnology promises to play an important role in the future development of diagnostic and therapeutic methods. However, many aspects of these nanodiagnostics techniques need to be evaluated further, especially the safety issues[96].

During this work, we tried to miniaturize the biosensor using screen-printed electrodes and make them biocompatible for *in vivo* measurements. Among other approaches, we started developing a biosensor that resorted to microfluidic devices as they possess great advantages as smaller quantities of test samples and reagents, drastic reductions in test times due to the possibility of automation of various process steps.

Our first approach for the immunosensor development was based on the immobilization of the protein of interest on magnetic microbeads modified with nitrilotriacetic acid (NTA).This technique resembles a flow cytometry protocol already established by our group[52].

In parallel, we focused on an exploratory study to evaluate the protein type and content present in exosomes, released in the different stages of disease (healthy humans, symptomatic patients, asymptomatic patients). These proteins could act as innovative biomarkers and be applied to biosensors, improving the sensitivity of these tools.

The main goal of this work is to develop an accurate, specific and sensitive diagnosis tool capable of detecting early stages of disease and asymptomatic cases of canine Leishmaniasis.

Methods

1. Reagents & Materials

De-ionized or ultrapure water (conductivity < 0,1 µS.cm^{-1}) was employed throughout this work and all chemicals were of analytical grade. Potassium hexacyanoferrate III, potassium hexacyanoferrate II trihydrate and sodium hydrogen phosphate dihydrate were obtained from Riedel-de Haën; acetonitrile and pyrrole were purchased from Sigma-Aldrich; lithium perchlorate anhydrous was obtained from Fluka Chemika; magnetic microbeads (5x10^{7} mg/mL particles in the suspension) were purchased from Kisker Biotech GmbH & Co. KG; screen-printed carbon electrode DS110 (SPCE) were from Dropsens and NdFeB magnet with 15,7 N force were obtained from Supermagnete, Germany. Fetal Bovine Serum (FBS) was obtained from Lonza; Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich; reagent A (copper tartrate) and reagent B (Folin reagent) were purchased from Bio-Rad; RPMI base was obtained from Gibco; SDM-79 base[97]; hemin was obtained from Sigma-Aldrich and the centriprep Ultracel YM-3 filtering units were purchased from Millipore. Nitrocellulose membranes were purchased from Bio-rad and ECL Plus chemiluminescent substrate was obtained from Amersham Bio-sciences. Polyacrylamide, secondary antibodies (anti-dog and anti-human IgG conjugated to horseradish peroxidase), Tween 20 and o-phenylenediamine (OPD) were obtained from Sigma-Aldrich; hydrogen peroxide (H_2O_2) was purchased from Merck; HCl was obtained from Fisher Scientific. Preparation of Citrate Buffer was performed with citric acid monohydrate and trisodium citrate dehydrate both obtained from Sigma-Aldrich. Carbonate buffer solution was performed with sodium bicarbonate and sodium carbonate decahydrate from Sigma-Aldrich. Beads Latex aldehyde-sulfate 4% w/v 4 µm Ref A37304 were purchased from Invitrogen.

2. Immunosensor development

2.1 Electrogeneration of a Poly(pyrrole)-NTA Chelator Film

NTA-pyrrole monomer was synthetized according to the supporting information of the article “Electrogeneration of a Poly(pyrrole)-NTA Chelator Film for a Reversible Oriented Immobilization of Histidine-Tagged Proteins”[98].

The NTA- pyrrole monomer solution was prepared with 4 mM of this monomer in a 0,5 M of lithium perchlorate anhydrous ($LiClO_4$) and acetonitrile.

A pyrrole solution (0,1 M) was prepared and dissolved in 0,5 M of lithium perchlorate anhydrous and acetonitrile.

Polymerization of both NTA- pyrrole monomer and pyrrole solutions were tested with chronoamperometry, in which a 0,9 V current was applied for 5 seconds or 20 seconds until a polypyrrole film with the thickness of 1 mm was formed; and cyclic voltammetry, in which a repeated cycling over of potentials (3 scan rates) in a range of -0,5 V to 1,5 V at a scan rate of 0,1 V/s was performed with the same purpose. For this, a screen-printed carbon electrode (SPCE) was used and 100 µL of the described pyrrole solutions were deposited on the electrode.

2.2 Development of an Electrochemical Immunosensor based on magnetic microbeads

The magnetic microbeads are 1,7 µm size, spherical and paramagnetic in nature. These microbeads are easily retained when a magnetic field is applied and quickly resuspended when released from the magnetic force. It is also important to mention that no significant magnetism is retained after exposure to the magnetic fields.

2.3 Preparation of antigen-coated magnetic microbeads

47,2 µL of ultrapure water and 2,8 µL of beads were pipetted to an eppendorf and vortexed for 30 seconds. The beads were retained in the eppendorf with the help of a magnet (at least 30 seconds of contact) and the supernatant was aspirated with a micropipette; the beads were resuspended in 100 µL of ultrapure water and vortexed for 10 seconds and the supernatant was removed as previously described. Then, the beads were resuspended again in 100 µL of PBS and the same procedure was followed. Finally, the beads were resuspended in 100 µL of PBS and posteriorly diluted 10 times to obtain $5x10^6$ particles/100 µL.

50 µL of the previously described beads suspension were pipetted to an eppendorf and 2,5 µg of CPX2 was added. Vortex gently for 20 seconds. The antigen incubation with the beads happened overnight (ON) at 4 ºC. Furthermore, the beads in each eppendorf were retained with the help of a magnet and the supernatant was removed. For blocking, 100 µL of FBS 10% (in PBS) were added to the eppendorf and incubated 1h at 37 ºC. After the incubation, the washing steps previously described were performed twice with 500 µL of PBS.

2.4 Sample incubation

100 µL of serum (diluted from a range of 1:15000 to 1:250 in PBS for canine sample, depending on the experiments) were added to each eppendorf containing the antigen-coated magnetic microbeads and the samples were left to incubate for 30 minutes at room temperature (RT).

The samples were kindly provided by Dr. Luís Cardoso (UTAD). The symptomatic canine serums were obtained from dogs naturally infected with *L. infantum* from Portuguese endemic areas.

2.5 Microfluidic platform incorporating magnetic field controller

A magnetic field controller was designed and produced in our laboratory, as it is shown in Figure 7:

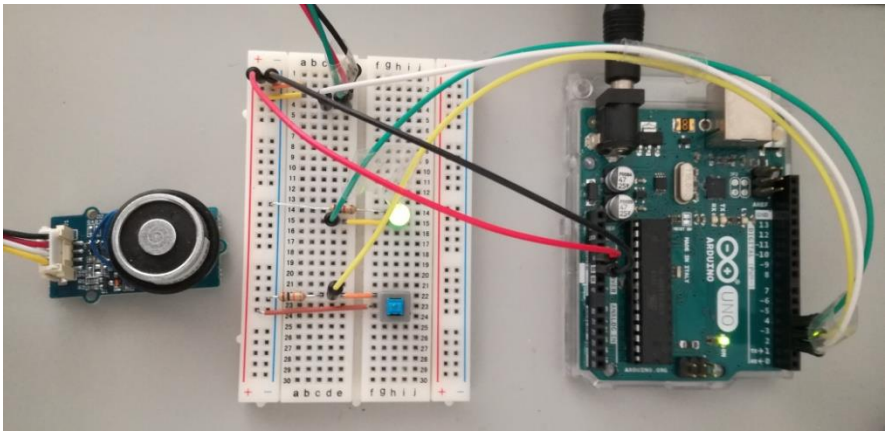

Figure 7 - Magnetic field controller

In parallel, a microfluidic platform was also designed and constructed.

One channel ($Ø_{int}$=0,5 mm) was drilled in a single acrylic plate (3 x 2,5 x 0,5 cm) in order to be used to introduce the samples in the microfluidic platform and confined to the SPE. Perpendicularly, another channel was drilled with the same diameter as waste. The SPE surface that contains the reference, the working and the counter electrode was accommodated in a chamber, with 0,07 mm of height and with a $Ø_{int}$ of 8 mm. This chamber was sealed with an O-ring with the same internal diameter. Another single acrylic plate with the same size and with the same dimensions of the chamber was coupled to the previous and the SPE was introduced in between.

Flow lines were made with PTFE tubing ($Ø_{int}$=0,5 mm). The solutions were introduced in the microfluidic device by peristaltic pump (P), using a PVC tube with $Ø_{int}$=1,3 mm. The flow injection valve with a loop of 90 µL was controlled manually. Flow injection analysis (FIA) system was chosen due to the simplicity of managing and as it provides reproducible volumes of samples, assuring the presence of the redox probe (Fe^{2+}/Fe^{3+}) to build the base line. Other aspect taken into account was the adjustment of the loop length (sample volume).

The antigen-coated magnetic microbeads incubated with serum were introduced in the system through the loop of the FIA valve, propelled by a peristaltic pump. The

microbeads were injected through the flow lines till the arrival to the working electrode surface, where a magnetic field was activated. Below it is represented the assembly (Figure 8).

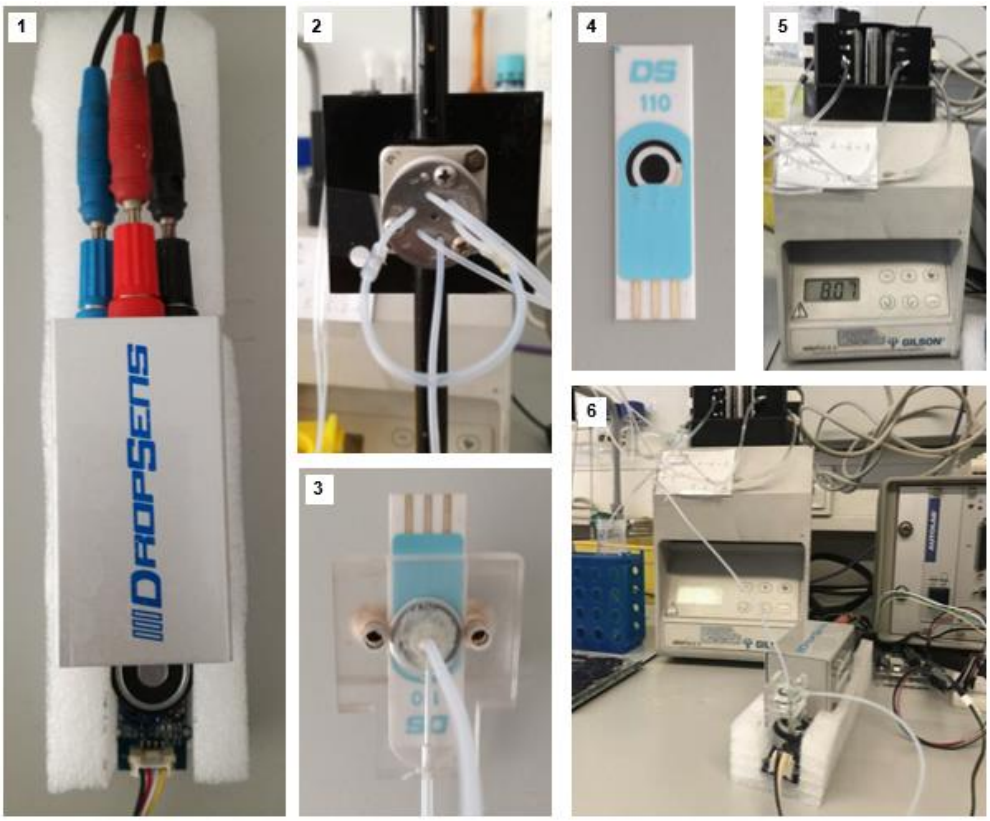

Figure 8 - 1. Dropsens switch box and electrical connections; 2. FIA valve; 3. Microfluidic Platform; 4. Screen-printed carbon electrode with silver pseudoreference electrode; 5. Peristaltic pump; 6. Assembled system

2.6 Measurements

The electrochemical measurements were performed using a potentiostat (Metrohm), model Autolab PGSTAT10 and was controlled by GPES 4.9.005 software. For these measurements, screen-printed carbon electrodes were used. Working and counter electrodes were made of carbon and the pseudoreference electrode and electrical contacts were made of silver. The diameter of the working electrode was 4 mm. For electrochemical assays, the SPEs inserted in the microfluidic platform, were placed in a switch box (DROPSENS) and the electrical connections were introduced in the potentiostat.

Square wave voltammetry (SWV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were conducted in a mixture of 1,0 mmol.L^{-1} of $[Fe(CN)6]^{3-/4-}$ prepared in PBS buffer, pH 7,0. In these, the potential was scanned from −0,5 to +0,7 V, at 50 mV.s^{-1}. In SWV studies potentials were changed from −0,5 to +0,7 V, at a 0,100 V.s^{-1}, corresponding to a frequency of 25 Hz.

Impedimetric analysis was conducted in an Autolab PGSTAT204 in NOVA 1.10 software, using the same probe solution, at 110 mV, with a number of frequencies equal to 50 and of amplitude 0,01 V. The frequency range was 0,1–100 KHz. Data were fitted to a Randles equivalent circuit using the implemented ANOVA software.

Below it is represented a scheme of the overall procedure (Figure 9):

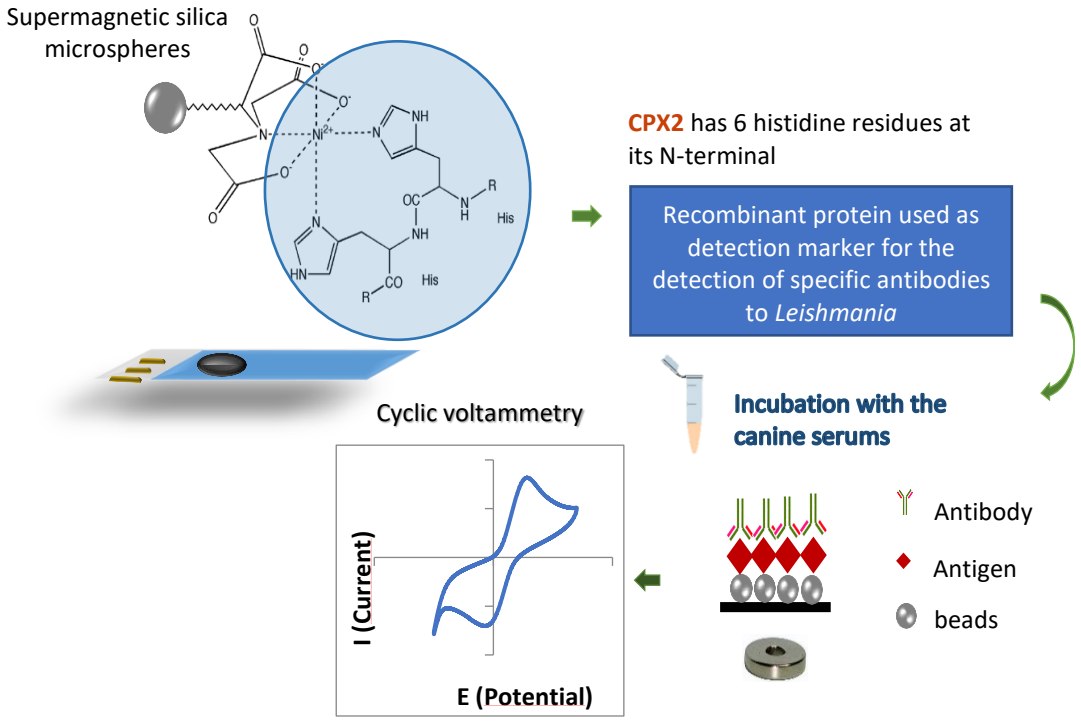

Figure 9 - Experimental procedure scheme. Incubation of supermagnetic silica microspheres coated with nitrilotriacetic acid and nickel ions with the histidine tail of CPX2. Posterior incubation of antigen-coated microbeads with canine serums for 30 minutes; injection in the microfluidic platform with the magnetic field on and reading of samples in the SPCE resorting to cyclic voltammetry.

2.7 Protein quantification

Protein quantification was based in a colorimetric assay similar to the well-Lowry assay. Several dilutions of a BSA containing from 0,125 mg/mL to about 4 mg/mL were performed to achieve a standard curve. The standard was prepared in the same buffer as the sample analyzed. 5 µL of standards and samples were pippeted to a microtiter plate and 25 µL of reagent A (copper tartrate) solution was added to each well. Then, 200 µL of reagent B (Folin reagent) was added into each well. Gently mix the samples and after 15 minutes, read absorbances at 750 nm.

3. Identification of new promising markers from human plasma exosomes to detect leishmaniasis asymptomatic cases

3.1 Recovery of extracellular material from *L. infantum*

Promastigotes used for exoproteome studies were maintained in cRPMI (RPMI base complemented with 10% SDM-79 base and 2,5 µg/mL of hemin), a medium developed by our group and validated for exoproteome studies. All cultures were grown with a starting inoculum of 1×10^6 parasites.

Logarithmic promastigotes with a startup inoculum of 1×10^6 in cRPMI were grown and the culture medium was recovered at 96 h. The parasites were removed by centrifugation followed by filtration through a 0,4 µm filter. The exoproteome was centrifuged at 10.000 g for 10 min at 4 °C to remove cellular debris, and further centrifuged at 100.000 g for 3 h. The vesicle depleted exoproteome (VDE) was recovered without disturbing the vesicle pellet, and then the final 1 mL of residual volume was decanted and discarded leaving only the vesicle pellet that was resuspended in 500 µL of PBS. The vesicles (EVs) were stored at either 4 °C or −90 °C depending on further intended use. The recovered VDE was concentrated to a residual volume of 500 µL using centriprep Ultracel YM-3 filtering units (Millipore). The samples were dialyzed twice against PBS using the same YM-3 filtering devices. After the second dialysis step, the VDE was again concentrated to a residual volume of 500 µL and stored at −90 °C (Figure 10).

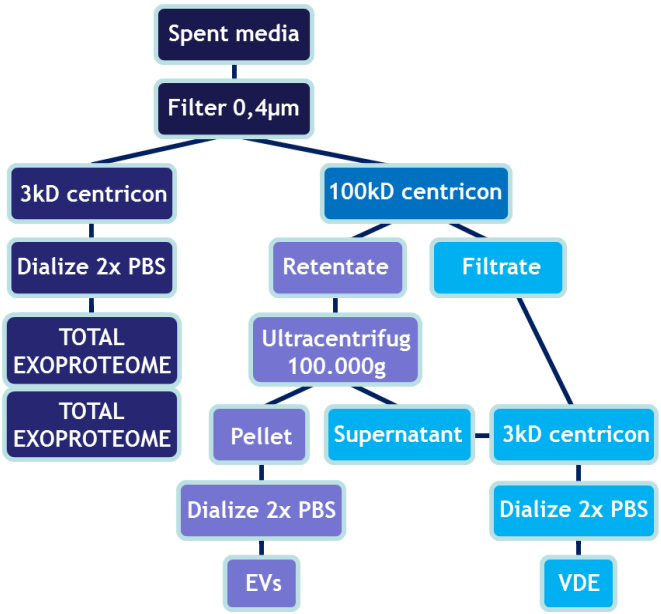

Figure 10 - Experimental procedure to recover extracellular material of *Leishmania infantum.*

3.2 Western blot analysis

Leishmania EVs and VDE were run on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. The blots were blocked overnight in 5% skimmed milk in PBS. Dog serums and human serums were diluted 1:250 in PBS containing 0,1% Tween 20 (PBS/Tween) and incubated for 1 h with the membranes. The blots were washed three times for 5 min in PBS/Tween and incubated with horseradish peroxidase-conjugated anti-dog/anti-human (1:500). The blots were washed as above, incubated with ECL Plus chemiluminescent substrate and exposed using the Chemidoc XRS system (Biorad).

3.3 ELISA - Enzyme-Linked Immunosorbent Assay

Flat-bottomed microtiter plate was coated with extracellular material (total exoproteome; extracellular vesicles; and vesicle depleted exoproteome) in a range of 0,0002 µL to 20 µL or soluble promastigote *Leishmania* antigens (SPLA) all diluted in 0,05 M carbonate buffer (pH=9,6) and dispensed 50 µL/well. The plates were incubated overnight at 4ºC. Posteriorly, the plates were washed several times with PBS-Tween (0,05%) and blocked with PBS-low-fat-milk (3%), for 1h at 37ºC. The plates were washed with PBS-Tween (0,05%) and positive and negative canine serums were diluted (1:1500) in PBS-Tween (0,05%)-low-fat-milk (1%) and incubated for 30 min at 37ºC. After washing the plates, the incubation was proceeded with secondary antibody (anti-dog/ anti-human IgG conjugated to horseradish peroxidase) diluted in PBS-Tween (0,05%)-low-fat-milk (1%) for 30 minutes at 37ºC, in dark. The plates were washed and the product was developed for 10 minutes in the dark at room temperature (RT) using o-phenylenediamine (OPD) in citrate buffer as substrate and hydrogen peroxide (H_2O_2) as reactive oxygen metabolic byproduct. Reaction was stopped with of HCl 3M and absorbance values were read at 492 nm in an automatic reader.

3.4 Sample collection

Plasma samples from symptomatic and asymptomatic *Leishmania* patients, were provided by Javier Morena from Institute de Salut Carlos III in Madrid, Spain (Table 1). As negative controls, it was used plasma from healthy donors, to compare the profiles of the infected and not infected patients.

Table 1 - Plasma samples from negative controls; asymptomatic; symptomatic and healed visceral leishmaniasis patients.

03/03/17	1AT	**Negative Control**	rK39-		
03/03/17	2CA	**Negative Control**	rK39-		
03/03/17	3AF	**Negative Control**	rK39-		
03/03/17	4A	**Negative Control**	rK39-		
02/02/17	608	**Asymptomatic**	rK39-	CPA+	Immunocompetent
16/02/17	621	**Asymptomatic**	rK39-	CPA+	Immunocompetent
16/02/17	8844	**Asymptomatic**	rK39-	CPA+	Immunocompetent
03/02/17	5878	**Active**	rK39+	Immunocompetent	
25/01/17	3935	**Active**	PCR +	rK39+	
25/01/17	4544	**Active**	PCR +	rK39+	
29/12/16	64047	**Healed VL**	rK39-	VIH+	
20/01/17	3081	**Healed VL**	rK39-	anti-TNF treatment	
20/01/17	3092	**Healed VL**	rK39-		

All the samples were stored in aliquots at -80 °C until the isolation of extracellular vesicles (EVs). Before performing the isolation of EVs, using Size Exclusion Chromatography (SEC), plasma samples were centrifuged twice at 2000 g for 10 min at 4 °C to avoid the presence of fibrins in the columns.

3.5 Plasma-EVs Isolation by SEC

A small aliquot of each sample before loading was kept to measure the total particle concentration by NTA.

Extracellular vesicles were isolated from 100 µL or 1 mL of platelet-free plasma samples using SEC. This method involved the preparation of a “homemade” Sepharose CL-2B column (1 mL or 10 mL) and the collection of serial fractions of the plasma samples. The SEC procedure was performed as previously described[99]. SEC would permit the separation of the extracellular vesicles by size and the first fractions would be enriched in the higher sized vesicles (microvesicles), then the exosomes enriched fractions would be obtained and further soluble protein fractions would be eluted.

Protein concentrations of the chromatographic fractions were measured by spectrophotometer NanoDrop (Thermo ScientificTM).

3.6 Exosome-characterization: Bead-based assay for FACS analysis

This method was performed based on conjugation of latex microbeads with extracellular vesicles of different sample origin and antibody coupling for FACS analysis. The exosomes obtained from plasma or culture, and isolated by SEC, were coupled to latex microbeads and then incubated with antibodies to detect the presence of exosome markers in our preparations.

The exosomes were labeled with fluorophore-conjugated antibodies and analyzed by FACS. For these characterizations, it was used CD5L and CD71 antibodies for the detection of exosome markers in our plasma samples, as they were previously used by the group for this type of identification. CD63 antibody was tried out to detect the presence of extracellular vesicles in the supernatant of promastigotes culture.

Samples were analyzed using a FACSverse flow cytometer (BD Biosciences, San Jose, CA, USA) and flow cytometry data were analyzed with the FlowJo software.

3.7 Nanosight

This technique measures the rate of Brownian motion of the nanoparticles using a NTA-Nanosight LM10 system (Malvern Instruments Ltd, Malvern, UK). The instrument is equipped with a 638 nm laser, a system of video capture and a particle-tracking software. It is used to determine EVs concentration and size. The fractions with highest fluorescence values to CD71 (exosome enriched fractions) were analyzed in this equipment. To obtain an accurate determination of the concentration and size of the vesicles the number of particles per frame must be in a range from 20 to 150. This implies making dilutions of the chosen samples to get the results.

Results

As mentioned before, one of the most crucial and important steps of building an immunosensor is to do the correct immobilization of the protein of interest. It was decided the use of CPX2 as the protein to be immobilized as it had already been synthetized in our laboratory and we had considerable volume to do several assays and run preliminary experiments. Although not being the more sensitive recombinant protein regarding symptomatic canine serums, CPX2 showed good results when it came to detect asymptomatic canine serums in ELISA. Furthermore, CPX2 alone could not be the best protein to immobilize in the immunosensor or could require the presence of other antigen but taking into account the resources available it was the best candidate to run the trials.

Regarding this matter, in this work three different approaches were followed:

1. Electrogeneration of a Poly(pyrrole)-NTA for Histidine-Tagged Proteins Immobilization

Conducting polymers are suited to the development of electrochemical biosensors as they provide immobilization of biomolecules maintaining its activity and also allow rapid electron transfer[100]. Among them, polypyrrole has good conductivity and good stability, which makes it a good polymer to produce conductive films[101,102,103].

Immobilized metal-ion affinity chromatography (IMAC) uses transition metal complexes of NTA to purify proteins with histidine tails. In resemblance to this technique, our aim was to immobilize an antigen through the formation of a complex between the ligands of the NTA and the protein histidine-tag[104].

NTA is a chelator that coordinates bivalent metal cations such as $Cu^{2+}$and Ni^{2+} leaving free coordination sites of the chelator-metal complex for the binding of histidine-tagged proteins[104]. Since NTA immobilization needs to be orientated to complexation occur, the synthesis of this derivate of polypyrrole should allow the special control of NTA without compromising conductivity and also the reversible ligation of histidine-tagged proteins.

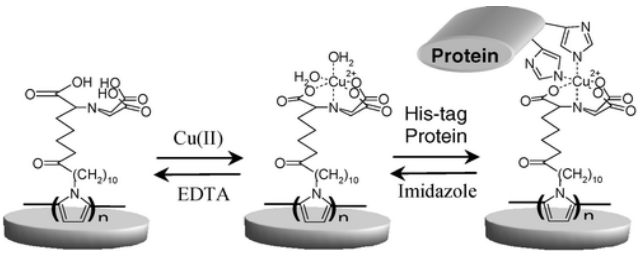

Figure 11- Schematic Representation of the Reversible Immobilization of Histidine-Tagged Biomolecules to an Electrogenerated Poly(pyrrole)-NTA Film[98].

After the synthesis and the characterization of the pyrrole NTA monomer by nuclear magnetic resonance, it was our aim to analyze the electrochemical behavior of the synthetized monomer.

According to "Electrogeneration of a Poly(pyrrole)-NTA Chelator Film for a Reversible Oriented Immobilization of Histidine-Tagged Proteins" a novel approach that enabled the protein immobilization via coordination of Cu^{2+} and histidine-tagged proteins through the synthesis and electropolymerization of a pyrrole N-substituted by a nitrilotriacetic acid was described. The electropolymerization of the monomer was described by doing repeated potential cycling over the range of 0-0,8 V and with the appearance of an oxidation peak at 0,18 V that clearly indicated the formation of a polymeric coating[98]. However, when performed in our lab, neither the formation of the peak or the polymer film by cyclic voltammetry was observed. Thereafter, it was decided to increase the amount of probe used to certify that there was enough amount of electron transfer to allow redox reactions and the formation of the polymer. An increased concentration of the monomer was also tested and none of the approaches lead to the formation of the polymer.

Several questions were considered concerning different aspects of preparation and polymerization of the synthetized polymer, such as purity/quality of the reagents and the organic synthesis that was followed by the detailed experimental part of the scientific article[98]. To guarantee that there weren't any problems concerning the electrode surface or electroanalytical procedure and/or equipment, an electrochemical coating of the conductive support on a SPE was performed with pyrrole by doing a chronoamperometry with the formation of a polypyrrole film with 1 mm of thickness. Moreover, we performed potential cycling over the range of -0,5-1,5 V and we still visualized the polymer film formation.

To overcome these problems new monomer solutions and different conditions in the chronoamperometry parameters were tested without any success. According the author[98] there were some mistakes in the experimental report and the synthesis of pyrrole NTA monomer was impossible to perform. No more work was invested on this immobilization strategy.

2. Immobilization of antigen in magnetic microbeads

As an alternative, it was followed the immobilization strategy used in cytometry by our group[52], using magnetic microbeads that allow the antigen binding (currently the recombinant protein CPX2).

Magnetic microbeads are becoming a great approach in biosensors, namely in immunosensors. They produce stable and inexpensive devices, rapid and easy to operate[105]. Electromagnets can pull immuno- substituted beads towards a binding site and the species that do not bind can be removed by rinse. The major challenge with magnet microbeads associated with immunosensors is the construction of the device. Once assembled, the operation of the system is simple[106].

This particular type of magnetic beads have a Fe_3O_4 core, which confers them the magnetic properties, covered by silica. This silica surface (with hydroxyl groups) is then derivatized with the chelating ligand nitrilotriacetic acid (NTA). In turn, NTA will allow the immobilization of divalent nickel ions (Ni^{2+}), essential for the formation of covalent bonds with the histidine tail of the protein (Figure 12).

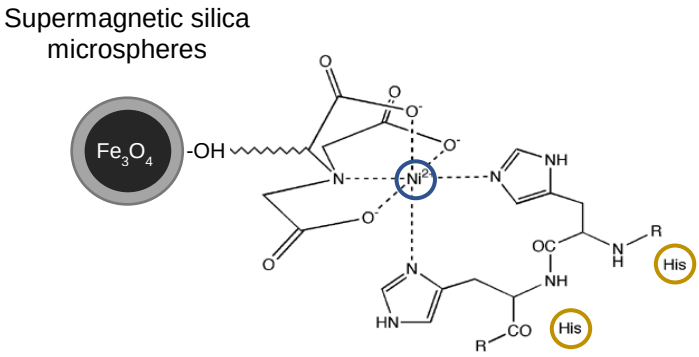

Figure 12 - Representation of the supermagnetic silica microspheres with Fe_3O_4 core covered by hydroxyl groups coated with nitrilotriacetic acid and nickel ions and respective chelation with histidine residues of proteins of interest.

The immobilized metal affinity beads offer high binding capacity and, as mentioned before, they can be used for small scale affinity purification as well as high-throughput screening of recombinant his-tagged proteins.

The polyhistidine tag is the most popular affinity tag and consists of six consecutive histidine residues[64]. The assays were initiated with CPX2 since previous results had shown good specificity and sensitivity for symptomatic and asymptomatic patients[23].

Our approach was to test the flow cytometry protocol adapted to an immunosensor with electrochemical transducer. Thus, it was investigated the electrochemical response of the prepared immunosensor against negative serum (non-infected dog for leishmaniasis), positive serum (symptomatic dog with leishmaniasis) and superpositive serum (symptomatic dog with high titer). For that purpose, electrochemical impedance spectroscopy was used and the results are presented in Figure 13:

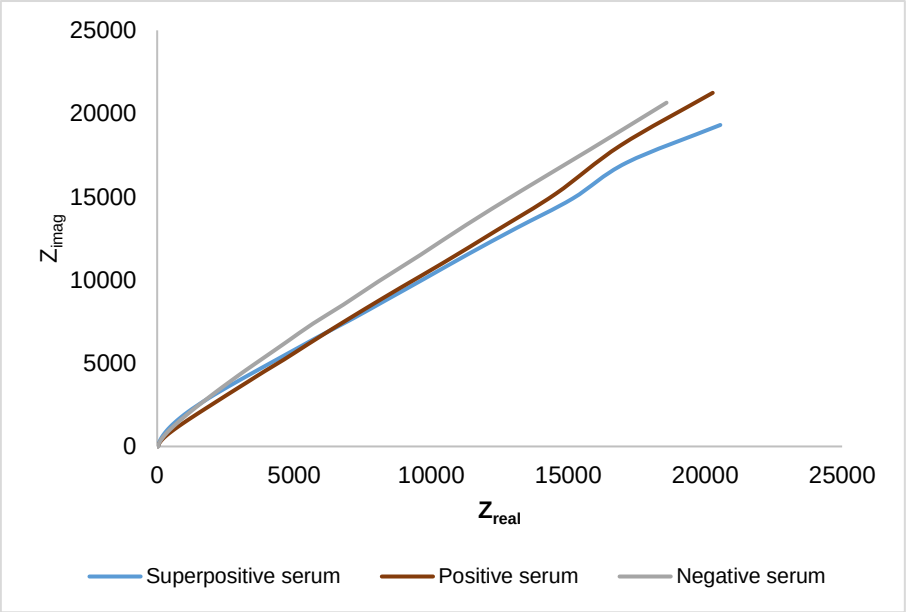

Figure 13 - Graphical representation of a negative, positive and superpositive serums measured by Electrochemical Impedance Spectroscopy (EIS). Z_{imag} represents the imaginary part of impedance and Z_{real} represents the real part of impedance.

According to the graphical representation, only the diffusional controlled region of the Nyquist plot was observed. There was no formation of a semi-circle, which might indicate that the sensing layer formed in the working electrode surface (magnetic microbeads layer) was not efficient to cause charge transfer resistance (Rct). Therefore, the steric barrier presented to the redox probe at the surface of the electrode was not enough to trigger a response[79]. On the other hand, this electrochemical technique must be more explored with biosensors incorporating magnetic microbeads, since there are few articles describing these procedures and the majority of them is applied to enzymatic biosensors[107,108].

For those reasons, it was decided the reproduction the same protocol but this time with cyclic voltammetry as measurement technique, to investigate if this method was sensitive enough to differentiate the samples (Figure 14).

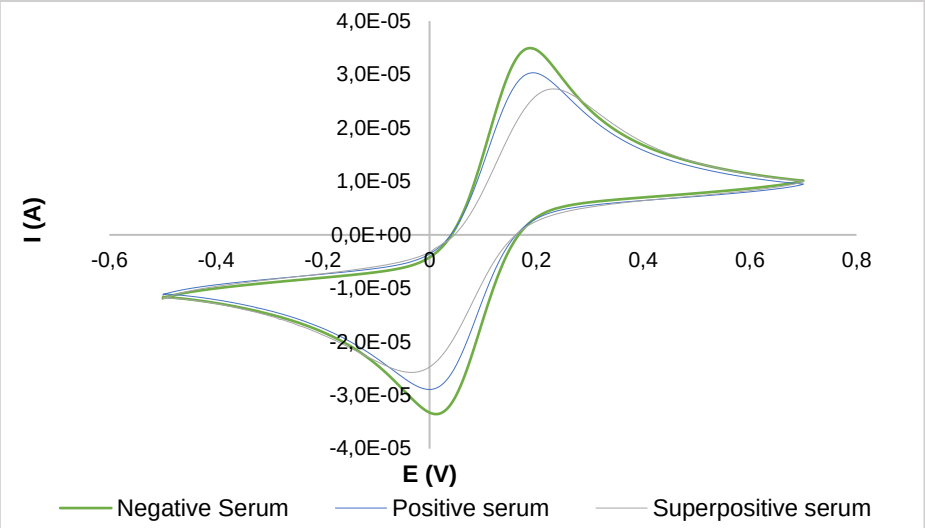

Figure 14 - Graphical representation of a negative, positive and superpositive serums measured by cyclic voltammetry. I (A) represents current measured in amperes and E (V) represents potential measured in volts.

It was clear the visualization of differences between negative, positive and superpositive samples. The absence of antibodies in the serum to bind to the CPX, immobilized in the magnetic microbeads in the sensor surface, results in a thinner layer that enables a higher oxidation/reduction peak intensity due to less charge transfer resistance. It is also noticeable that higher titer samples result in more anti-*Leishmania* antibodies binding to CPX2. The layer presented to the redox probe is thicker which presents itself as minor current (less electron transfer and electrons achieving the electrode surface).

For comparison purpose, square wave voltammetry was considered. In Figure 15 it is presented a compilation of voltammograms that were obtained by using different positive serum dilutions, a negative serum and as “blank” PBS (used to dilute samples).

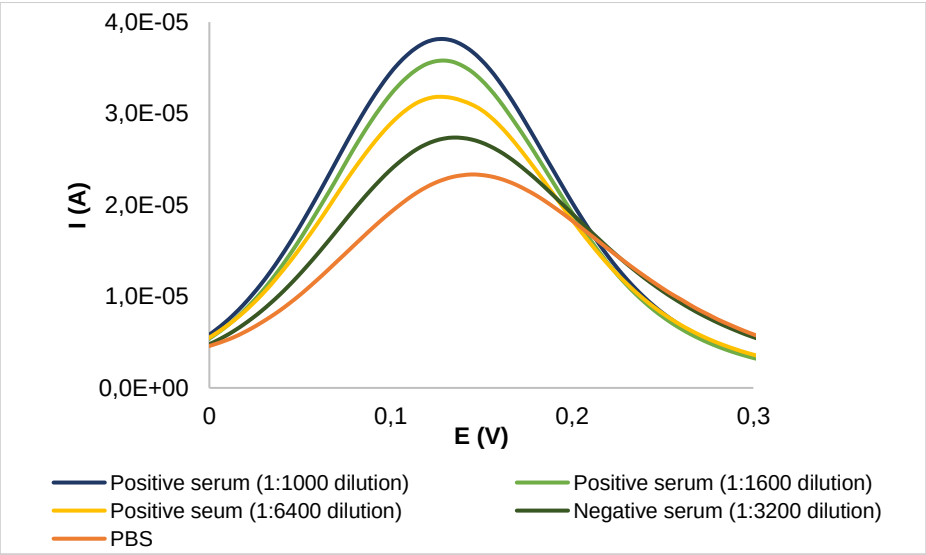

Figure 15 - Graphical representation of several dilutions of a positive serum obtained by Square Wave Voltammetry (SWV). I represents current measured in amperes (A) and E represents potential measured in volts (V).

Once again, it is possible to distinguish the positive and negative serums as well as differentiate different antibody titers of a positive serum. After the serum incubation there was a change in the potential of the electrode probably because of the established kinetics barrier between $[Fe(CN)6]^{4-}/^{3-}$ and the charge of the antigen/antibody complex. Even more, the intensity of the current is proportional to the antibody concentration. The electron transfer resistance at this structure was expected to be higher relative to high antibody concentration. However, a higher oxidation peak current was observed at high antibody concentrations. More studies must be done to understand these results.

To improve the assembly of a stable and accurate immunosensor based on magnetic particles, a flow device to be coupled in a flow injection analysis was evaluated to guarantee reproducible volumes of magnetic microbeads suspension.

Development of a magnetic field controller coupled to a microfluidic platform

First, it was designed and built an electrical circuit (magnetic field controller) that allows the creation of a magnetic field (on/off configuration) (Figure 7). In parallel, a microfluidic platform was designed and manufactured to allow the samples feeding to the electrode (propelled by a peristatic pump). Coupling the magnetic field controller to the base of the working electrode (confined in the microfluidic device) will permit the retention of the magnetic microbeads with further serum incubation. This strategy will leverage a more sustainable procedure because of the reutilization of the electrode (screen-printed electrode) through several samples, decreasing also the cost of the analysis, more precision and in “green chemistry” concept.

The coupling of the microfluidic platform to the flow injection analysis system (FIA) enables reproducible volumes of sample and therefore minimize the waste of reagents.

In this phase of the development of a biosensor, optimization of many parameters is essential to the good function of the same. Antigen-beads proportion and incubation were already optimized, as well as blocking, by our group in “Development of a Fluorescent Based Immunosensor for the Serodiagnosis of Canine Leishmaniasis Combining Immunomagnetic Separation and Flow Cytometry”[52]. Other parameters such as the hydrodynamic conditions through the SPCE (flow rate, volume of the loop, time); the voltammetry scan rate and the ON/OFF time period of the magnetic field applied to the magnetic microbeads still had to be studied and stablished.

Following the assembly of the system, the first trial was performed with the serum of a non-infected dog and with several dilutions of an infected dog serum. The immobilization of CPX2 and wash steps were performed outside the system. Then, the magnetic

microbeads immobilized with CPX2 antigen were introduced in non-studied size loop of the FIA valve (30 µL) and were injected into the microfluidic device while the magnet was active. The incubation of the serum on the magnetic microbeads previously prepared was done in the flow system by injecting the samples (serums containing or not antibodies anti-*Leishmania*). The system was stopped for 30 minutes while the magnet was active. As the serum samples were diluted in the redox probe used in that assay (Fe^{2+}/Fe^{3+}) it was possible to obtain a voltammogram after the period of incubation. The results can be seen in the figure bellow (Figure 16).

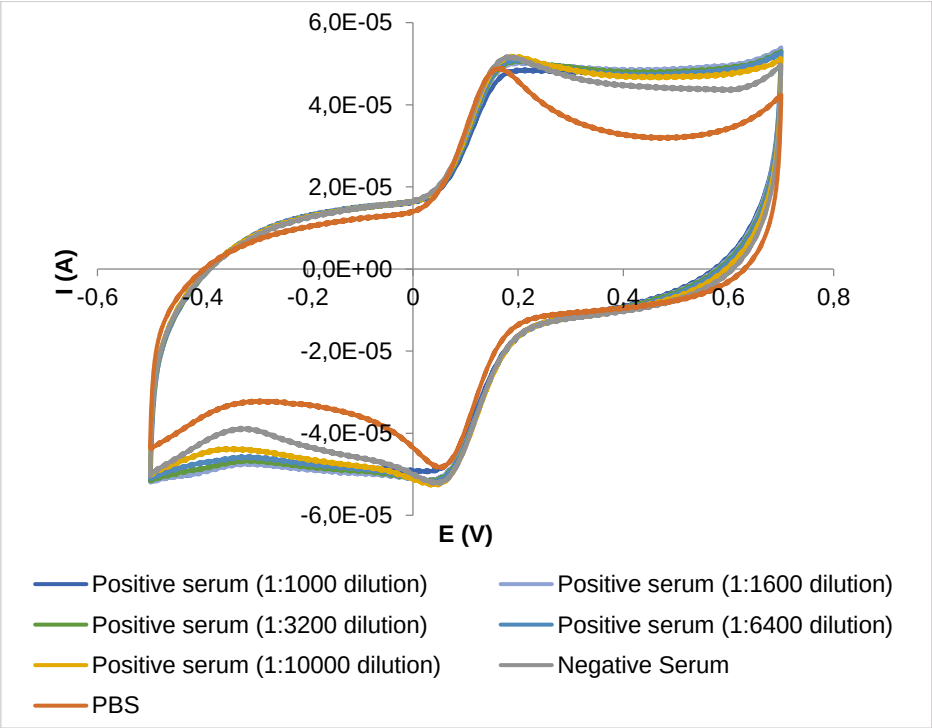

Figure 16 - Graphical representation of several dilutions of a positive serum obtained by cyclic voltammetry (CV). I represents current measured in amperes (A) and E represents potential measured in volts (V).

As we can observe, there was a tremendous increase of the capacitive currents. This type of current doesn't obey to Faraday's law and is not proportional to the concentration of analyte. The contact of the serum with the carbon surface promotes the unspecific adsorption of the antibodies, leading to the formation of a biochemical layer that increases the electron transference resistance.

As this increase in the capacitive currents was probably due to the binding of antibodies to the electrode surface, a different approach was thought and executed. Thereby, the preparation of the magnetic microbeads and the coupling of the antibodies to CPX2 were performed outside the system. Once the incubation of the beads was done outside, they were resuspended in PBS to guaranty the conformational stability of the proteins and the redox probe was used as carrier. Thus, the samples were in contact with the SPCE

during the measurement time and were rinsed after obtaining the voltammogram, by the deactivation of the magnetic field.

Moreover, an experiment with the serums incubation outside the system was performed to try to avoid high capacitive currents. Several dilutions of a symptomatic canine Leishmaniasis serum were characterized by cyclic voltammetry, as seen in Figure 17:

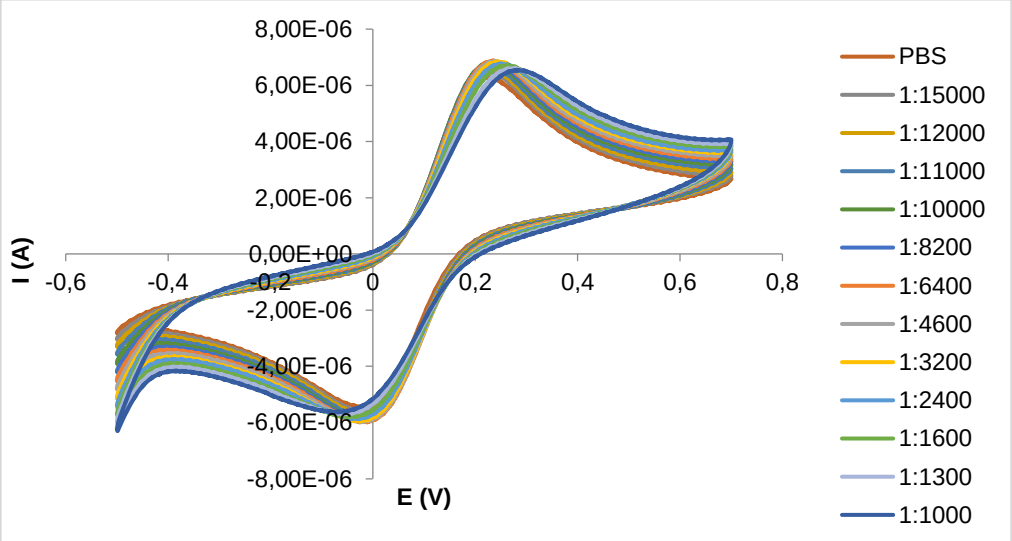

Figure 17 - Voltammograms obtained with several dilutions of a positive serum resorting to cyclic voltammetry. I represents current measured in amperes (A) and E represents potential in volts (V).

According to the voltammograms (Figure 17) it was possible to identify a shift of the potential. More concentrated samples lead to bigger shifts and higher values of potential looking to be related with Nernstian response. Thus, these results were analyzed according equation 1, in which the potential follows a linear regression with the logarithm of the activity (antibodies concentration) (Figure 18).

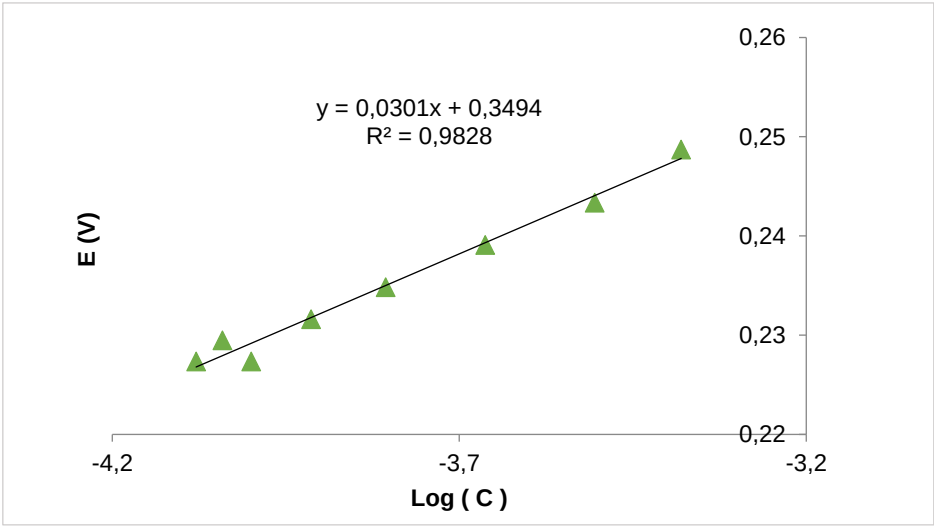

Figure 18 -Calibration curve obtained with Nernst equation, in which potential (E) in volts (V) is dependent of the logarithm of concentration (Log (C)). The linear regression was obtained with several dilutions of the same positive sample.

There is in fact a correlation of potential against the concentration of the several dilutions of the positive serum. This correlation is linear up to the 1:10000 dilution. The titer of each sample will depend on the quantity of antibodies anti-*Leishmania* and the fact that response is obtained with such diluted samples indicates that this method may be quite sensitive.

This experiment was followed by the optimization of some parameters since it was crucial to establish the conditions that lead to the best response possible.

The first optimized parameter was the volume of sample. Different volumes of sample were tested in a range from 45 µL to 540 µL, to evaluate which was the most appropriate to use with this type of system and electrode and which one gave the best correlation.

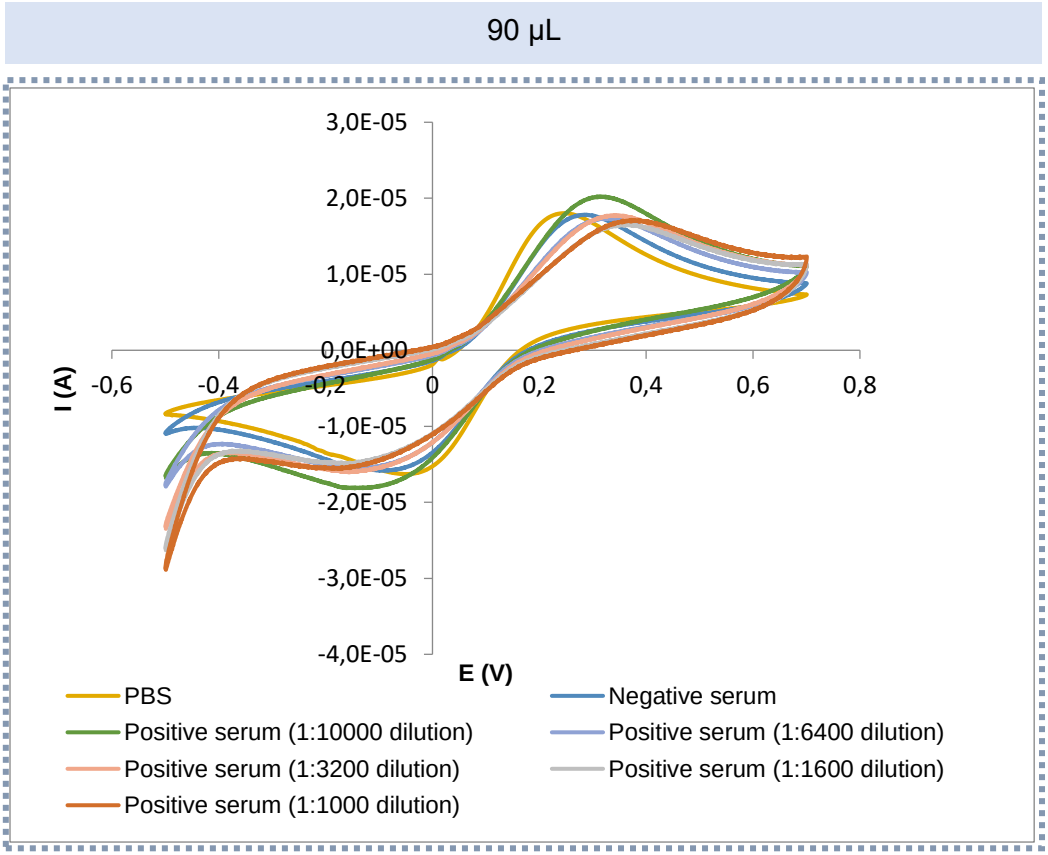

Figure 19 - Voltammograms of a negative serum and several dilutions of a positive serum obtained with 90 µL of sample resorting to cyclic voltammetry.

The optimal volume of sample was 90 µL, since it was the one in which the shift in the potential was more accentuated. Higher volumes didn't introduce any improvements in the potentiometric response.

Once again, the voltammograms present a shift in the potential against the concentration of the several dilutions of the positive serum, correlating with the Nernst equation (Figure 20).

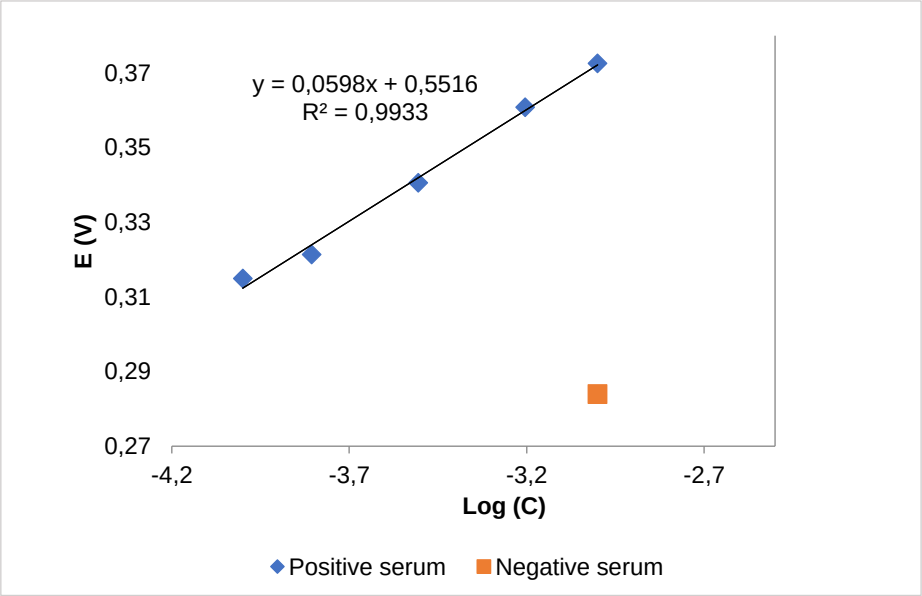

Figure 20 - curve obtained with Nernst equation, in which potential (E) in volts (V) is dependent of the logarithm of concentration (Log (C)). The linear regression was obtained with several dilutions of the same positive sample.

Moreover, we could clearly distinguish the negative serum from the positive serum dilutions as the potential of the negative serum was lower than the more diluted sample of the infected canine serum, due to the lack of antibodies anti-*Leishmania* in the negative serum sample.

The slope was very close to the theoretical value (59,2 mV) which is in accordance with the type of redox probe used (Fe^{2+}/Fe^{3+}). Nevertheless, it was verified that the slope was quite different from the previous one. Further studies were performed to investigate this irreproducibility of the slope.

Other parameter to be optimized was the scan rate, since it influences electrochemical response. This concept is defined as the time that takes to sweep the potential range. This parameter can influence the interpretation of results since, for example, high scan rates peaks have large current and high resistance that might result in distortions and misinterpretations of results.

So, different scan rates (50 mV; 100 mV; 200 mV) were evaluated to perceive which one gave the best correlations.

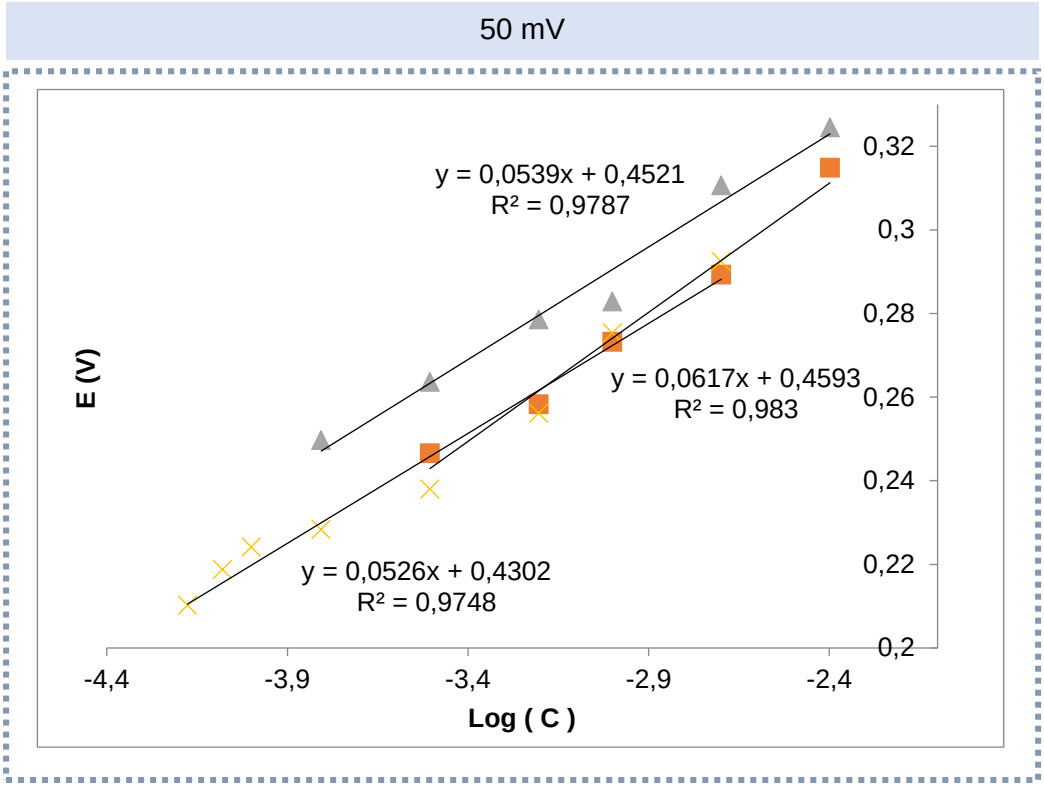

Figure 21 - Three calibration curves obtained with several dilutions of a positive canine serum at a scan rate of 50 mV. They were obtained with Nernst equation, in which potential (E) in volts (V) is dependent of the logarithm of concentration (Log (C)).

After proceeding to the same protocol several times it was concluded that the scan rate of 50 mV/s was the more adequate to our experiments (Figure 21) since the coefficient of determination (r^2) was closer to 1, the slope was near the theoretical nernstian slope (59mv.dec^{-1}) and a higher concentration range was identified.

Meanwhile, background noise appeared during calibration measurements due to the damage of the electrical circuit (magnetic field controller). A new magnetic field controller and a new microfluidic platform were constructed in a more robust approach.

Several calibrations with positive and negative serums were performed but were always irreproducible. Most of the time no linear relation was found between potential shift and titer of antibodies. It was discussed possible causes that were affecting the results.

We wondered if the change of the miniaturized device could influence them, but after using the system carefully and with the appropriate management the results were still irreproducible.

Two main questions are related with the biochemical stability of the proteins used in this work (the effect of storage time and freeze-thaw cycles) and the type of serum containers used to aliquot the sample (the containers could adsorb protein to the surface). The quantification of the proteins was made according section 2.7 of Methods; and an experiment was performed with the serum from the original containers and from one of the aliquots. Potentiometric calibrations were performed and the results were almost overlapping. That excluded the hypothesis that the irreproducibility between calibrations was due to the protein content in the serum.

To overcome random errors special attention was given to the dilutions of the samples and this change in the preparation of samples slightly improved the results, although with fluctuations in slope, as it is possible to observe in Figure 22.

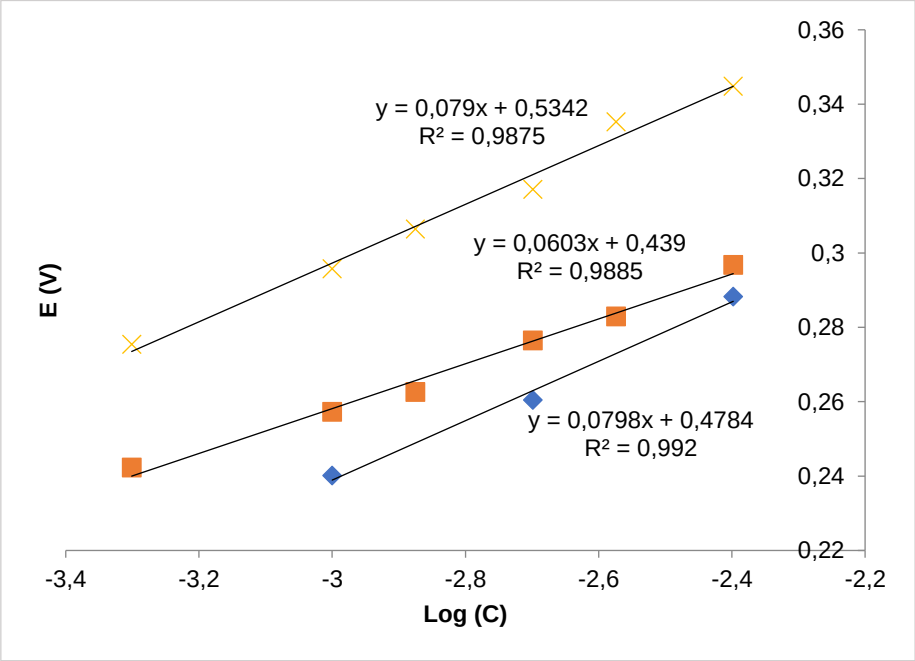

Figure 22 - Calibration curves obtained with several dilutions of a positive canine serum after optimizing some parameters. They were obtained with Nernst equation, in which potential (E) in volts (V) is dependent of the logarithm of concentration (Log (C)).

One of the main reasons to build a microfluidic platform was to ensure reproducible volumes, as well as to reduce the cost of the analysis by using the same SPCE to analyze several samples. Thus, two consecutive calibrations were performed with the same electrode. It was noticed that the slope changed a lot from the first calibration to the second. Also, we noticed that the second calibration started with a similar potential to the last sample of the first calibration (Figure 23).

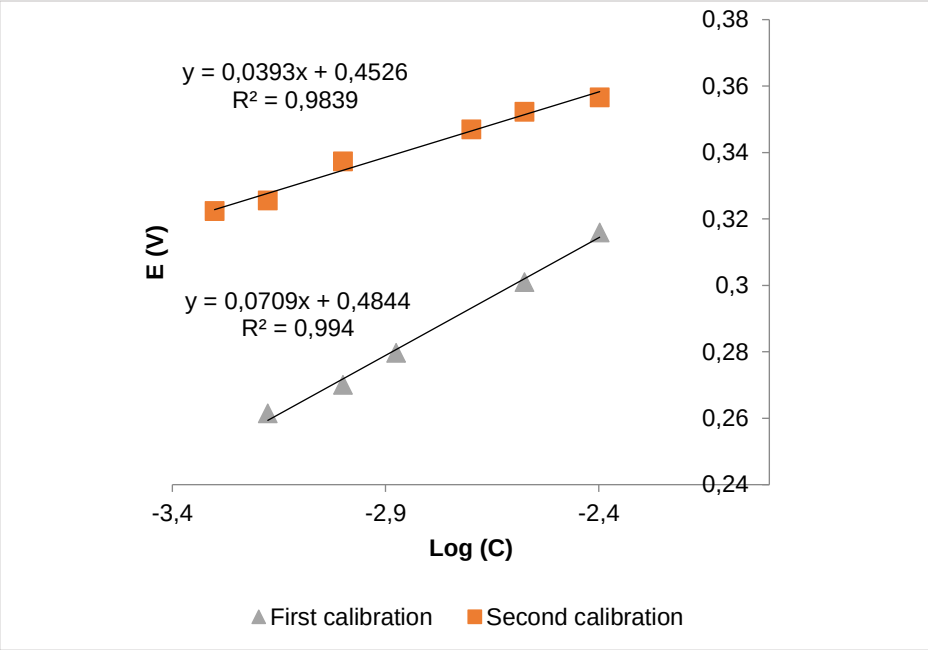

Figure 23 - Two consecutive calibration curves with the same sample dilutions of a positive canine serum. They were obtained with Nernst equation, in which potential (E) in volts (V) is dependent of the logarithm of concentration (Log (C)).

The experiment was repeated and the same situation was observed; the second calibration began with the potential of the last sample of the first calibration. That meant that some phenomena was promoting a change in the electrode surface. Even though the system was washed between samples with the magnet off (to allow the rinse of all magnetic nanoparticles and other impurities that the sample might contain), something was shifting the potential. So, two experiments were performed to see what was causing the potential shifting. First, the non-coated magnetic microbeads were used and the measurements were done exactly in the same way as it was done with the previous samples (Figure 24).

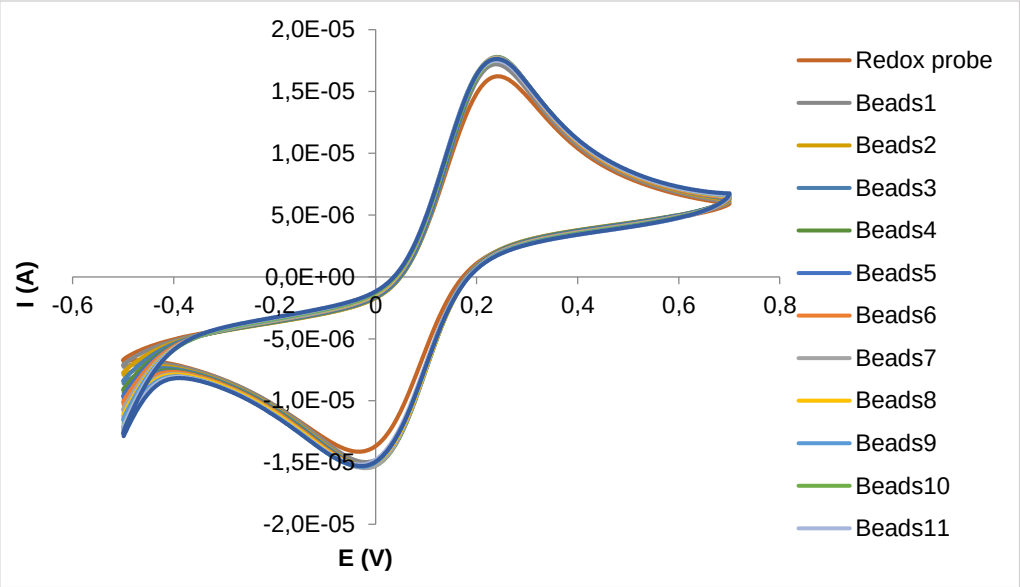

Figure 24 - Voltammograms corresponding to the results of a cyclic voltammetry of the same sample – beads. I represents current measured in amperes (A) and E represents potential in volts (V).

As observed, there was no alteration in the potential from one sample to the next one. The absence of potential shift indicates that the magnetic beads are not the responsible element for change of the electrode surface.

After excluding the possibility of the magnetic microbeads as source of the electrode surface damage, a similar approach with CPX2 coated magnetic microbeads (overnight incubation) was performed. This would allow the evaluation of the antigen influence on the potential shifting and the reuse of the electrode (Figure 25).

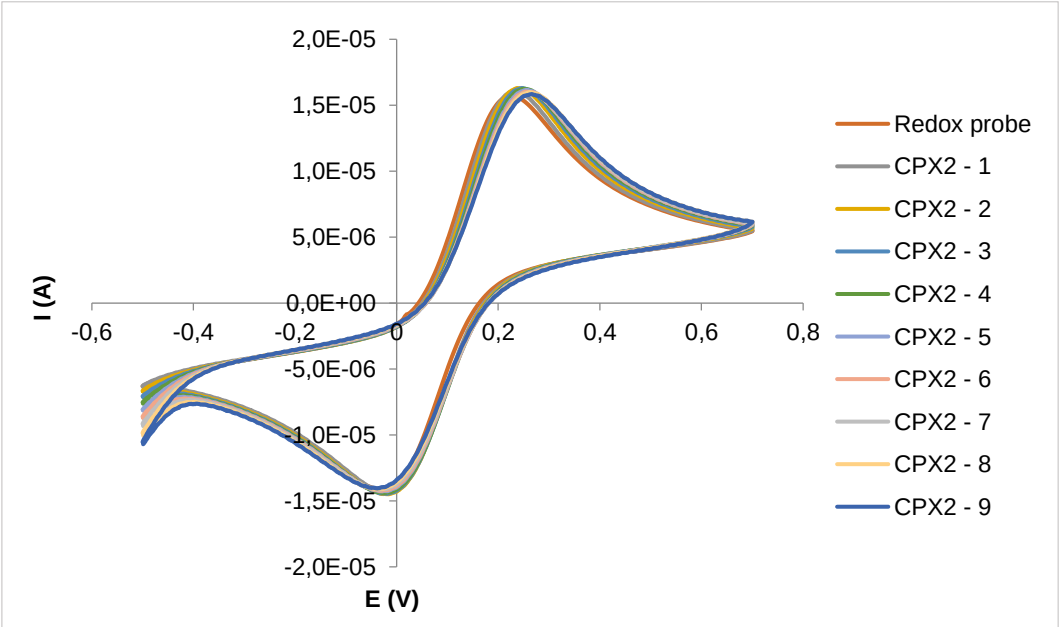

Figure 25 - Voltammograms corresponding to several readings of the same sample - microbeads immobilized with CPX2. I represents current measured in amperes (A) and E represents potential in volts (V).

It is evident that after passing the same sample through the electrodes multiple times, there is a potential shift. Even when the washing steps are properly executed, this still leads to a shift in potential. This completely explains the different responses from the first to the second calibration and the fact that samples with the same concentration don't have the same potential. In this case CPX2 looks to be adsorbed to the electrode, changing the potential. This makes the measurement of samples and the calibration impossible. Therefore, approaches to enable the cleaning of the electrode surface were thought. Trypsin is known for its capability of splitting cells and detaching cells and other biomolecules from flasks and other plastic/glass surfaces. So, this reagent was evaluated for its ability of revert the adsorption occurring in the electrode surface. After testing this option, it was realized that trypsin does not reverse the electrode surface (potential shift cannot be reversed). It is our aim to evaluate the use of an acetate buffer at pH 4 to clean the carbon surface of the electrode; blocking of the carbon surface with BSA or even to do a direct covalent binding of the antigen to the carbon electrode surface.

In parallel, experimental work in the identification of new biomarkers was performed, namely by recovering exosomes from human plasm samples.

3. Identification of new promising markers from human plasma exosomes to detect Leishmaniasis asymptomatic cases

The proteic material in extracellular vesicles has been used as novel biomarkers in the diagnosis of several diseases. Thus, in this work extracellular material from *L. infantum* was recovered to access the potential of the different exoproteome fractions of this parasite and evaluate its use as markers in this infectious disease.

To respond to these questions, it was decided to perform an ELISA with the several fractions recovered from the *L. infantum* culture: total exoproteome (EXO); extracellular vesicles (EVs) and vesicle depleted exoproteome (VDE). These fractions were coated in the plates and soluble promastigotes *Leishmania* antigens (SPLA) were used as a control to the experiment. Different volumes of extracellular material (20 µL corresponding to 1x; 2 µL to 10 times dilution – 10x; and 0,2 µL corresponding to 100 times dilution - 100x) were used and each of them incubated with a well characterized positive canine leishmaniasis serum (+) and a negative canine leishmaniasis serum (-).

Canine serums were used to do the preliminary experiments because human samples are a lot more difficult to get and we have limited volumes of samples.

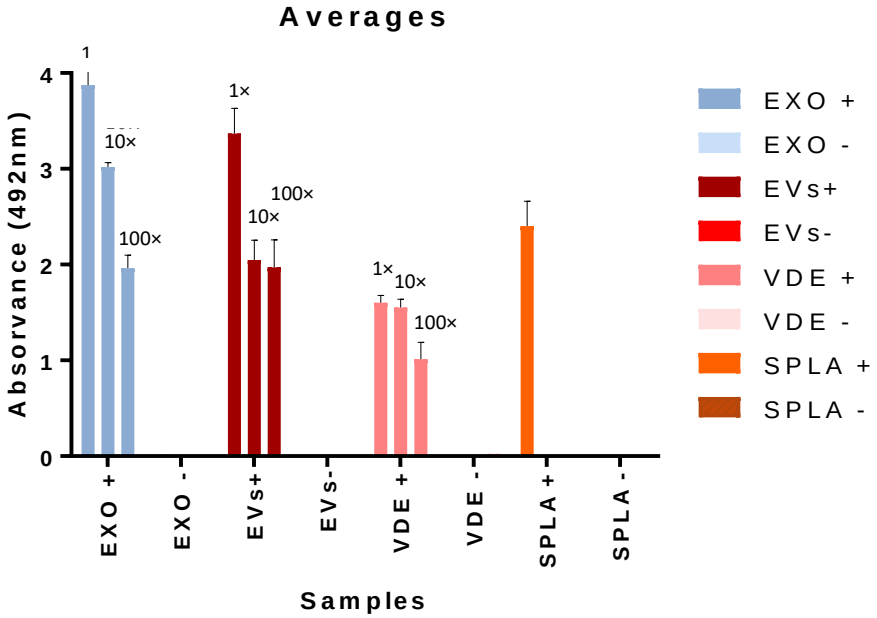

Figure 26 - Averages of the absorvance using ELISA technique of different exoproteome fractions (Total exoproteome - EXO; Extracellular vesicles - EVs; Vesicle depleted exoproteome - VDE) and Soluble Promastigote *Leishmania* antigens (SPLA) against positive canine serum (+) and negative canine serum (-) for Leishmaniasis. The second column of each fraction was diluted 10 x and the third column was diluted 100 x.

In Figure 26, it is observed high reactivity of the secreted material against positive canine serum and nearly no response is seen when the fractions are incubated with a non-infected dog serum (negative serum). Also, the same response is obtained with SPLA which corroborates our experiment. Furthermore, the positive canine serum has a higher reaction with the EVs fraction when compared to the VDE fraction.

Regarding ratio between positive (+) and negative (-) canine serum samples, we can conclude that better correlation is achieved with more diluted samples (100 x) (except for the VDE fraction, but this might be due to the washing steps of ELISA that weren't performed with the same efficacy when compared with the other fractions) (Figure 27).

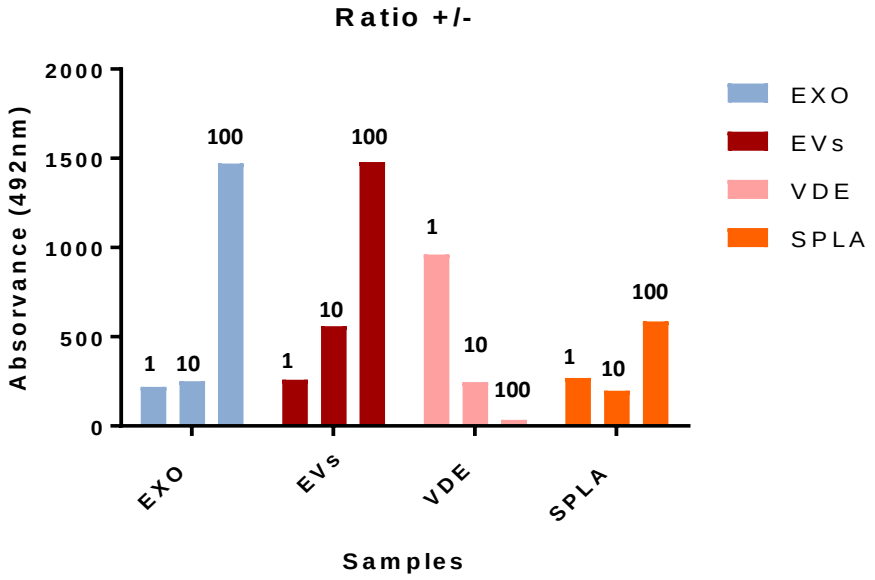

Figure 27 - Ratio of the absorvance between the positive (+) and the negative (-) canine serum responses for different exoproteome fractions (Total exoproteome - EXO; Extracellular vesicles - EVs; Vesicle depleted exoproteome - VDE) and against Soluble Promastigote *Leishmania* antigens (SPLA). The second column of each fraction was diluted 10 x and the third column was diluted 100 x.

In order to get more information, a western blot was performed with the EVs and the VDE fractions. Different volumes of these fractions were used to investigate which one was the best, as this type of assay had never been done by the group before with this type of samples. The experiments were done with two different membranes, one of them incubated with a positive dog for Leishmaniasis and the other membrane was incubated with a negative serum (healthy canine).

As it is visualized in Figure 28, two bands appeared around 90 kD and 120 kD. There is some background, as it is possible to see in the membrane regarding the negative canine serum. One possible explanation is the quite a lot of extracellular material (50 µL of EVs/VDE) used in those wells. Furthermore, the blur circled in blue is characteristic of the presence of some abundant proteins released by *Leishmania infantum* involved in its virulence factors such as GP63 and also a protein known as beta-fructofuranosidase involved in carbohydrate metabolism.

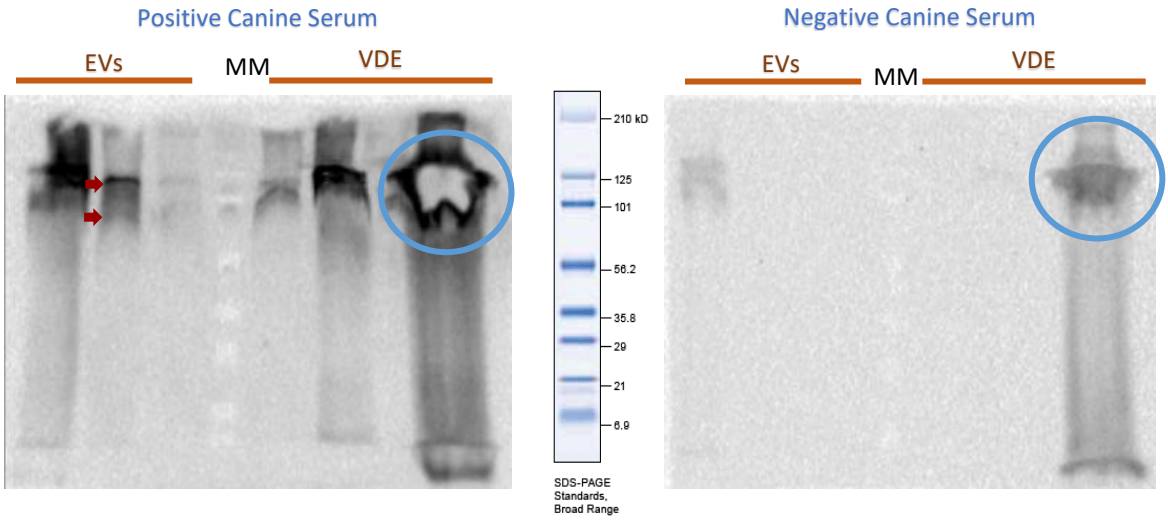

Figure 28 - Results of the Western Blot with different volumes (50 µL; 5 µL and 0,5 µL) of EVs and VDE fractions against different serums - positive canine serum (left image) and negative canine serum (right image)

Stripping of the membrane was performed and two different serums were incubated. The results are presented in Figure 29. The negative canine serum didn't give any background and the positive serum presented two bands in the well with 5 µL of extracellular vesicles. However, these two bands are not overlapping with the bands from the previous western blot, which indicates that for different serums the profile of the bands is different.

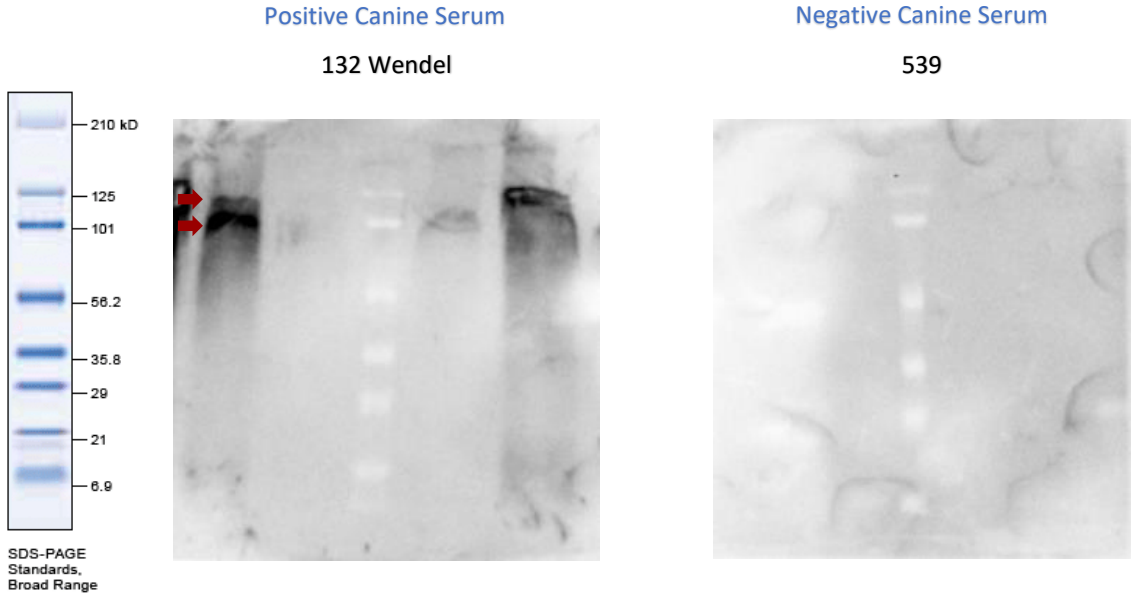

Figure 29 - Western blot after stripping the membrane and doing incubation with different serums for the wells corresponding to EVs and VDE fractions (5 µL and 0,5 µL of extracellular material).

In order to evaluate the optimal quantity of extracellular material to be plated in an ELISA, different volumes (0,0002 µL; 0,002 µL; 0,02 µL; 0,2 µL; 2 µL) were considered. The better OD ratios were obtained with 0,2 µL of extracellular material (Figure 30). Once more, EVs present a higher response against the canine positive serum when compared to VDE fraction.

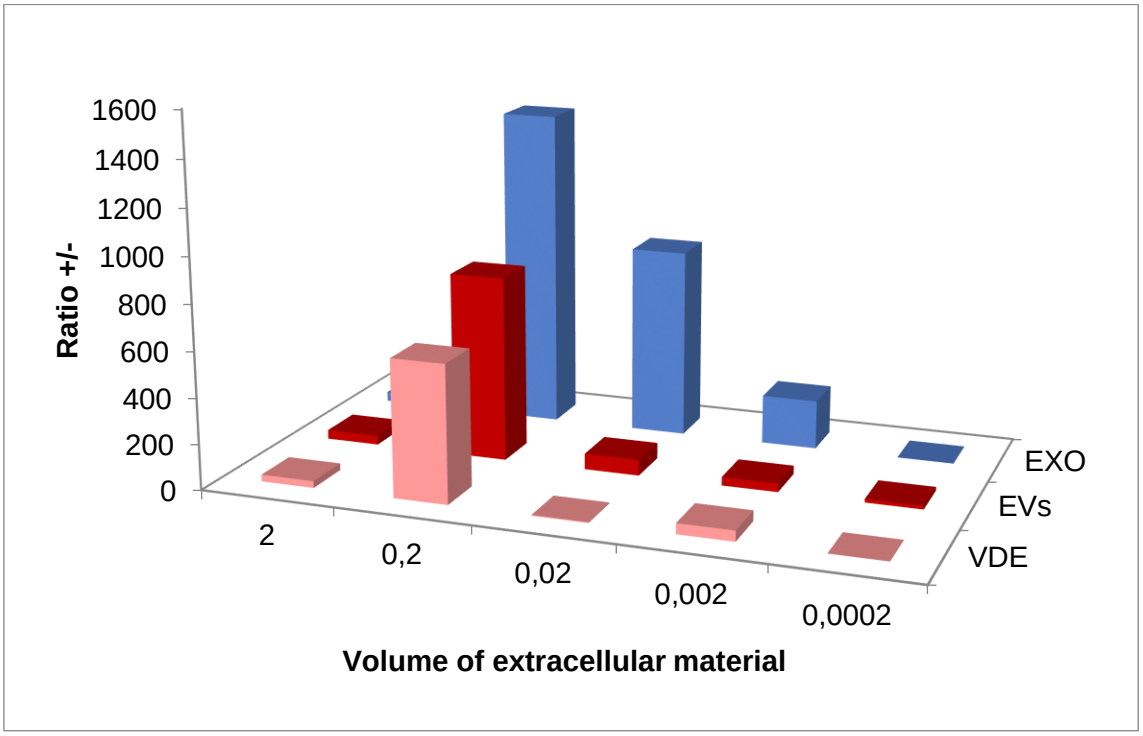

Figure 30 - Ratio between response against positive canine serum (+) and negative canine serum (-) with different fractions of exproteome of *Leishmania infantum* (Total exoproteome - EXO; EVs - extracellular vesicles; VDE - vesicle depleted exoproteome) for different volumes of extracellular material.

Since the exploratory research with canine serums was encouraging it was our aim to proceed the study with human samples against extracellular material obtained from the culture of *L. infantum,* EXO, EVs and VDE, and against SPLA. As a preliminary experiment, an ELISA was performed with 2 µL of the same extracellular material against human samples. This volume increment was to guarantee the signal achievement (Figure 31).

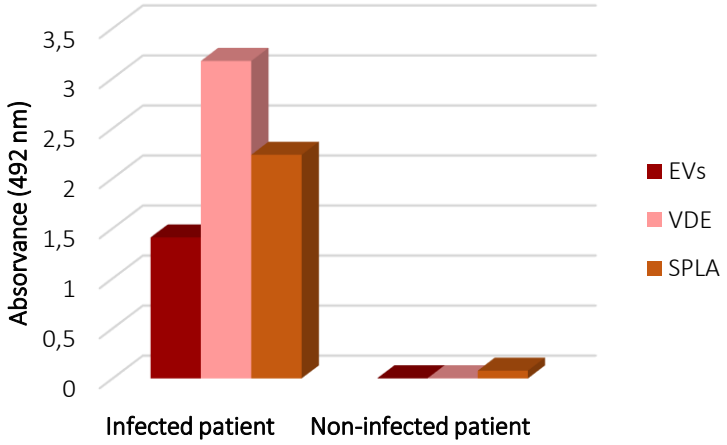

Figure 31 - Results of an ELISA for exoproteome fractions (EVs - extracellular vesicles; VDE - vesicle depleted exoproteome) and SPLA - soluble promastigotes *Leishmania* antigens against human samples from an infected and a non-infected patient.

Over again there was reactivity of SPLA and exoproteome fractions (EVs and VDE) against the infected Leishmaniasis patient whereas no response was obtained with non-infected Leishmaniasis patient plasma. This indicates that there are proteins in the extracellular material of *L. infantum* reacting against Leishmaniasis infected patients sample, showing once more the potential of EVs as possible biomarkers for Leishmaniasis diagnosis. EXO is not presented in Figure 31 since the assay resulting color was too saturated and optical density could not be measured.

Proteins associated with EVs (including Exosomes) secreted by infective *L. infantum* have been previously identified in cell culture supernatants by our group, but never in human samples. Thus, it is our goal to identify proteins in Exosomes isolated from healthy patients (negative controls) and plasma of Leishmaniasis symptomatic and asymptomatic patients.

3.1. Isolation and characterization of exosomes from negative controls

Firstly, isolation and characterization of the exosomes enriched fractions from negative controls were performed. The exosomes (EXos) were isolated from 100 µL plasma samples. The isolated fractions were obtained with a 1 mL sepharose column through size exclusion chromatography (SEC) and analyzed by bead-exosome FACS assay using CD71 marker, a marker used for the detection of exosomes in plasma (Figure 32-34):

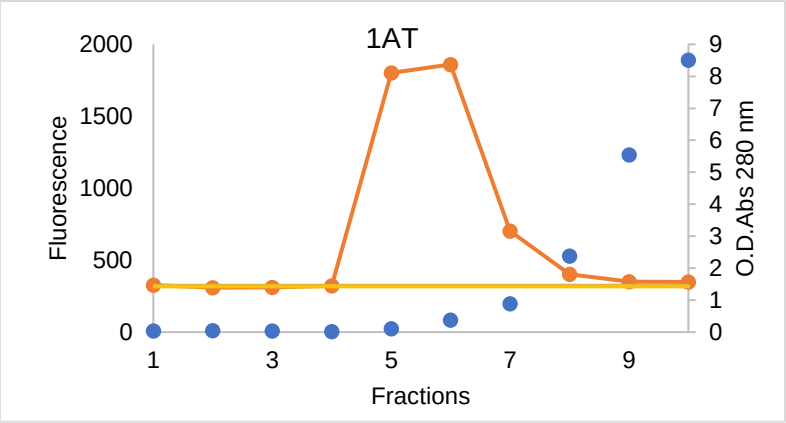

Figure 32 - Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 1AT (negative control) and respective controls. Fr5 and Fr6 represent fractions 5 and 6 with a concentration of proteins of 0,109 and 0,375 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

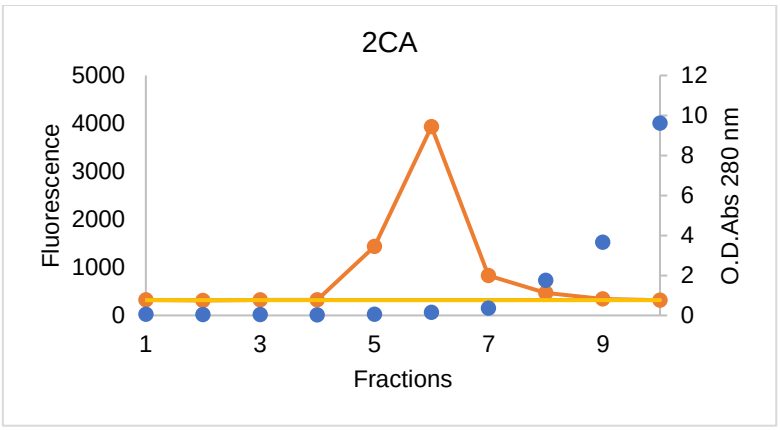

Figure 33- Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 2CA (negative control) and respective controls. Fr5 and Fr6 represent fractions 5 and 6 with a concentration of proteins of 0,049 and 0,153 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

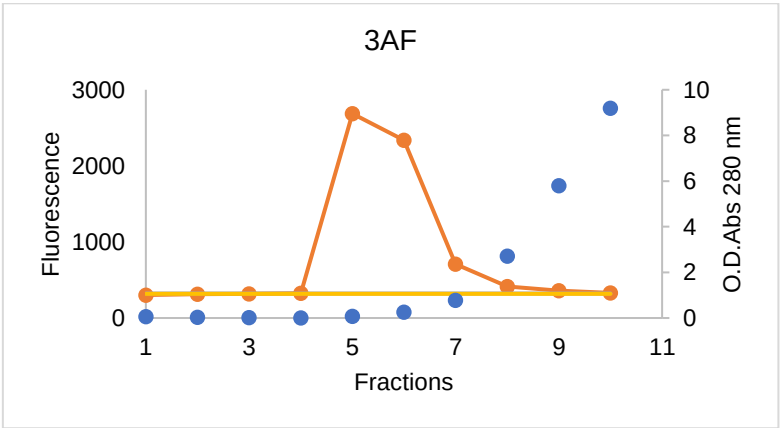

Figure 34 - Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 3AF (negative control) and respective controls. Fr5 and Fr6 represent fractions 5 and 6 with a concentration of proteins of 0,082 and 0,266 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

From the results of nanodrop, similar profiles in all negative controls were visualized. The protein concentration starts to rise in fractions 5/6 which indicates that fraction 5 is probably enriched in EXos, since previous results showed that the fractions with more EXos are the ones before the accentuated rise in protein concentration.

As expected, the fractions with more isolated EXos were fractions 5 and 6, as it was confirmed from the beads based assay performed with CD71 marker.

The same assay with CD5L marker was also performed but the control with the isotype had high background making impossible to interpret the results (data not shown).

From the negative controls, the fractions that had the highest response to CD71 were analyzed by nanosight (Figure 35).

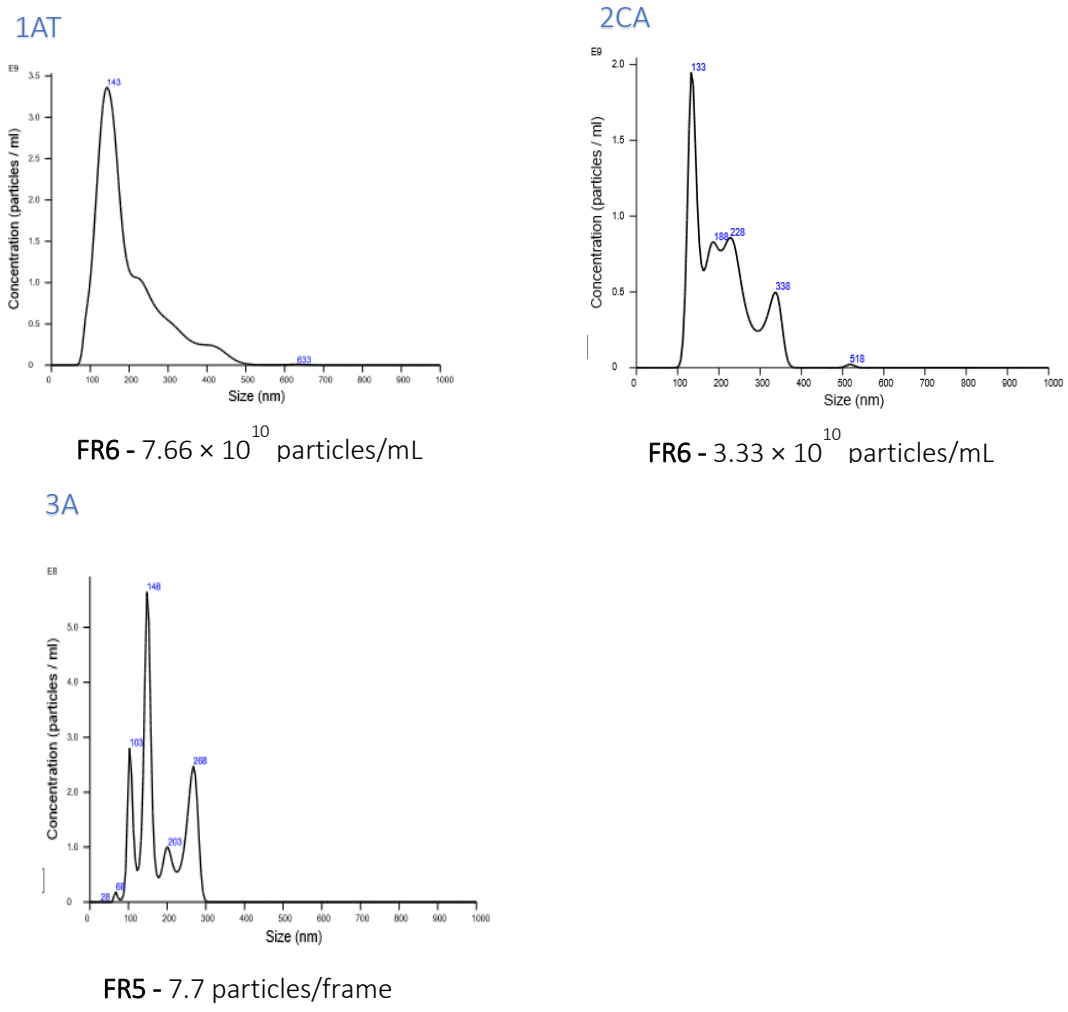

Figure 35 - Nanosight profile for the negative controls (1AT, 2CA, 3AF). Size and concentration information for the fraction with more EVs.

The size and concentration of the analyzed samples for the fractions that were most enriched in exosomes can be observed in Table 2:

Table 2 - Size (mode) and concentration (particles/mL or particles/frame) of the fractions (FR) enriched in EXos for negative controls.

Samples	Mode	Concentration	
2CA FR6	134,1 nm	3,33 x 10^{10} particles/mL	21,1 particles/frame
1AT FR6	143,0 nm	7,66 x 10^{10} particles/mL	48,0 particles/frame
3AF FR5	148,7 nm	7,7 particles/frame	

The size of these samples varies between 134,1-148,7 nm and the concentration is in the order of 10^{10} particles/mL. It wasn't possible to determine the concentration of fraction 5 (FR5) of 3AF sample because the number of particles for frame was too low. Another dilution was not performed (a more concentrated one) due to the volume necessary for proteomics.

3.2. Isolation and characterization of exosomes from Active-Infected Patients

For the active group, Exos were isolated from 5 different plasma samples (100 µL). EXos enriched fractions from samples 5878, 3935 and 4544 were obtained by SEC using sepharose columns of 1 mL and characterized by bead-exosome FACS assay using CD71 marker, nanodrop and NTA. Results are shown in figures 36-40.

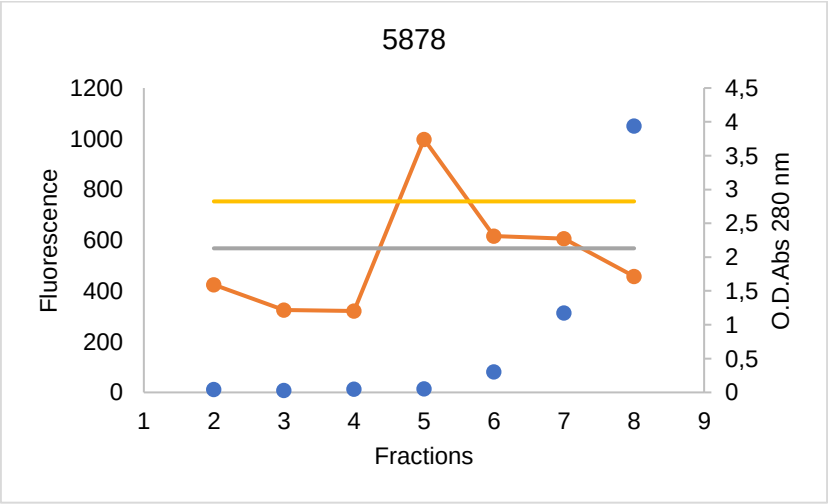

Figure 36- Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 5878 (active) and respective controls. Fr5 and Fr6 represent fractions 5 and 6 with a concentration of proteins of 0,056 and 0,307 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

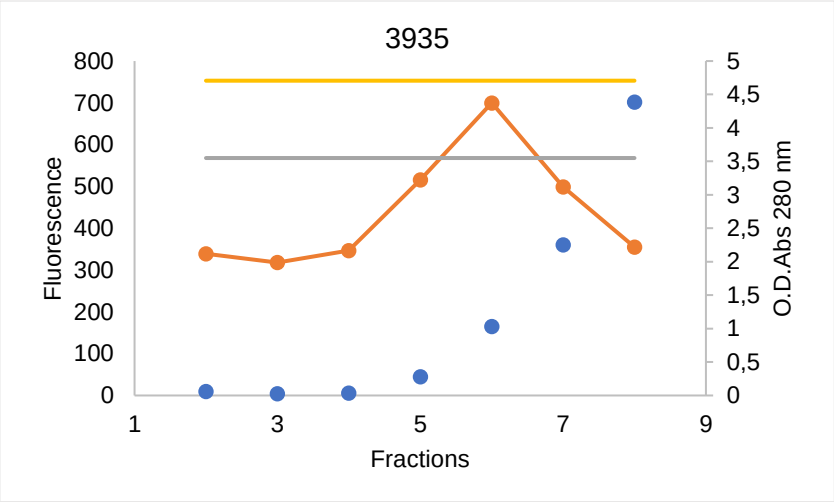

Figure 37 - Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 3935 (active) and respective controls. Fr5 and Fr6 represent fractions 5 and 6 with a concentration of proteins of 0,283 and 1,034 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

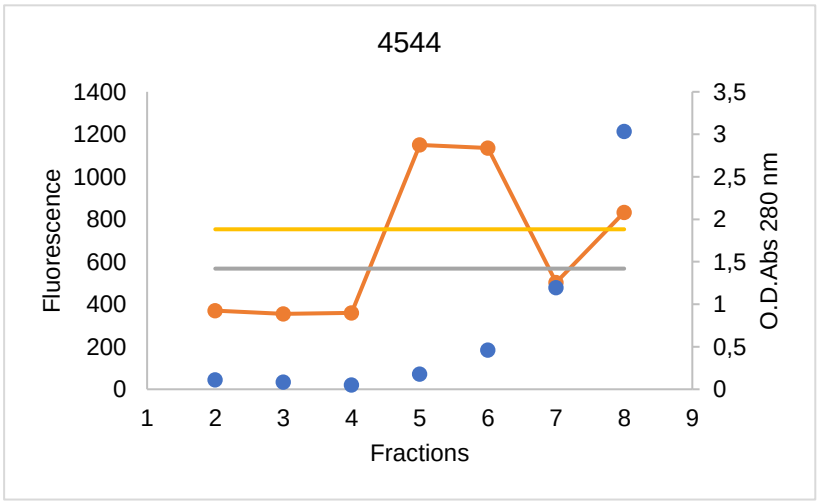

Figure 38 - Protein quantification (●) by nanodrop (Abs 280 nm); Bead-based assay for FACS analysis (●) for fractions 1-10 obtained from SEC for sample 4544 (active) and respective controls. Fr4, Fr5 and Fr6 represent fractions 4, 5 and 6 with a concentration of proteins of 0,052; 0,178 and 0,472 µg/µL, respectively. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

For 5878 and 4544 samples, the fraction with EXos was fraction 5. In the case of 3935 samples, the fraction enriches in exosomes was fraction 6. The fractions that presented the highest response to CD71, a marker used for the detection of EXos in plasma, were analyzed by nanosight (Figure 39).

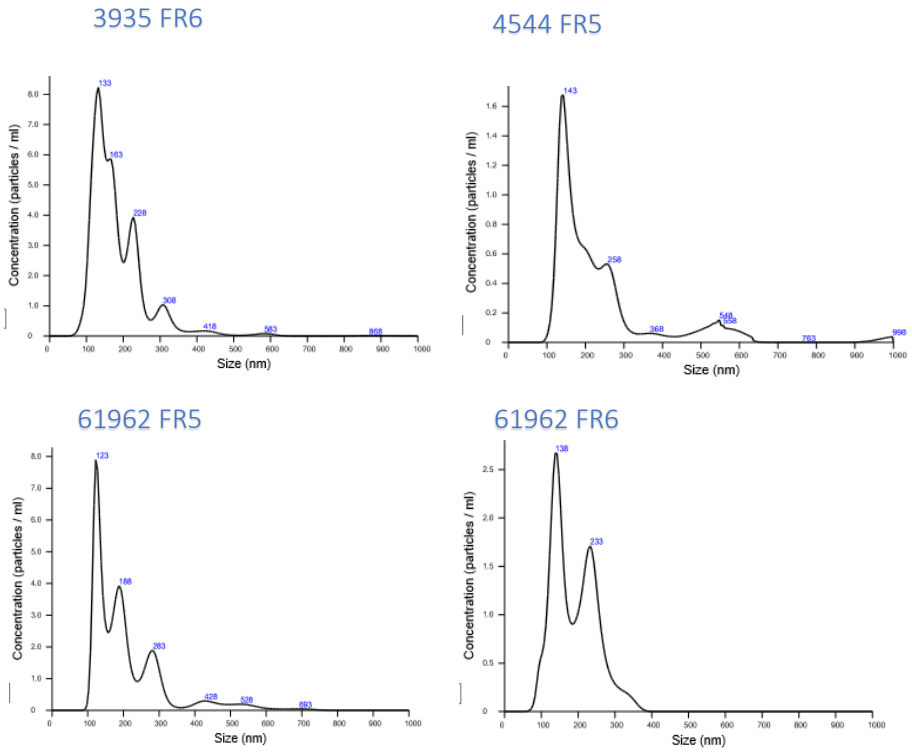

Figure 39 - Nanosight profile for the active Leishmaniasis patients (3935 FR6; 4544 FR5; 61962 FR5 & FR6). Size and concentration information for the fractions with more EXos.

Bellow we can observe the size and concentration of the samples for the fractions that were most enriched in exosomes (Table 3):

Table 3- Size (mode) and concentration (particles/mL or particles/frame) of the fractions (FR) enriched in EXos for active Leishmaniasis plasmas

Samples	Mode	Concentration	
3935 FR6	131,8 nm	1,63 x 10^{11} particles/mL	137,5 particles/frame
4544 FR5	140,2 nm	3,21 x 10^{10} particles/mL	54,3 particles/frame

The size of these samples varies between 131.8-140.2 nm and the concentration is in the order of 10^{10} or 10^{11} particles/mL, similarly to the negative controls. We used both fraction 5 and fraction 6 of sample 61962 for nanosight analysis because the bead-based assay wasn't performed and we were not sure which fraction was enriched in exosomes.

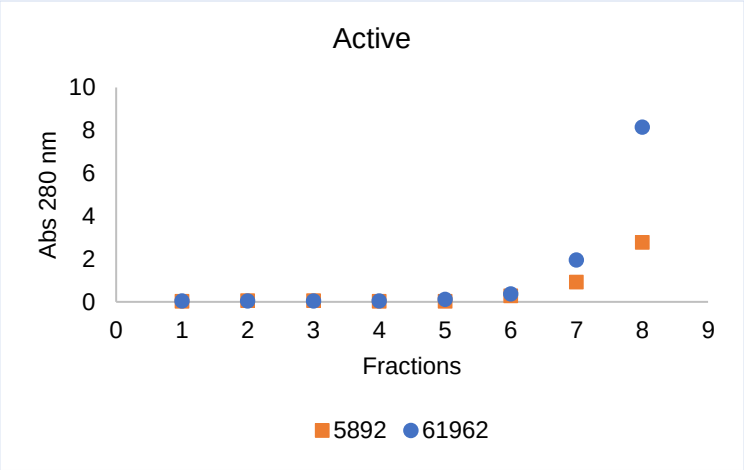

Figure 40 - Results of nanodrop (Abs 280 nm) for fractions 1-8 obtained from SEC for samples 5892 and 61962 (active). Fractions 5 and 6 from sample 5892 contained 0,0346 and 0,288 µg/µL. Fractions 5 and 6 from sample 61962 contained 0,128 and 0,379 µg/µL

3.3. Isolation and characterization of extracellular vesicles from Supernatant from *L. infantum* culture

To evaluate the efficiency of different size sepharose columns (1 and 10 mL) on the recovery of EVs, SEC was performed with the supernatant from a culture with *Leishmania infantum* (Figure 41-42).

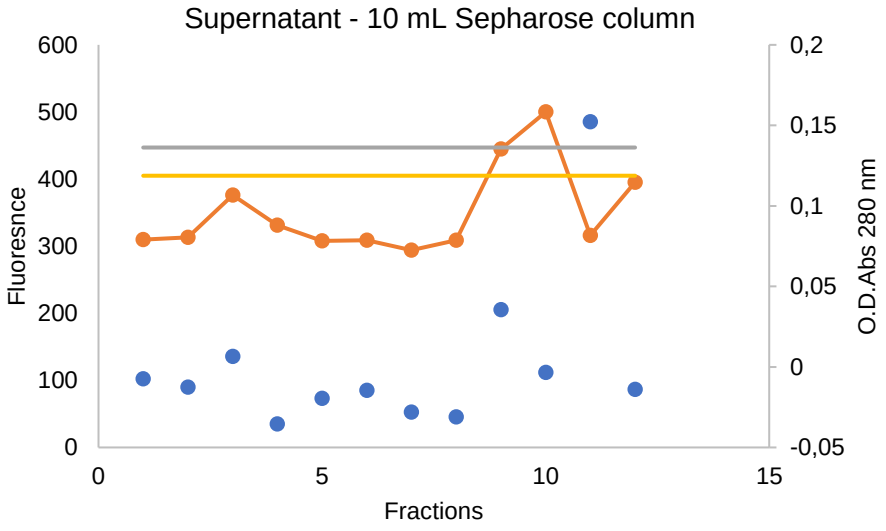

Figure 41 - Results of nanodrop (●) (Abs 280 nm), bead-based assay for FACS analysis (●) and respective controls (fluorescence) for fractions 1-12 obtained from 10 mL column SEC for supernatant sample from *Leishmania infantum* culture. Fr10 represents fraction 10 with 0,01 µg/µL. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

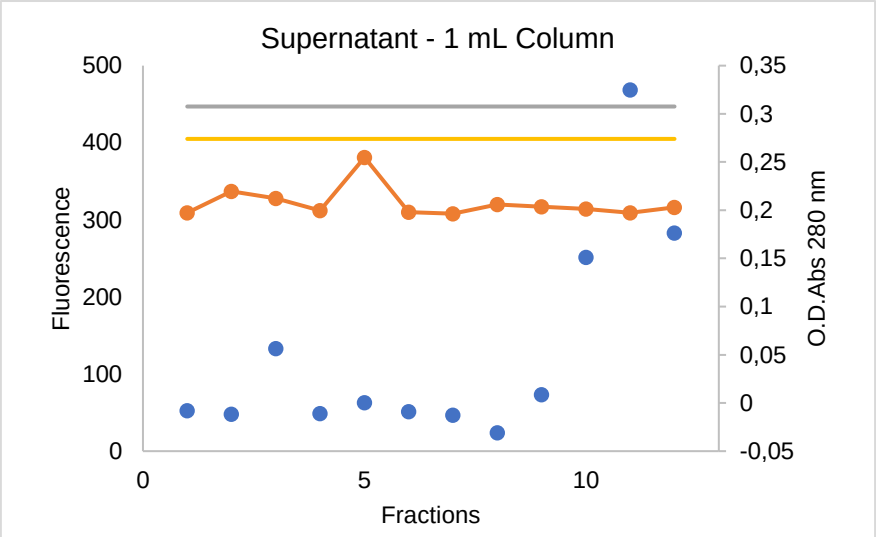

Figure 42 - Results of nanodrop (●) (Abs 280 nm), bead-based assay for FACS analysis (●) and respective controls (fluorescence) for fractions 1-12 obtained from 1 mL column SEC for supernatant sample from *Leishmania infantum* culture. Fr5 represents fraction 5 with 0,01 µg/µL. C1 (-) - exosomes + beads + isotype (1:5000) + secondary antibody; C2 (-) - beads + Isotype (1:5000) + secondary antibody

We can observe a peak in fraction 5 in the SEC performed with 1 mL column, suggesting that the most of the EVs were isolated in this fraction. In the 10 mL column, it seems that the EVs were isolated in fraction 9 and 10. In both cases, the protein concentration of these fractions is really low. These results also indicate that with 1 mL columns we can isolate the most of the EVs in a single fraction.

3.4. Isolation and characterization of exosomes from Asymptomatic – Infected Patients

For asymptomatic patients, we isolated the exosomes (EXos) from 3 different plasma samples. The isolated fractions were obtained with a 1 mL sepharose column through size exclusion chromatography (SEC):

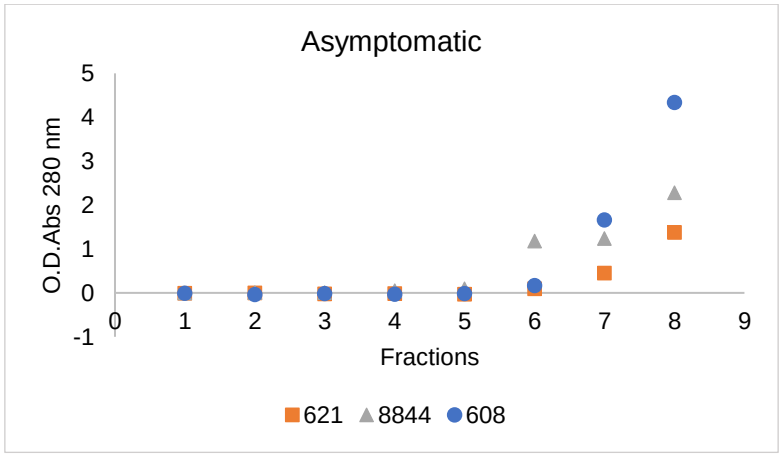

Figure 43 - Results of nanodrop (Abs 280 nm) for fractions 1-8 obtained from SEC for samples 608, 8844 and 621 (asymptomatic).

Table 4 - Results of nanodrop for samples 608, 8844 and 621 (asymptomatic) in µg/µL.

Samples	Fraction 5	Fraction 6
608	0	0,164
8844	0,091	1,177
	Fraction 6	**Fraction 7**
621	0,09	0,452

3.5. Isolation and characterization of exosomes from Healed patients

For healed patients, we isolated the exosomes enriched fractions from 3 different plasma samples through SEC:

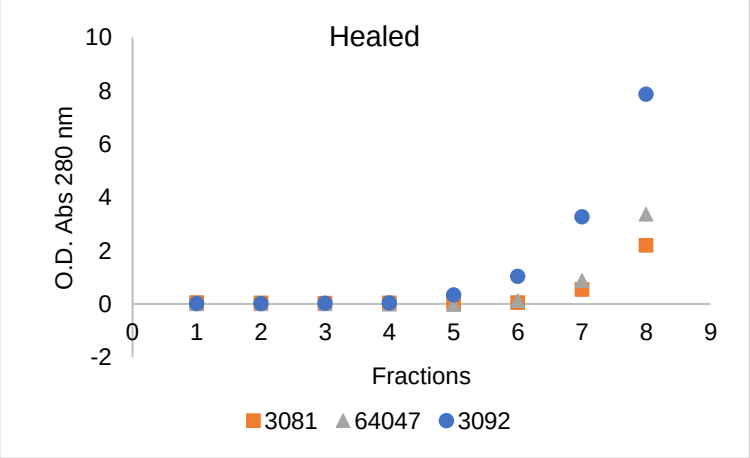

Figure 44- Results of nanodrop (Abs 280 nm) for fractions 1-8 obtained from SEC for samples 3081, 64047 and 3092 (healed).

Table 5 - Results of nanodrop for samples 3081, 64074 and 3092 (healed) in µg/µL.

Samples	Fraction 4	Fraction 5
3092	0,045	0,335
3081	0	0,128
	Fraction 6	**Fraction 7**
64047	0,053	0,546

Both the asymptomatic group and the healed group present a similar profile (Figure 43-44) to the other two groups, which indicate that the exosomes should be in fraction 5 or fraction 6. Nevertheless, bead-based assay for FACS analysis with CD71 marker should be performed in every sample to confirm the presence of EXos. After that, nanosight should also be performed to give us information about size and concentration.

Discussion

The aim of this project was to develop an immunosensor for *Leishmania* diagnosis. For that, different immobilization strategies were tested. The first approach based on a work developed by Haddour N. *et al.*[98] was inefficient due to the wrong description of the organic synthesis of NTA-pyrrole. To overcome this unsuccessful procedure, the antigen immobilization was done in magnetic microbeads, according the protocol used in cytometry[52]. The first results showed to be promising and further studies were done on the described system, such as the optimization of the hydrodynamic conditions to be used in Flow Injection Analysis by using a microfluidic platform. However, it was observed a change in the base line of the electrode potential due to a change of the surface because of unspecific protein immobilization. So different strategies are still under study to overcome the described problems.

Other important goal to achieve in this project was the isolation of exosomes from human plasmas to do further characterization and proteomic analysis. Regarding the isolation of exosome enriched fractions with size exclusion chromatography (SEC), when we compare the results, it seems that the 1 mL sepharose columns can isolate de exosomes in only one fraction. Although the concentration of proteins is higher than usual, the fractions isolated in these columns are also more concentrated. So, it seems an advantage to use these columns to isolate samples, especially when there is little volume of samples, as these columns require ten times less sample to do the experiment (100 µL of plasma).

Other important aspect of this project was to characterize the samples with bead-based assay for FACS analysis and nanosight. Unfortunately, we couldn't get the results from the last bead-based assay due to a failure in the FACSverse flow cytometer. Consequently, not all the fractions were analyzed in nanosight. We were able to do all the EXos isolation through SEC and to evaluate the concentration of protein in each sample in nanodrop.

In the bead-based assays, the controls were quite high and in some cases, were higher than the signal of the fractions. It might be due to the isotype antibody, that is giving for some reason a high background. However, the pique is well defined and in most cases, is higher than the controls.

For a matter of time and resources, the proteomic analysis will be performed in the next weeks, starting with 3 negative controls and 3 active patients for Leishmaniasis. From these results, we will be able to compare the two group and see if we can identify some

possible biomarker. If that happens, the other samples will also be sent to proteomic analysis to get a more concise and accurate study.

References

1. Report WH. The Wo World rld Health Report 2004 changing history. 2005.

2. Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. 2012;7(5). doi:10.1371/journal.pone.0035671.

3. Organization WH. Leishmaniasis. http://www.who.int/leishmaniasis/en/.

4. Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *NatRevMicrobiol*. 2007;5(1740-1534 (Electronic)):873-882. doi:10.1038/nrmicro1748.

5. Srinivasan B, Tung S. Development and Applications of Portable Biosensors. *J Lab Autom*. 2015;20(4):365-389. doi:10.1177/2211068215581349.

6. Daniell H. NIH Public Access. 2012;76(October 2009):211-220. doi:10.1007/s11103-011-9767-z.Plastid.

7. Cecílio P, Pérez-Cabezas B, Santarém N, Maciel J, Rodrigues V, da Silva AC. Deception and manipulation: The arms of Leishmania, a successful parasite. *Front Immunol*. 2014;5(OCT):1-16. doi:10.3389/fimmu.2014.00480.

8. Roberts MTM. Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *Br Med Bull*. 2005;75-76(1):115-130. doi:10.1093/bmb/ldl003.

9. WHO. Technical Report of the TDR Disease Reference Group on Chagas Disease HT and L. Research Priorities for Chagas Disease , Human African Trypanosomiasis and Leishmaniasis. 2012:100.

10. Strazzulla A, Cocuzza S, Pinzone MR, et al. Mucosal leishmaniasis: An underestimated presentation of a neglected disease. *Biomed Res Int*. 2013;2013. doi:10.1155/2013/805108.

11. Gomes YM, Paiva Cavalcanti M, Lira R a., Abath FGC, Alves LC. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. *Vet J*. 2008;175(1):45-52. doi:10.1016/j.tvjl.2006.10.019.

12. Andrade R a D, Reis AB, Gontijo CMF, et al. Clinical value of anti-Leishmania (Leishmania) chagasi IgG titers detected by flow cytometry to distinguish infected from vaccinated dogs. *Vet Immunol Immunopathol*. 2007;116(1-2):85-97. doi:10.1016/j.vetimm.2007.01.002.

13. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. [Review] [199 refs]. *Lancet*. 9496;366(9496):1561-1577. doi:https://dx.doi.org/10.1016/S0140-6736(05)67629-5.

14. Pintado, V., Martín-Rabadán, P., Rivera, ML., Moreno, S., Bouza E. Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. *Med*. 2001;80(1):54-73. doi:10.1097/00005792-200101000-00006.

15. Distribution of Endemic Visceral Leishmaniasis worldwide 2015. World Health Organization. http://www.who.int/leishmaniasis/burden/en/. Published 2015.

16. Célia Maria Ferreira Gontijo; Melo Norma Maria. Laboratory Diagnosis of Visceral Leishmaniasis Laboratory Diagnosis of Visceral Leishmaniasis. *Clin Diagn Lab Immunol*. 2002;9(5):951-958. doi:10.1128/CDLI.9.5.951.

17. Kedzierski L. Leishmaniasis Vaccine: Where are We Today? *J Glob Infect Dis*. 2010;2(2):177-185. doi:10.4103/0974-777X.62881.

18. Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *Int J Parasitol*. 2007;37(10):1097-1106. doi:10.1016/j.ijpara.2007.04.003.

19. Kaye P, Scott P. Leishmaniasis: complexity at the host–pathogen interface. *Nat Rev Microbiol*. 2011;9(8):604-615. doi:10.1038/nrmicro2608.

20. Carson C, Quinnell RJ, Holden J, Garcez LM, Deborggraeve S, Courtenay O. Comparison of Leishmania OligoC-TesT PCR with conventional and real-time PCR for diagnosis of canine Leishmania infection. *J Clin Microbiol*. 2010;48(9):3325-3330. doi:10.1128/JCM.02331-09.

21. Schallig HDFH, da Silva ES, van der Meide WF, Schoone GJ, Gontijo CMF. Didelphis marsupialis (common opossum): a potential reservoir host for zoonotic leishmaniasis in the metropolitan region of Belo Horizonte (Minas Gerais, Brazil). *Vector Borne Zoonotic Dis*. 2007;7(3):387-393. doi:10.1089/vbz.2006.0651.

22. Coelho E a. F, Ramírez L, Costa M a. F, et al. Specific serodiagnosis of canine visceral leishmaniasis using Leishmania species ribosomal protein extracts. *Clin Vaccine Immunol*. 2009;16(12):1774-1780. doi:10.1128/CVI.00295-09.

23. Santarém N, Silvestre R, Cardoso L, Schallig H, Reed SG, Cordeiro-da-Silva A. Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. *J Clin Microbiol*. 2010;48(5):1866-

1874. doi:10.1128/JCM.02402-09.

24. Molina R, Amela C, Nieto J, et al. Infectivity of dogs naturally Phlebotomus perniciosus infected with Leishmania infantum to colonized Phlebotomus perniciosus. *Trans R Soc Trop Med Hyg*. 1994;88:491-493. doi:10.1016/0035-9203(94)90446-4.

25. Solcà MDS, Bastos LA, Guedes CES, et al. Evaluating the accuracy of molecular diagnostic testing for canine visceral leishmaniasis using latent class analysis. *PLoS One*. 2014;9(7). doi:10.1371/journal.pone.0103635.

26. Palatnik-De-Sousa CB, Dos Santos WR, França-Silva JC, et al. Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. *Am J Trop Med Hyg*. 2001;65(5):510-517.

27. Sousa S, Lopes AP, Cardoso L, et al. Seroepidemiological survey of Leishmania infantum infection in dogs from northeastern Portugal. *Acta Trop*. 2011;120(1-2):82-87. doi:10.1016/j.actatropica.2011.06.003.

28. Alvar J, Molina R, Andres MS, et al. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. *Ann Trop Med Parasitol*. 1994;88(February):371-378. doi:10.1080/00034983.1994.11812879.

29. Tayeh A, Jalouk L, Cairncross S. Twenty years of cutaneous leishmaniasis in Aleppo, Syria. *Trans R Soc Trop Med Hyg*. 1997;91(6):657-659. doi:10.1016/S0035-9203(97)90509-5.

30. Bern C, Joshi AB, Jha SN, et al. Factors associated with visceral leishmaniasis in Nepal: Bed-net use is strongly protective. *Am J Trop Med Hyg*. 2000;63(3-4):184-188.

31. Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. *J Trop Med*. 2012;2012. doi:10.1155/2012/892817.

32. Gradoni L. Canine Leishmania vaccines: Still a long way to go. *Vet Parasitol*. 2015;208(1-2):94-100. doi:10.1016/j.vetpar.2015.01.003.

33. Pejcic B, De Marco R, Parkinson G. The role of biosensors in the detection of emerging infectious diseases. *Analyst*. 2006;131(10):1079-1090. doi:10.1039/b603402k.

34. Jani I V., Janossy G, Brown DWG, Mandy F. Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor

settings. *Lancet Infect Dis*. 2002;2(4):243-250. doi:10.1016/S1473-3099(02)00242-6.

35. Vallur AC, Reinhart C, Mohamath R, et al. Accurate sero-detection of asymptomatic *Leishmania donovani* infection using defined antigens. *J Clin Microbiol*. 2016;54(February):JCM.02620-15. doi:10.1128/JCM.02620-15.

36. Soper S a., Brown K, Ellington A, et al. Point-of-care biosensor systems for cancer diagnostics/prognostics. *Biosens Bioelectron*. 2006;21(10):1932-1942. doi:10.1016/j.bios.2006.01.006.

37. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: Current status and future applications. *J Clin Microbiol*. 2007;45(1):21-25. doi:10.1128/JCM.02029-06.

38. Silvestre R, Santarém N, Teixeira L, Cunha J, Schallig H, Cordeiro-da-Silva A. Evaluation of Leishmania species reactivity in human serologic diagnosis of leishmaniasis. *Am J Trop Med Hyg*. 2009;81(2):202-208. doi:81/2/202 [pii].

39. Siddig M, Ghalib H, Shillington DC, Petersen EA. Visceral leishmaniasis in the Sudan: Comparative parasitological methods of diagnosis. *Trans R Soc Trop Med Hyg*. 1988;82(1):66-68. doi:10.1016/0035-9203(88)90265-9.

40. Srividya G, Kulshrestha A, Singh R, Salotra P. Diagnosis of visceral leishmaniasis: Developments over the last decade. *Parasitol Res*. 2012;110(3):1065-1078. doi:10.1007/s00436-011-2680-1.

41. Schallig HDFH, Canto-Cavalheiro M, Da Silva ES. Evaluation of the direct agglutination test and the rK39 dipstick test for the sero-diagnosis of visceral leishmaniasis. *Mem Inst Oswaldo Cruz*. 2002;97(7):1015-1018. doi:10.1590/S0074-02762002000700015.

42. Coura-Vital W, Marques MJ, Veloso VM, et al. Prevalence and factors associated with Leishmania infantum infection of dogs from an urban area of Brazil as identified by molecular methods. *PLoS Negl Trop Dis*. 2011;5(8). doi:10.1371/journal.pntd.0001291.

43. Islam MZ, Itoh M, Mirza R, et al. Direct agglutination test with urine samples for the diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg*. 2004;70(1):78-82.

44. Adams ER, Jacquet D, Schoone G, Gidwani K, Boelaert M, Cunningham J. Leishmaniasis Direct Agglutination Test: Using Pictorials as Training Materials to Reduce Inter-Reader Variability and Improve Accuracy. *PLoS Negl Trop Dis*.

2012;6(12):1-6. doi:10.1371/journal.pntd.0001946.

45. Pattabhi S, Whittle J, Mohamath R, et al. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. *PLoS Negl Trop Dis*. 2010;4(9). doi:10.1371/journal.pntd.0000822.

46. Mettler M, Grimm F, Capelli G, Camp H. Evaluation of Enzyme-Linked Immunosorbent Assays , an Immunofluorescent-Antibody Test , and Two Rapid Tests and Gel Tests) for Serological Diagnosis of Symptomatic and Asymptomatic Leishmania Infections in Dogs Evaluation of Enzyme-Linked Immunosorbent A. *J Clin Microbiol*. 2005;43(11):5515-5519. doi:10.1128/JCM.43.11.5515.

47. Alvar J, Cañavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. *Adv Parasitol*. 2004;57(4):1-88. doi:10.1016/S0065-308X(04)57001-X.

48. Otranto D, Paradies P, Sasanelli M, Spinelli R, Brandonisio O. Rapid Immunochromatographic Test for Serodiagnosis of Canine Leishmaniasis Rapid Immunochromatographic Test for Serodiagnosis of. *J Clin Microbiol*. 2004;42(6):2769-2771. doi:10.1128/JCM.42.6.2769.

49. Otranto D, Paradies P, De Caprariis D, et al. Toward diagnosing Leishmania infantum infection in asymptomatic dogs in an area where leishmaniasis is endemic. *Clin Vaccine Immunol*. 2009;16(3):337-343. doi:10.1128/CVI.00268-08.

50. Nolan JP, Mandy F. Multiplexed and microparticle-based analyses? Quantitative tools for the large-scale analysis of biological systems. *Cytom Part A*. 2006;69(5):318-325. doi:10.1002/cyto.a.20266.

51. Silvestre R, Santarém N, Cunha J, et al. Serological evaluation of experimentally infected dogs by LicTXNPx-ELISA and amastigote-flow cytometry. *Vet Parasitol*. 2008;158(1-2):23-30. doi:10.1016/j.vetpar.2008.09.001.

52. Sousa S, Cardoso L, Reed SG, et al. Development of a Fluorescent Based Immunosensor for the Serodiagnosis of Canine Leishmaniasis Combining Immunomagnetic Separation and Flow Cytometry. *PLoS Negl Trop Dis*. 2013;7(8):1-7. doi:10.1371/journal.pntd.0002371.

53. Lemos EM, Gomes IT, Carvalho SFG, et al. Detection of anti-Leishmania (Leishmania) chagasi immunoglobulin G by flow cytometry for cure assessment following chemotherapeutic treatment of american visceral leishmaniasis. *Clin Vaccine Immunol*. 2007;14(5):569-576. doi:10.1128/CVI.00354-06.

54. Sudarshan M, Weirather JL, Wilson ME, Sundar S. Study of parasite kinetics with antileishmanial drugs using real-time quantitative PCR in Indian visceral leishmaniasis. *J Antimicrob Chemother*. 2011;66(8):1751-1755. doi:10.1093/jac/dkr185.

55. Bastien P, Procop GW, Reischl U. Quantitative real-time PCR is not more sensitive than " conventional" PCR. *J Clin Microbiol*. 2008;46(6):1897-1900. doi:10.1128/JCM.02258-07.

56. Porrozzi R, Santos Da Costa M V., Teva A, et al. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic Leishmania infantum visceral infections in dogs. *Clin Vaccine Immunol*. 2007;14(5):544-548. doi:10.1128/CVI.00420-06.

57. Espinosa D, Boggild AK, Deborggraeve S, et al. Leishmania OligoC-TesT as a simple, rapid, and standardized tool for molecular diagnosis of cutaneous leishmaniasis in Peru. *J Clin Microbiol*. 2009;47(8):2560-2563. doi:10.1128/JCM.00259-09.

58. Espy MJ, Uhl JR, Sloan LM, et al. Real-Time PCR in Clinical Microbiology : Applications for Routine Laboratory Testing. *Clin Microbiol Rev*. 2006;19(1):165-256. doi:10.1128/CMR.19.1.165.

59. Cañavate C, Herrero M, Nieto J, et al. Evaluation of two rK39 dipstick tests, direct agglutination test, and indirect fluorescent antibody test for diagnosis of visceral leishmaniasis in a new epidemic site in highland Ethiopia. *Am J Trop Med Hyg*. 2011;84(1):102-106. doi:10.4269/ajtmh.2011.10-0229.

60. Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A, Ferroglio E. Asymptomatic Leishmania infantum infection in an area of Northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. *J Clin Microbiol*. 2010;48(1):131-136. doi:10.1128/JCM.00416-09.

61. Goto Y, Coler RN, Guderian J, Mohamath R, Reed SG. Cloning, characterization, and serodiagnostic evaluation of Leishmania infantum tandem repeat proteins. *Infect Immun*. 2006;74(7):3939-3945. doi:10.1128/IAI.00101-06.

62. Fonseca AM, Faria AR, Rodrigues FTG, et al. Evaluation of three recombinant Leishmania infantum antigens in human and canine visceral leishmaniasis diagnosis. *Acta Trop*. 2014;137:25-30. doi:10.1016/j.actatropica.2014.04.028.

63. Coelho EAF, Costa LE, Lage DP, et al. Evaluation of two recombinant Leishmania proteins identified by an immunoproteomic approach as tools for the serodiagnosis of canine visceral and human tegumentary leishmaniasis. *Vet Parasitol*. 2016;215:63-71. doi:10.1016/j.vetpar.2015.11.006.

64. Santarém N, Tomás A, Ouaissi A, et al. Antibodies against a Leishmania infantum peroxiredoxin as a possible marker for diagnosis of visceral leishmaniasis and for monitoring the efficacy of treatment. *Immunol Lett*. 2005;101(1):18-23. doi:10.1016/j.imlet.2005.04.006.

65. Boarino A, Scalone A, Gradoni L, et al. Development of recombinant chimeric antigen expressing immunodominant B epitopes of Leishmania infantum for serodiagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol*. 2005;12(5):647-653. doi:10.1128/CDLI.12.5.647-653.2005.

66. Yáñez-Mó M, Siljander PRM, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;4(2015):1-60. doi:10.3402/jev.v4.27066.

67. Santarém N, Racine G, Silvestre R, Cordeiro-da-Silva A, Ouellette M. Exoproteome dynamics in Leishmania infantum. *J Proteomics*. 2013;84(April):106-118. doi:10.1016/j.jprot.2013.03.012.

68. Fais S, O'Driscoll L, Borras FE, et al. Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. *ACS Nano*. 2016;10(4):3886-3899. doi:10.1021/acsnano.5b08015.

69. Corrales RM, Sereno D, Mathieu-Daudé F. Deciphering the Leishmania exoproteome: What we know and what we can learn. *FEMS Immunol Med Microbiol*. 2010;58(1):27-38. doi:10.1111/j.1574-695X.2009.00608.x.

70. Rogers KR. Recent advances in biosensor techniques for environmental monitoring. *Anal Chim Acta*. 2006;568(1-2):222-231. doi:10.1016/j.aca.2005.12.067.

71. Tudorache M, Bala C. Biosensors based on screen-printing technology, and their applications in environmental and food analysis. *Anal Bioanal Chem*. 2007;388(3):565-578. doi:10.1007/s00216-007-1293-0.

72. Micheli L, Grecco R, Badea M, Moscone D, Palleschi G. An electrochemical immunosensor for aflatoxin M1 determination in milk using screen-printed electrodes. *Biosens Bioelectron*. 2005;21(4):588-596.

doi:10.1016/j.bios.2004.12.017.

73. Zhang Y, Heller A. Reduction of the nonspecific binding of a target antibody and of its enzyme-labeled detection probe enabling electrochemical immunoassay of an antibody through the 7 pg/mL-100 ng/mL (40 fM-400 pM) range. *Anal Chem*. 2005;77(23):7758-7762. doi:10.1021/ac051218c.

74. Makaraviciute A, Ramanaviciene A. Site-directed antibody immobilization techniques for immunosensors. *Biosens Bioelectron*. 2013;50:460-471. doi:10.1016/j.bios.2013.06.060.

75. Fu X, Wang J, Li N, Wang L, Pu L. Label-free electrochemical immunoassay of carcinoembryonic antigen in human serum using magnetic nanorods as sensing probes. *Microchim Acta*. 2009;165(3-4):437-442. doi:10.1007/s00604-009-0159-x.

76. Ansari AA, Alhoshan M, Alsalhi MS, Aldwayyan AS. Prospects of nanotechnology in clinical immunodiagnostics. *Sensors*. 2010;10(7):6535-6581. doi:10.3390/s100706535.

77. Tang DP, Yuan R, Chai YQ, et al. Novel potentiometric immunosensor for hepatitis B surface antigen using a gold nanoparticle-based biomolecular immobilization method. *Anal Biochem*. 2004;333(2):345-350. doi:10.1016/j.ab.2004.06.035.

78. Qiu JD, Huang H, Liang RP. Biocompatible and label-free amperometric immunosensor for hepatitis B surface antigen using a sensing film composed of poly(allylamine)-branched ferrocene and gold nanoparticles. *Microchim Acta*. 2011;174(1):97-105. doi:10.1007/s00604-011-0585-4.

79. Cecchetto J, Carvalho FC, Santos A, Fernandes FCB, Bueno PR. An impedimetric biosensor to test neat serum for dengue diagnosis. *Sensors Actuators, B Chem*. 2015;213(February):150-154. doi:10.1016/j.snb.2015.02.068.

80. Perinoto ÂC, Maki RM, Colhone MC, et al. Biosensors for efficient diagnosis of leishmaniasis: Innovations in bioanalytics for a neglected disease. *Anal Chem*. 2010;82(23):9763-9768. doi:10.1021/ac101920t.

81. Ramos-Jesus J, Carvalho K a., Fonseca R a S, et al. A piezoelectric immunosensor for Leishmania chagasi antibodies in canine serum. *Anal Bioanal Chem*. 2011;401(3):917-925. doi:10.1007/s00216-011-5136-7.

82. Souto DEP, Silva J V., Martins HR, et al. Development of a label-free

immunosensor based on surface plasmon resonance technique for the detection of anti-Leishmania infantum antibodies in canine serum. *Biosens Bioelectron*. 2013;46:22-29. doi:10.1016/j.bios.2013.01.067.

83. Putzbach W, Ronkainen NJ. Immobilization techniques in the fabrication of nanomaterial-based electrochemical biosensors: a review. *Sensors (Basel)*. 2013;13(4):4811-4840. doi:10.3390/s130404811.

84. Wang J. Electrochemical biosensors: Towards point-of-care cancer diagnostics. *Biosens Bioelectron*. 2006;21(10):1887-1892. doi:10.1016/j.bios.2005.10.027.

85. Hammond JL, Formisano N, Estrela P, Carrara S, Tkac J. Electrochemical biosensors and nanobiosensors. *Essays Biochem*. 2016;60(1):69-80. doi:10.1042/EBC20150008.

86. Monzó J, Insua I, Fernandez-Trillo F, Rodriguez P. Fundamentals, achievements and challenges in the electrochemical sensing of pathogens. *Analyst*. 2015:7116-7128. doi:10.1039/c5an01330e.

87. Johnson A, Song Q, Ko Ferrigno P, Bueno PR, Davis JJ. Sensitive affimer and antibody based impedimetric label-free assays for C-reactive protein. *Anal Chem*. 2012;84(15):6553-6560. doi:10.1021/ac300835b.

88. Ferreira NS, Sales MGF. Disposable immunosensor using a simple method for oriented antibody immobilization for label-free real-time detection of an oxidative stress biomarker implicated in cancer diseases. *Biosens Bioelectron*. 2014;53:193-199. doi:10.1016/j.bios.2013.09.056.

89. Holford TRJ, Davis F, Higson SPJ. Recent trends in antibody based sensors. *Biosens Bioelectron*. 2012;34(1):12-24. doi:10.1016/j.bios.2011.10.023.

90. Amorim CMPG. Avaliação da utilização de sensores renováveis miniaturizados na análise de drogas e tóxicos em diversas matrizes.

91. Billah M, Hays HCW, Millner PA. Development of a myoglobin impedimetric immunosensor based on mixed self-assembled monolayer onto gold. *Microchim Acta*. 2008;160(4):447-454. doi:10.1007/s00604-007-0793-0.

92. Campanella L, Martini E, Tomassetti M. Determination of HIgG and anti-HIgG using a single potentiometric immunosensor and two different “competitive methods”: Application to the analysis of globulin G in human serum. *Sensors Actuators, B Chem*. 2008;130(1):520-530. doi:10.1016/j.snb.2007.09.046.

93. Jaffrezic-Renault N, Dzyadevych S V. Conductometric microbiosensors for environmental monitoring. *Sensors*. 2008;8(4):2569-2588. doi:10.3390/s8042569.

94. Jain KK. Applications of nanobiotechnology in clinical diagnostics. *Clin Chem*. 2007;53(11):2002-2009. doi:10.1373/clinchem.2007.090795.

95. Keay RW, McNeil CJ. Separation-free electrochemical immunosensor for rapid determination of atrazine. *Biosens Bioelectron*. 1998;13(9):963-970. doi:10.1016/S0956-5663(98)00008-6.

96. Azzazy HME, Mansour MMH, Kazmierczak SC. Nanodiagnostics: A new frontier for clinical laboratory medicine. *Clin Chem*. 2006;52(7):1238-1246. doi:10.1373/clinchem.2006.066654.

97. Schönenberger BR. Cultivation and in vitro cloning or procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Short communication. *Acta Trop*. 1979;36(3):289-292.

98. Haddour N, Cosnier S, Gondran C. Electrogeneration of a poly(pyrrole)-NTA chelator film for a reversible oriented immobilization of histidine-tagged proteins. *J Am Chem Soc*. 2005;127(16):5752-5753. doi:10.1021/ja050390v.

99. de Menezes-Neto A, Sáez MJ, Lozano-Ramos I, et al. Size-exclusion chromatography as a stand-alone methodology identifies novel markers in mass spectrometry analyses of plasma-derived vesicles from healthy individuals. *J Extracell Vesicles*. 2015;4(1):1-14. doi:10.3402/jev.v4.27378.

100. Teles FRR, Fonseca LP. Applications of polymers for biomolecule immobilization in electrochemical biosensors. *Mater Sci Eng C*. 2008;28(8):1530-1543. doi:10.1016/j.msec.2008.04.010.

101. Ouyang J, Li Y. Great improvement of polypyrrole films prepared electrochemically from aqueous solutions by adding nonaphenol polyethyleneoxy (10) ether. *Polymer (Guildf)*. 1997;38(15):3997-3999.

102. Myers RE. Chemical oxidative polymerization as a synthetic route to electrically conducting polypyrroles. *J Electron Mater*. 1986;15(2):61-69. doi:10.1007/BF02649904.

103. Omastová M, Trchová M, Kovářová J, Stejskal J. Synthesis and structural study of polypyrroles prepared in the presence of surfactants. *Synth Met*. 2003;138(3):447-455. doi:10.1016/S0379-6779(02)00498-8.

104. Hart SR, Waterfield MD, Burlingame AL, Cramer R. Factors governing the solubilization of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI TOFMS. *J Am Soc Mass Spectrom*. 2002;13(9):1042-1051. doi:10.1016/S1044-0305(02)00432-4.

105. Haun JB, Yoon TJ, Lee H, Weissleder R. Magnetic nanoparticle biosensors. *Wiley Interdiscip Rev Nanomedicine Nanobiotechnology*. 2010;2(3):291-304. doi:10.1002/wnan.84.

106. Paleček E, Fojta M. Magnetic beads as versatile tools for electrochemical DNA and protein biosensing. *Talanta*. 2007;74(3):276-290. doi:10.1016/j.talanta.2007.08.020.

107. Chou J-C, Yan S-J, Liao Y-H, et al. Fabrication of Flexible Arrayed Lactate Biosensor Based on Immobilizing LDH-NAD+ on NiO Film Modified by GO and MBs. *Sensors*. 2017;17(7):1618. doi:10.3390/s17071618.

108. Khalilzadeh B, Shadjou N, Afsharan H, Eskandani M, Charoudeh HN, Rashidi MR. Reduced graphene oxide decorated with gold nanoparticle as signal amplification element on ultra-sensitive electrochemiluminescence determination of caspase-3 activity and apoptosis using peptide based biosensor. *BioImpacts*. 2016;6(3):135-147. doi:10.15171/bi.2016.20.

109. Clinical forms of the leishmaniases. World Health Organization. http://www.who.int/leishmaniasis/clinical_forms_leishmaniases/en/.

Appendix 1 – Commercial Immunochromatographic Tests

Manufacturer	Product name	Bound Antigen	Application	Time	Leishmaniasis type	Test	Web site
Bio-Rad Laboratories	DiaMed-IT LEISH	rK39	Human	na	Visceral L.	IC	http://www.diamed.com.br/cmi/Pagina.aspx?306
CTK Biotech, Inc	OnSite Leishmania Ab Rapid Test	rK39	Human	15 min	Visceral L.	IC	http://www.grupolabca.com.mx/laboratorios%20clinicos/pruebas%20rapidas/ctk%20inserts/R0122C.pdf
Span Diagnostics, Lda	Crystal KA (Kala azar)	rKE16	Human	15 min	MucoCutânea	IC	http://www.dpcleb.com/product/view/204
InBios International Inc	Kalazar Detect™	rK39	Human	10 min	Visceral L.	IC	http://www.inbios.com/rapid-tests/kalazar-detect
InBios International Inc	Canine Visceral Leishmaniasis Dipstick*	rK39	Dog	na	Visceral L.	IC	http://www.inbios.com/veterinary-reagent-controls/canine
Vtrade	Anigen Rapid Leishmania Ab Test Kit	na	Dog	20 min	Visceral L.	IC	http://www.vtrade.be/nl/main/gamma/rapid-tests/test-rapide-kitvia-v600920-leishmaniose-eng.pdf
BIONOTE	Anigen Rapid Leishmania Ab Test Kit	na	Dog	20 min	Visceral L.	IC	http://www.bionote.co.kr/ANIMAL/ENG/Production/canine.asp?LT=2&MODE=V&bIdx=165&bcIdx=9&P=2&BLT=L&PC=20&gAIdx=&gSearchText=
Virbac Animal Health	Speed Leish K	Kinesin Capture Complex	Dog	20 min	Visceral L.	IC	http://en.bvt.fr/p-bvtfrpuben/display.aspx?srv=p-bvtfr&typ=pub&lang=en&cmd=view&style=styles/specie.xsl&select=PRODUCT%5B@IDeqPRODUCT_98%5D
Bio-Manguinhos/Fiocruz	DPP® Canine Leishmaniasis	rK39	Dog	15 min	Visceral L.	IC	https://www.bio.fiocruz.br/en/index.php/products/ivd-tests/rapid-tests/dppr-canine-leishmaniasis
Biogal, Galed Labs.	ImmunoRun	na	Dog	5-20	Visceral L.	IC	file:///C:/Users/camorim/Downloads/ircli155.01.12.11%20(2).pdf
Quicking Biotech	Canine Leishmania Antibody Test	HRPO Conjugate	Dog	10 min	Visceral L.	IC	http://en.quicking.cn/Products/Pet/Canine/Enquicking12.html
EcoDiagnóstica	Leishmania Ab	na	Dog	10 min	Visceral L.	IC	http://www.ecodiagnostica.com.br/produto/leishmania-ab