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Extracellular vesicles as a tool for identification of new biomarkers in Chagas disease

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Espero que us mogui un desig de coneixement, l'anhel de saber què en pot sorgir, de l'exhalació de l'univers. Perquè, encara que la duració d'un cert univers sigui calculable, la varietat de la vida que s'hi crea no ho és. Els edificis que hem aixecat, l'art, la música i la poesía que hem compost, la vida mateixa que hem tingut: res d'això es podría haver previst, perquè res d'això era inevitable.

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ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
ADA	Adenosine deaminase
AGC	Auto gain control
ANP	Atrial natriuretic peptide
APOA1	Apolipoprotein A1
BBA	Bead-based assay flow cytometry
BCA	Bicinchoninic acid
BNP	Brain natriuretic peptide
BPM	Beats per minute
BSA	Bovine serum albumin
BZD	Benznidazole
CCL	Chemokine C-C ligands
CD	Chagas disease
CEADES	Ciencia y Estudios Aplicados para el Desarrollo En Salud y medioambiente
CKMB	Creatine kinase–MB
CRP	C-reactive protein
cryoEM	Cryo-electron microscopy
CT	Computed tomographic scan
cTnT	Cardiac troponin T
DMEM	Dulbecco modified eagles minimal essential medium
DNA	Deoxyribonucleic acid
DTU	Discrete typing unit
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
ETP	Endogenous thrombin potential
EV	Extracellular vesicle
F1+2	Fragment 1+2
FBN	Fibronectin
FBS	Fetal bovine serum
FDR	False discovery date
GAL-1	Galectin-1
GPI-mucins	Glycosylphosphatidylinositol-anchored mucin glycoproteins
GPx	Glutathione peroxidase
HCD	Higher-energy collisional dissociation
HCF	Human cardiac fibroblasts
HCM	Human cardiac myocytes
HLA	Human leukocyte antigen
HSF	Human spleen fibroblast
HUVEC	Human umbilical vein endothelial cells
IgG	Immunoglobulin G
IFN-γ	Interferon gamma
IL	Interleukin
ISEV	International society for extracellular vesicles
kDNA	*T. cruzi*'s kinetoplast deoxyribonucleic acid
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
MAPK	Mitogen-activated protein kinases

MASP	Mucin-Associated Surface Proteins
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MMP	Matrix metalloproteinase
MS	Mass spectrometry
NFX	Nifurtimox
NO	Nitric oxide
NTA	Nanoparticle track analysis
NT-proBNP	N-terminal portion of brain natriuretic peptide
PAHO	Pan American Health Organization
PAP	Plasmin -antiplasmin complexes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Proteome discover
PGE2	Prostaglandin E2
PLSDA	Partial least squares-discriminant analysis
PPDK	Pyruvate phosphate dikinase
PS	Penicillin/streptomycin
P. vivax	*Plasmodium vivax*
qPCR	Quantitative PCR
RHS	Retrotransposon hot dpot
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
Se	Selenium
SEC	Size exclusion chromatography
SMGC	Smooth muscle growth complement
SN	Supernatant
SNO	S-nitrosylation
SNP	Single nucleotide polymorphism
T. cruzi	*Trypanosoma cruzi*
TcTASV	*Trypanosoma cruzi* Trypomasigote Alanine, Valine and Serine family
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TLR2	Toll-Like Receptor 2
TNF-α	Tumor necrosis factor alpha
TTM	Treatment
UC	Ultracentrifugation
UP	Unique peptide
VCAM	Vascular cell adhesion molecule
WHO	World Health Organization

ABSTRACT

Introduction

American tripanosomiasis or Chagas Disease (CD), caused by the parasite *Trypanosoma cruzi (T. cruzi),* remains one of most neglected tropical diseases. Endemic from 21 countries in Latin America, it is the most important infection in the region in terms of public health and economic impact (1) . Updated information from the Pan American Health Organization (PAHO) indicates that 12.000 people die from CD annually in the Americas (2). However, these figures may be highly conservative estimates, as other studies mention that as many as 200.000 people living with *T. cruzi* infection may die over the next five years from heart disease and related complications (3). Moreover, about 70 million people are exposed to the parasite, six to seven million now live with *T. cruzi* infection, and 30.000 new infections occur annually in the Americas (1). Furthermore, in the last decades CD has become a global health concern due to the migration flows from Latin America to United States, Europe, Canada and Japan (4,5).

Many challenges remain regarding CD control and prevention in endemic and non-endemic countries. There is an urgent need of more practical and useful diagnostic methods, there are no preventive vaccines, and the two available treatments present several adverse drug reactions and limited efficacy during the chronic phase of the disease (6–10). Since there are no prognosis markers, drugs should be administered to all *T. cruzi* infected individuals that fulfill treatment criteria (1,11). Additionally, there are no tests-of-cure either, which limits patients´ follow-up and the search of safer and more efficacious drugs. Thus, the finding of reliable biomarkers of disease progression and/or treatment response would mean the greatest leap forward in the history of CD since its discovery in 1909 (12).

In this context, research on the role of extracellular vesicles (EVs) for biomarkers discovery has grown exponentially in the last decades. EVs are small double membrane vesicles of cellular origin, present in most biological fluids and secreted by all kind of cells (13). The different roles of EVs are still being explored, and include multiple biological functions, such as intercellular signaling and cell-to-cell communication (14). As the study of EVs is an active area of research, many biomedical utilities are still being explored, such as carriers for drug and gene therapy, antigen presentation, or therapeutic properties. Importantly, EVs present a huge potential as biomarkers in clinical diagnosis: they present highly specificity and sensitivity, excellent stability, and can be easily obtained in biofluids (14,15).

This thesis explores the potential of EVs secreted during *T. cruzi* infection as potential biomarkers for therapeutic response and disease outcome in CD.

Hypothesis and objectives

Our hypothesis is that circulating EVs from *T. cruzi* infection, contain human and parasite proteins that are present or upregulated before treatment and disappear or are downregulated post-treatment. Moreover, we propose that circulating EVs present a particular signature specific of the disease stage. Therefore, EVs isolated from *T. cruzi* infected patients biofluids could be used as potential biomarkers for therapeutic response and disease outcome.

To gain insights into the use of EVs secreted during *T. cruzi* infection as novel biomarkers for CD and its role in CD pathology, we have established three specific objectives:

1. To identify EVs biomarkers in plasma of different clinical groups of chronic *T. cruzi* infected patients.

2. To validate such biomarkers in the context of therapeutic response, and its possible correlation with the progression to cardiological clinical manifestations.

3. To determine the role of circulating EVs in CD pathology.

Methods and key results

A total of 77 individuals were recruited for this study. Participants were allocated in four different groups based in their serological test and cardiological complications:

A) *T. cruzi*-seropositive individuals without evidence of organ involvement (called as individuals with the indeterminate form of the disease)

B) *T. cruzi*-seropositive individuals presenting cardiopathy

C) *T. cruzi*-seronegative individuals presenting cardiopathy

D) *T. cruzi*-seronegative individuals without evidence of organ involvement

Several methods for isolating EVs from plasma have been performed: size-exclusion chromatography (10 mL and one mL columns), ultracentrifugation (UC) as a pre-isolation step, and immunoaffinity capture using CD9, CD63 and CD81 positive beads; being the latter the best method to purify circulating EVs from *T. cruzi* infected cells. Similarly, several methods for EVs characterization have been explored: bicinchoninic acid (BCA) and microBCA, bead-based assay flow cytometry (BBA), nanoparticle track analysis (NTA), western blot and electron-microscopy. Moreover, mass-spectromery proteomics analysis have been performed to determine the composition of the EVs.

Through the proteomic analysis of the EVs purified by immunoaffinity capture, we have identified a cluster of 62 parasite proteins detected with at least 2 unique peptides (UP) in EVs from *T. cruzi* infected patients. Curiously, more parasite proteins have been found in the group of *T. cruzi* infected patients presenting the indeterminate form, compared to those CD patients presenting the cardiac form. As expected, more *T. cruzi* proteins have been identified in EVs isolated from plasma samples of patients before treatment compared to those collected after treatment. On the other hand, we have also detected 375 human proteins with at least 2 UP, several of those specifically identified in *T. cruzi* infected patients.

We have also isolated the vesicles secreted by infected and uninfected cardiac cells by immunoaffinity capture using CD9, CD63 and CD81 beads, characterized these EVs by western blot, and performed mass-spectrometry proteomics in order to study its proteomic profile. Interestingly, we have identified 13 parasite proteins in EVs from *T. cruzi* infected cardiac cells, most of these detected in vesicles secreted by human cardiac fibroblasts. Moreover, we have identified a cluster of human proteins specific from EVs of infected cardiac cells.

Finally, to determine a potential function of EVs derived from *T. cruzi* infection, an angiogenic assay using HUVEC cells and VEs derived from three patients with cardiac CD was performed. Interestingly, we found that HUVEC cells angiogenic response was dependent on the EV stimulus. HUVEC cells treated with EVs from *T. cruzi* infected human cardiac myocytes presented a decreased angiogenic response compared to cells treated with EVs from uninfected cells. On the other hand, HUVEC cells treated with EVs isolated from plasma of cardiac CD presented an increase in the angiogenesis compared to cells treated with EVs from healthy donors.

Discussion

Our study represents the first mass spectrometry proteomics analysis of EVs from *T. cruzi* infected patients presenting several forms of the disease. First, we have shown that the immunoaffinity capture is the best method to enrich circulating EVs from *T. cruzi* infected cells in the different groups of patients presenting chronic CD from our study. The proteomic analysis of these EVs has identified parasite proteins, finding important among samples collected before and after BZD treatment. Moreover, some of the parasite proteins identified are disease specific, being exclusively detected in patients presenting the cardiac or the indeterminate form of the disease. Interestingly, we have also described a specific group dependent human proteomic profile. Curiously, several of the human proteins identified in EVs from *T. cruzi* infected patients

are associated to inflammation, and might be of interest for a better understanding of the disease pathology. All together, these results reinforce the potential of EVs as novel biomarkers for CD.

We also performed the first LC-MS/MS proteomic analysis of EVs derived from *T. cruzi* infected and uninfected human cardiac cells (strain CL Brener). Interestingly, we observed a clear enrichment of parasite proteins, together with specific human proteins in EVs secreted by infected cells.

Finally, the preliminary results of our functional assay have shown that EVs found in circulation in plasma of CD patients increase the angiogenic response compared to EVs from healthy donors. This effect in the increase of the angiogenesis is not related to potential EVs secreted by infected human cardiac myocytes. Further studies including a larger sample size are needed to understand the role of circulating EVs in CD pathology.

Conclusions

- To our knowledge, this is the first molecular characterization and proteomic profiling of plasma-derived EVs purified directly from plasma of chronic CD patients with different clinical manifestations.
- Immunoaffinity capture using CD9, CD63, and CD81 is presented as the best method to purify circulating EVs from chronic CD patients.
- Circulating EVs derived from *T. cruzi* infected cells (amastigote forms) and purified by immunoaffinity capture showed a clear enrichment of parasite proteins.
- We have identified human and parasite proteins which are present or upregulated in plasma-derived EVs from cardiac chronic CD patients before treatment that are absent or downregulated following treatment, showing the potential of EVs to identify new biomarkers of therapeutic response for chronic CD.
- We have identified human and parasite proteins specifically associated to the indeterminate or cardiac form of the disease. These results encourage the use of circulating EVs as potential biomarkers of disease progression.
- EVs derived from in vitro *T. cruzi*-infected cardiac cells and purified by immunoaffinity capture using CD9, CD63, and CD81 showed a clear enrichment of parasite proteins. Molecular cargo present in EVs secreted from infected cells is different from the EVs secreted directly by the parasite.

- Specific human proteins have also been identified in EVs secreted by cardiac infected cells, which could be used to immunoaffinity capture this particular subpopulation of EVs in biological samples of *T. cruzi* infected patients.

- A preliminary functional assay using EVs derived from three cardiac CD patients showed a clear tendency to increase angiogenesis. Further studies using samples of patients presenting the indeterminate form of the disease and increasing the sample size are needed.

RESUMEN

Introducción

La enfermedad de Chagas, causada por el parásito *Trypanosoma cruzi (T. cruzi),* es todavía hoy una de las enfermedades más desatendidas. Endémica de 21 países en América Latina, es la infección más importante en la región en cuanto a la salud pública e impacto económico (1). Información actualizada de la Organización Panamericana de la Salud indica que cada año 12.000 personas mueren de enfermedad de Chagas en América Latina (2). No obstante, estas cifras pueden ser estimaciones muy conservadoras, ya que otros estudios mencionan que unas 200.000 personas viviendo con infección por *T. cruzi* podrían morir en los próximos cinco años debido a problemas cardíacos y complicaciones derivadas de estos (3). Además, hay unos 70 millones de personas expuestas a la enfermedad, de seis a siete millones de personas viven hoy en día con infección por *T. cruzi,* y 30.000 nuevas infecciones ocurren anualmente en América Latina (1). Por otro lado, en las últimas décadas la enfermedad de Chagas se ha convertido en un problema de salud global debido a los flujos migratorios de América Latina a Estados Unidos, Europa, Canadá y Japón (4,5).

Quedan muchos desafíos pendientes con respecto al control y la prevención de la enfermedad de Chagas en países endémicos y no endémicos. Por un lado, hay una necesidad urgente de métodos de diagnóstico más prácticos y útiles, no existen vacunas preventivas y los dos tratamientos disponibles causan varias reacciones adversas a los medicamentos y presentan una eficacia limitada durante la fase crónica de la enfermedad (6–10). Por otro lado, como no existen marcadores de pronóstico, los medicamentos deben administrarse a todas las personas infectadas por *T. cruzi* que cumplan con los criterios de tratamiento (1,11). Además, tampoco existen marcadores de curación, lo que limita el seguimiento de los pacientes y la búsqueda de fármacos más seguros y eficaces. Por consiguiente, el hallazgo de biomarcadores fiables de progresión de la enfermedad y/o respuesta al tratamiento supondría el mayor salto en la historia de la enfermedad de Chagas desde su descubrimiento en 1909 (12).

En este contexto, la investigación sobre el rol de las vesículas extracelulares (VE) para el descubrimiento de biomarcadores ha crecido exponencialmente en las últimas décadas. Las VEs son pequeñas vesículas de doble membrana de origen celular, presentes en la mayoría de los fluidos biológicos y secretadas por todo tipo de células (13). Los diferentes roles de las VEs aún se están explorando e incluyen múltiples funciones biológicas (14). Dado que el estudio de las VEs

es un área activa de investigación, todavía se están explorando las potenciales utilidades biomédicas de estas, tales como transporte de fármacos y terapia génica, presentación de antígenos o propiedades terapéuticas. En este contexto, es importante destacar que las VEs presentan un gran potencial como biomarcadores: presentan alta especificidad y sensibilidad, excelente estabilidad y pueden aislarse fácilmente de biofluidos (14,15).

Esta tesis explora el potencial de las VEs secretadas durante la infección por *T. cruzi* como potenciales biomarcadores para la respuesta terapéutica y el desarrollo de la enfermedad de Chagas.

Hipótesis y objetivos

Nuestra hipótesis es que las VEs que encontramos en circulación en la infección por *T. cruzi* contienen proteínas humanas y del parásito que están presentes o sobreexpresadas antes del tratamiento y desaparecen o disminuyen después del tratamiento. Además, proponemos que las VEs circulantes presentan una firma particular específica de la etapa de la enfermedad. Por lo tanto, las VEs aisladas de biofluidos de pacientes infectados con *T. cruzi* podrían usarse como potenciales biomarcadores para la respuesta terapéutica y el desarrollo de la enfermedad de Chagas.

Para obtener información sobre el uso de las VEs secretadas durante la infección por *T. cruzi* como nuevos biomarcadores para la enfermedad de Chagas, y su papel biológico en esta patología, hemos establecido tres objetivos específicos:

1. Identificar biomarcadores de VEs en plasma de diferentes grupos clínicos de pacientes con infección crónica por *T. cruzi.*

2. Validar dichos biomarcadores en el contexto de la respuesta terapéutica y su posible correlación con la progresión de la enfermedad y las manifestaciones clínicas cardiológicas.

3. Determinar el papel de las VEs circulantes en la patología de la enfermedad de Chagas.

Métodos y resultados claves

Un total de 77 individuos han sido reclutados para este estudio. Los participantes fueron asignados a cuatro grupos diferentes en función de sus resultados serológicos y complicaciones cardiológicas:

A) Individuos *T. cruzi*-seropositivos que no presentan evidencia de afectación orgánica (individuos que presentan la forma indeterminada de la enfermedad)

B) Individuos *T. cruzi*-seropositivos que presentan cardiopatía

C) Individuos *T. cruzi*-seronegativos que presentan cardiopatía

D) Individuos *T. cruzi*-seronegativos sanos

A lo largo del trabajo, se han utilizado varios métodos para aislar las VEs del plasma de los pacientes: cromatografía de exclusión por tamaño (columnas de 10 mL y de un mL), ultracentrifugación como paso previo al aislamiento de las VEs, y captura por inmunoafinidad directa para el enriquecimiento de VEs positivas por CD9, CD63 y CD81; siendo este último método el mejor para purificar VEs circulantes procedentes de células infectadas con *T. cruzi*. Del mismo modo, se han explorado varios métodos para la caracterización de las VEs, tales como el ácido bicinconinico (BCA) y microBCA, la citometría de flujo, el *nanoparticle track analysis*, western blot y microscopía electrónica. Además, se ha realizado un análisis por espectrometría de masas para determinar la composición proteómica de las VEs.

A través de los análisis proteómicos de las VEs purificadas por inmunocaptura, hemos identificado un grupo de 62 proteínas del parásito con al menos 2 péptidos únicos (PU) en VEs de pacientes infectados con *T. cruzi*. Curiosamente, se han encontrado más proteínas del parásito en el grupo de pacientes infectados por *T. cruzi* que presentan la forma indeterminada de la enfermedad que en los pacientes con enfermedad de Chagas que presentan la forma cardíaca. Como cabía esperar, se han identificado más proteínas del parásito en las VEs aisladas de muestras de plasma de pacientes antes del tratamiento en comparación con las colectadas después del tratamiento. A nivel de proteínas humanas, se han identificado 375 proteínas con más de 2 PU, varias de las cuales se encuentran específicamente en pacientes infectados por *T. cruzi*.

También hemos aislado las VEs secretadas por células cardíacas infectadas y no infectadas mediante la técnica de captura por inmunoafinidad directa para el enriquecimiento de VEs positivas por CD9, CD63 y CD81. Estas VEs fueron caracterizadas por western blot, y posteriormente se realizó proteómica para estudiar su perfil, encontrando 13 proteínas del parásito secretadas en VEs de células cardíacas infectadas con *T. cruzi*, la mayoría de estas descritas en VEs secretadas por fibroblastos cardíacos humanos. Además, se identificó un grupo de proteínas humanas específicas de VEs secretadas por células cardíacas infectadas.

Finalmente, para determinar una posible función de las VEs derivadas de la infección, realizamos un experimento de angiogénesis, usando células HUVEC y VEs derivadas de tres pacientes con enfermedad de Chagas cardíaca. Curiosamente, encontramos que la respuesta es dependiente del estímulo. Por un lado, las células HUVEC tratadas con VEs de cardiomiocitos humanos infectados con *T. cruzi* presentaron una respuesta angiogénica disminuida en

comparación con las células tratadas con VEs de células no infectadas. Contrariamente, las células HUVEC tratadas con VEs aisladas de plasma de pacientes de Chagas con afectación cardíaca presentaron un aumento en la angiogénesis en comparación con las células tratadas con VEs de donantes sanos.

Discusión

Nuestro estudio representa el primer análisis proteómico de VEs de pacientes infectados con *T. cruzi* que presentan varias formas de la enfermedad. En primer lugar, hemos demostrado que la captura por inmunoafinidad es el mejor método para enriquecer VEs circulantes procedentes de celulas infectadas con *T. cruzi*, en los diferentes grupos de pacientes con enfermedad crónica de Chagas que componen nuestro estudio. Los análisis proteómicos de dichas VEs han permitido identificar proteínas del parásito, encontrando diferencias importantes en términos de proteínas del parásito entre las muestras antes y después del tratamiento con benznidazole. Por otro lado, algunas de las proteínas del parásito identificadas son específicas de pacientes que presentan la forma cardíaca y otras con la forma indeterminada de la enfermedad. Asimismo, hemos encontrado un perfil de proteínas humanas grupo específico. Curiosamente, varias de las proteínas humanas identificadas en las VEs de pacientes infectados por *T. cruzi* están asociadas a la respuesta inflamatoria y podrían ser de interés para una mejor comprensión de la patología. En conjunto, estos resultados refuerzan el potencial de las VEs como nuevos biomarcadores para la enfermedad de Chagas.

Por otro lado, hemos realizado el primer análisis proteómico de VEs secretadas por células cardíacas humanas, infectadas o no, con *T. cruzi* (cepa CL Brener). Curiosamente, hemos identificado un claro enriquecimiento en proteínas del parásito y un grupo de proteínas humanas expresadas exclusivamente en VEs de células infectadas.

Finalmente, los resultados preliminares de nuestro ensayo funcional muestran que las VEs que se encuentran en circulación en plasma de pacientes con enfermedad de Chagas aumentan la respuesta angiogénica en células HUVEC en comparación con las VEs de donantes sanos. Este efecto en el aumento de la angiogénesis no está relacionado con las VEs secretadas por las células cardíacas infectadas. Más estudios incluyendo un número mayor de muestras son necesarios para comprender el papel de las VEs circulantes en la patología de Chagas.

Conclusiones

- Hasta donde sabemos, esta es la primera caracterización molecular y proteómica de VEs aisladas de plasma de pacientes con infección por *T. cruzi* presentando diferentes manifestaciones clínicas de la enfermedad.

- La immunocaptura usando CD9, CD63 y CD81 se presenta como el mejor método para capturar VEs en circulación de pacientes crónicos con enfermedad de Chagas.

- VEs en circulación derivadas de células infectadas (con la forma amastigota del parásito) y purificadas por immunocaptura muestran un claro enriquecimiento en proteínas del parásito.

- Hemos identificado proteínas humanas y del parásito que están presentes o sobreexpresadas en VEs derivadas de plasma de pacientes con enfermedad de Chagas presentando la forma cardíaca de la enfermedad antes de tratamiento que están ausentes o presentan baja expresión después del tratamiento, demostrando que las VEs pueden ser potenciales biomarcadores de respuesta terapéutica para la enfermedad de Chagas.

- Hemos identificado proteínas humanas y del parásito específicas de pacientes que presentan la forma indeterminada o cardíaca de la enfermedad. Estos resultados apoyan la idea del uso de VEs en circulación como potenciales biomarcadores de enfermedad.

- Las VEs derivadas de células cardíacas infectadas por *T. cruzi* in vitro purificadas por immunocaptura usando los marcadores CD9, CD63, y CD81 mostraron un claro enriquecimiento en proteínas del parásito. La carga molecular presente en las VEs secretadas por células infectadas es diferente a la carga de las VEs secretadas directamente por el parásito.

- Se han identificado proteínas humanas exclusivamente expresadas en VEs de células cardíacas infectadas, que pueden ser usadas para imunocapturar esta subpoblación particular de VEs en muestras biológicas de pacientes con infección por *T. cruzi.*

- Un ensayo funcional preliminar usando VEs derivadas de tres pacientes cardíacos con enfermedad de Chagas mostró una clara tendencia a incrementar la angiogénesis de las células HUVEC. Es necesario realizar estudios adicionales usando muestras de pacientes presentando la forma indeterminada de la enfermedad y aumentando el número de muestras.

1. INTRODUCTION

1.1. CHAGAS DISEASE

1.1.1. Historical perspective of Chagas Disease

Chagas Disease (CD) is an ancient disease. The first evidences of *Trypanosoma cruzi* (*T. cruzi*) infection in the American continent come from approximately 9000 years ago: a study of exhumed mummies from archaeological sites in southern Peru and northern Chile revealed the presence of *T. cruzi*'s kinetoplast deoxyribonucleic acid (kDNA) by Polymerase Chain Reaction (PCR), demonstrating the antiquity of the disease in humans (16–19). Further evidence of CD in pre-Columbian times comes from Peruvian ceramics, showing possible representations of individuals suffering the disease, such as a head with an eyelid presenting the Romaña's sign (20). From the 16th century onwards, patients presenting symptoms compatible with CD began to be described; together with the presence of triatomine bugs, long before discovering its role in the transmission of *T. cruzi* infection (20,21). Probably the most famous report of the vector comes from Charles Darwin, who on the 25th of March 1835 described on his diary the "attack" of a kissing bug during the trip aboard the Beagle (20). However, it was not until 1909 that the scientific understanding of this disease began.

CD gets its name from its discoverer, Carlos Chagas, a young physician from Brazil. At the beginning of the 20th century, Dr. Carlos Chagas was sent to the state of Minas Gerais (Brazil), to control an outbreak of malaria that was delaying all the efforts of railroad construction. There, a railroad worker showed him a triatomine, a hematophagous insect common in the area and known as "barbeiro", which bit them at night. Observing the insect through the microscope, he observed the presence of flagellated parasites in its gut. The parasites were inoculated in healthy monkeys and, after 30 days, were observed in the blood of the infected animals. Dr. Carlos Chagas named the parasites *T. cruzi* in honor of his mentor, the Brazilian physician and bacteriologist Oswaldo Cruz (16,22). Meanwhile, Dr. Carlos Chagas was persistently looking for human hosts. He extracted blood from several people in the area, and finally found the parasite in the blood of a 2-year-old-girl, called Berenice, who presented fever, facial edema, hepatosplenomegaly and swollen lymph nodes (16,23–25). Thus, Dr. Carlos Chagas performed what it is known as a "reverse triple discovery": he was the first in discovering the vector, the parasite, and to describe the disease. Even though Dr. Carlos Chagas was the main contributor to the CD discovery, many

other researchers also improved the knowledge of the parasite and vector cycle in these early times (26,27).

In 1909, the first publication describing the disease was reported (Figure 1) (23). Since its discovery, several important physicians and researchers inside Oswaldo Cruz Institute showed a clear motivation against Dr. Carlos Chagas and CD, denying its existence or emphasizing its insignificance. Unfortunately, this behavior, based on professional rivalry, caused that Chagas' discoveries were discredited and nearly forgotten for almost 20 years. Dr. Carlos Chagas was nominated twice for the Nobel Prize, in 1913 and 1921, but he was never awarded (20).

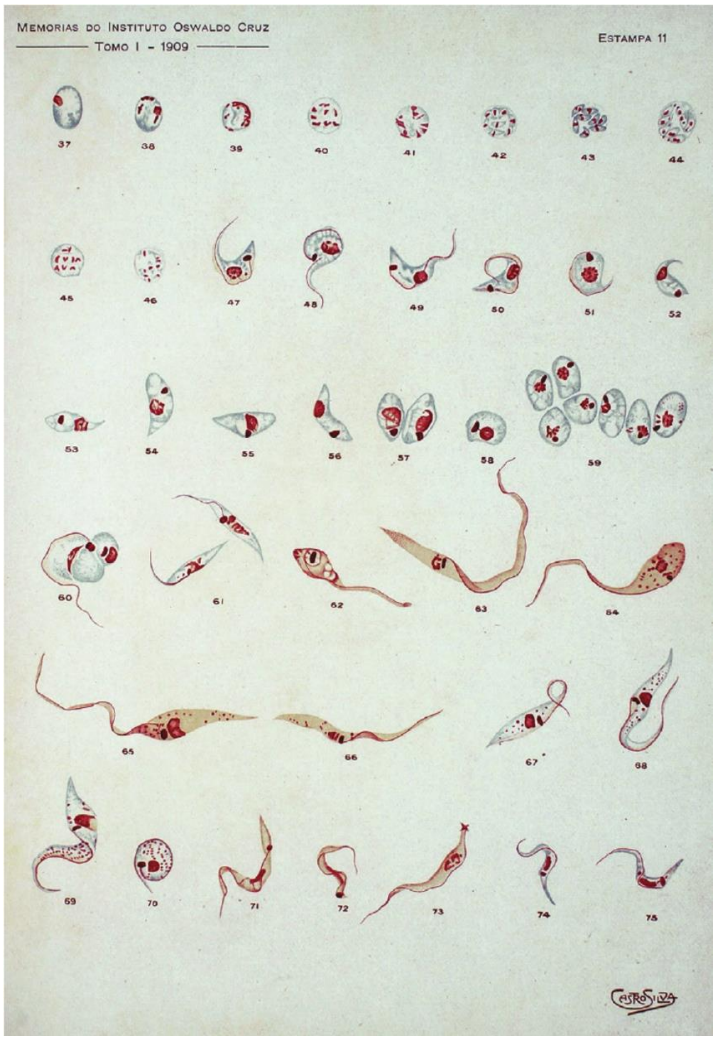


Figure 1. Reproduction of the first drawings describing *T. cruzi* by Dr. Carlos Chagas in 1909. Source of figure: (23)

Between 1909 and 1940, *T. cruzi* infection was linked to rural settings. However, the internal migration movements occurring since the mid-twentieth century within all Latin American countries from rural areas to large urban centers switched the characteristics of the

infection. CD, malaria and leishmaniasis arrived to big cities such as Buenos Aires or Rio de Janeiro. In urban healthcare centers, serological tests, electrocardiograms (ECG) and chest radiographs were performed, and studies of the digestive tract allowed doctors to recognize the esophageal and colonic involvement related to CD (16). In this context, new ways of transmitting the disease were discovered. During the first half of the 20th century, the main route of transmission was still the vectorial one. Although some non-vectorial mechanisms of transmission were suspected or reported in a few cases, it was not until the population increased its healthcare access that these were properly detected and described (16,28–31).

The first therapeutic option for *T. cruzi* infection was nifurtimox (NFX), commercialized by Bayer in 1965. Six years later, in 1971, benznidazole (BZD) was released (32). To this day, NFX and BZD continue to be the only two therapeutic options for *T. cruzi* infection, being the latter one the most commonly used globally (33). Trypanocidal treatment is currently indicated for acute cases, congenital infections, reactivations and patients in the chronic phase without symptomatology or with mild cardiac or digestive involvement (34,35). However, the efficacy of the treatment is highly variable, and depends on multiple factors, such as age of the patient, disease stage, drug dose and treatment duration, and the infecting *T. cruzi* strain or genotype (36). Moreover, both treatments are known to be associated with adverse drug reactions, which can lead to treatment discontinuation (7–9,37,38). More information regarding *T. cruzi* infection treatment can be found in the following section “Chagas Disease control: major challenges”.

In the eighties, the first reports of CD in immunosuppressed patients emerged, and it was found that *T. cruzi* reactivations are dependent on the type and severity of the immune deficiency and its association with high parasitemia (16).

In recent years, millions of people have moved across international borders, disseminating the infection from endemic to non-endemic areas. The change in migration trends and the detection of the first cases of locally transmitted *T. cruzi* infection in non-endemic countries (by non-vectorial mechanisms) raised the international alarm between 2000 and 2010, and created new epidemiological, economic, social and political challenges (39).

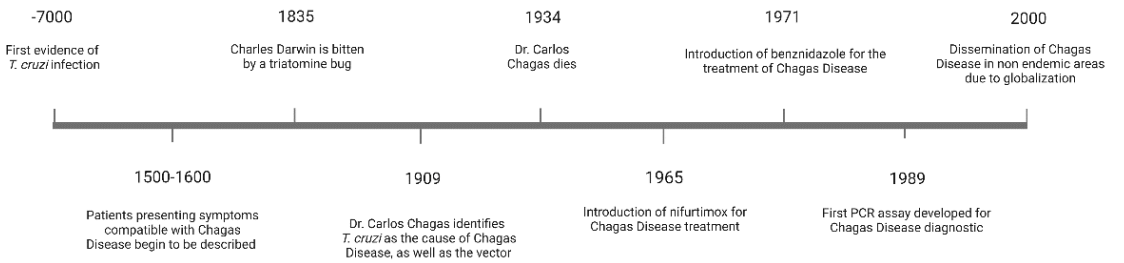


Figure 2 illustrates some of the most important events in CD history. Thus, even though CD knowledge has rapidly and extensively spread since the early findings, many challenges remain.

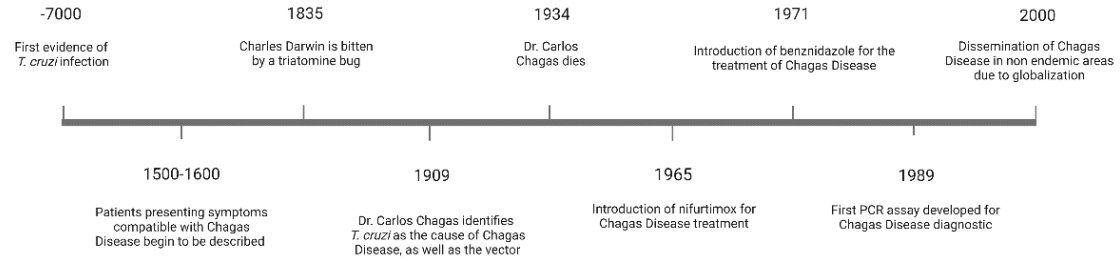


Figure 2. Timeline of the CD history. Created with BioRender.com.

1.1.2. *T. cruzi* infection and Chagas Disease burden

T. cruzi infection / CD is recognized by the World Health Organization (WHO) as one of the world's most neglected tropical diseases (1). Neglected tropical diseases are a diverse group of 20 diseases, mainly prevalent in tropical areas, that mostly affect impoverished and vulnerable communities. Importantly, CD is the most prevalent poverty-related parasitic disease in Latin America, and has serious health, social and economic consequences (1).

T. cruzi infection / CD is endemic from 21 countries in Latin America, where it presents the major burden (1). Due to the limitations of the existing surveillance systems, epidemiological data remains mainly based on models and assumptions, and not on real data (40). Nowadays, the WHO estimates that there are six to seven million people worldwide presenting the disease, with about 15.000 deaths occurring every year (1). Moreover, 75 million people in the Americas live in areas of exposure and are currently at risk of infection (1). Thus, *T. cruzi* infection still remains the most important parasitic disease in Latin America.

Because of globalization and human migration, the disease has become a global health problem, and it is now present in non-endemic areas, such as North America, Japan, Australia, and Europe (4,41). In non-endemic areas, most estimations rely on epidemiological models based on the number of migrants from each country at risk multiplied by the average infection rates. With an estimate of 300.000 persons living with *T. cruzi* infection, the United States is the country with higher number of cases outside endemic areas (4,5). In Europe, Spain has received the largest number of migrants from endemic areas, with around 55.367 migrants originally from endemic countries living with *T. cruzi* infection (42). Figure 3 shows the global distribution of *T. cruzi* infection cases.

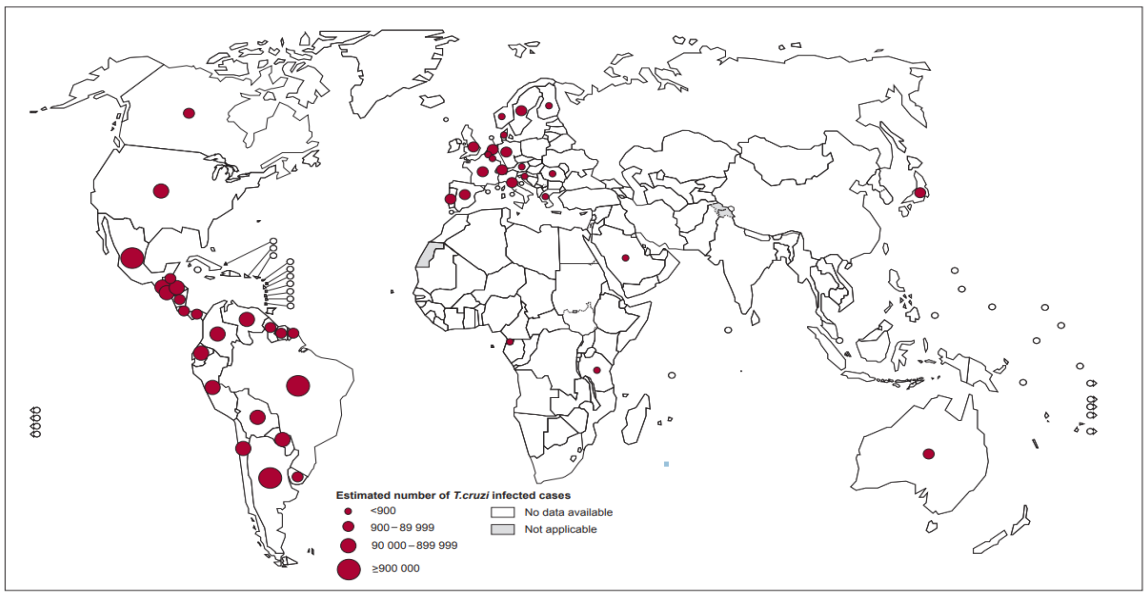


Figure 3. Global distribution of *T. cruzi* infection cases, based on official estimates (2018). Source of figure: (43)

1.1.3. Parasite characteristics, life cycle of *T. cruzi* and transmission

Chagas Disease is caused by the protozoan parasite *T. cruzi* (family Trypanosomatidae, order Kinetoplastida). The parasite is highly heterogeneous, and presents various morphological, biochemical and genetic changes along its life cycle.

The life cycle of the parasite is complex and involves several development stages in vertebrate (mammals) and invertebrate hosts (triatomines). There are more than 100 mammals acting as reservoirs, and more than 140 triatomine species that can be vectors in nature (44). Non-replicative forms are trypomastigotes, found in the bloodstream of mammalian hosts and in the vector's gut. The replicative forms are epimastigotes and amastigotes, which are found in the insect midgut and mammalian cells, respectively. The life cycle begins when the triatomine vector ingests circulating trypomastigotes in a blood meal from an infected mammalian host. Trypomastigotes transform into epimastigotes in the midgut of the vector, which differentiate into infective metacyclic trypomastigotes and are excreted with vector's feces. These metacyclic trypomastigotes enter to the mammalian host through a bite wound or any accessible open wounds, mucous membranes, or conjunctiva. Once in the blood stream, metacyclic trypomastigotes invade many types of nucleated cells. In the cytoplasm, trypomastigotes differentiate into the intracellular amastigote form, where amastigotes replicate over a period of four to five days. At the end of this period, the amastigotes transform into trypomastigotes, the host cell ruptures, and the trypomastigotes are released into the circulation, where they will invade new cells, initiating new replicative cycles (Figure 4) (45).

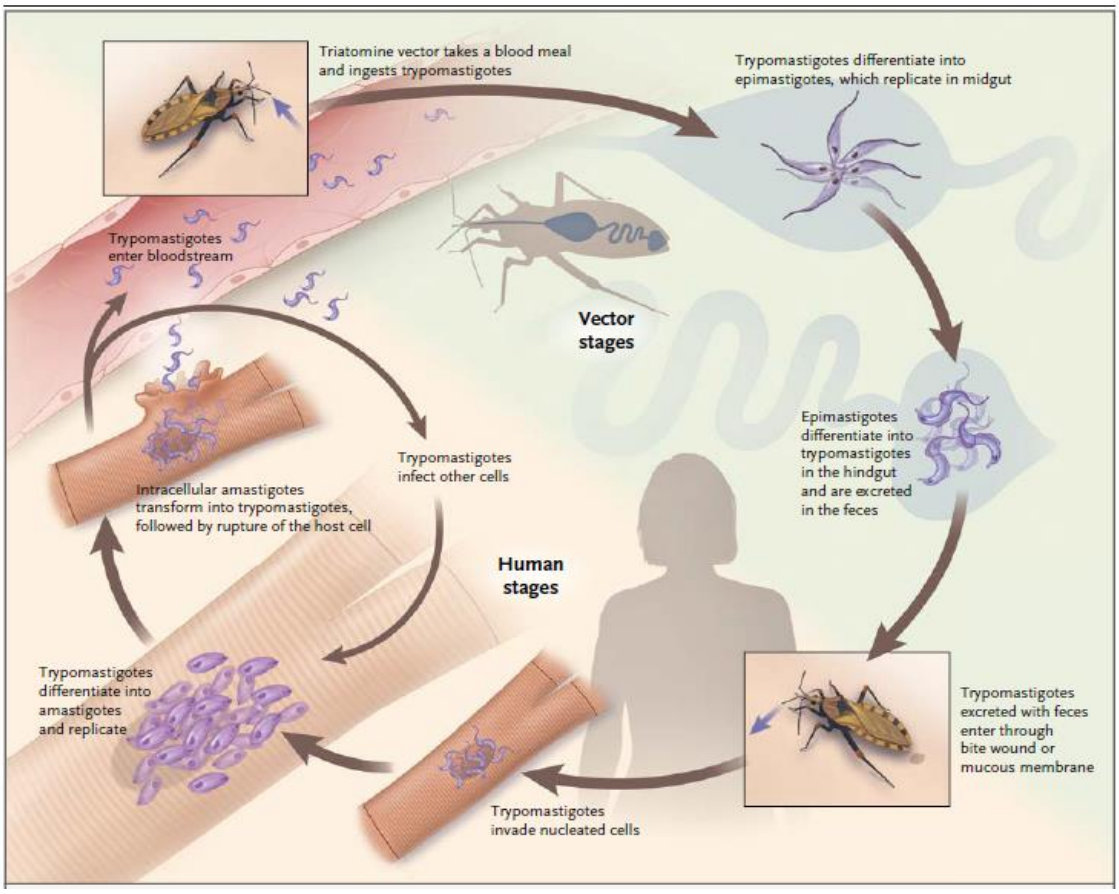


Figure 4. *T. cruzi* life cycle. Source of figure: (45)

Natural populations of *T. cruzi* have a high level of genetic diversity. The taxonomy of the parasite strains has been always difficult, but finally an international consensus was reached, and now are classified in discrete typing units (DTUs) according to their genotype. This genotype is associated with specific geographical distribution, host, and vectors (46–48). Moreover, parasites classified in the same DTU share genetic, molecular or immunological markers (46–48).

Historically, *T. cruzi* infection was transmitted to human mainly by large, bloodsucking reduviid bugs of the subfamily Triatominae. Although more than 130 species of triatomine bugs have been identified, maintaining *T. cruzi* transmission among mammals all over the Americas, only a handful are competent vectors for *T. cruzi. Triatoma infestans, Rhodnius prolixus* and *Triatoma dimidiata* are the most important vectors in the transmission of the parasite to humans. These domestic vectors can be commonly found in the cracks and holes in mud walls and roofs of rural houses, where habitants remain exposed to the vector and parasite over many years. Thus, disease prevention and control require strong and long-term vector control surveillance systems (49–51).

Vector control is carried out by chemical and physical measures. Chemical control consists of using insecticides in infested houses, while physical measures are carried through

repairs or reconstruction of infested dwellings. The effectiveness of the response depends on two major conditions: temporal continuity and spatial contiguity. These measures began at the end of 1950s in Venezuela, Brazil and Argentina, and were implemented in most Latin American countries in the 1970s and 1980s. However, no large-scale action was taken until the 1990s, with the creation of the "Cono Sur" Sub-regional Initiatives (1991) and the Andean and Central American Countries Initiative (1997), coordinated by the Pan American Health Organization (PAHO)/WHO The results from these last campaigns were very satisfactory, and almost all of the 18 Latin American countries declared themselves free of vectorial transmission (52,53).

Apart from the vector, non-vectorial mechanisms are also relevant. Those include oral transmission (after consumption of food contaminated with triatomine feces) (54), mother to child transmission (by congenital transplacental infestation, occurring in approximately 5-10% of the cases) (55), blood transfusion (56), and less frequently, by organ transplants and laboratory accidents (57). Sexual transmission has also been described (58). With the successful control measures implemented for vectorial transmission, mother to child transmission has become an important source of new cases, and nowadays remains as one of the main priorities in endemic and non-endemic regions (59). In this particular case, the risk of transmission is determined by several factors, such as maternal parasitemia levels, age, anti- *T. cruzi* immune response, and parasite strain (60). Serological screening of pregnant women at risk of infection and examination of babies born from seropositive mothers is critical to detect and treat *T. cruzi* positive babies, preventing congenital cases (61). Regarding control of transmission by transfusion or organ transplant, screening programs of blood and organ donors were implemented in Latin American countries since the 1970s, although the achievement of universal blood screening for *T. cruzi* infection of blood donors did not occur until 2015. More recently, these programs have also been implemented in non-endemic countries (62).

1.1.4. Chagas Disease: clinical features

T. cruzi infection/CD is a complex condition. It courses in two phases: the acute and the chronic phase. In the acute phase, more than 90% of cases are asymptomatic (63) and when symptoms appear, lasting for one or two weeks to manifest, these are usually mild and nonspecific (such as fever, malaise, headache, cough, swelling and abdominal pain). During this phase, the person with the infection presents high parasitemia, which induces a strong activation of the immune system, including high levels of plasmatic cytokines and activation of B and T lymphocytes. Although the activation of the host immune response reduces the parasite load, it is not able to eliminate the parasite in the most of the cases. Without treatment, the host will

now enter in a new phase of the infection: the chronic one (63). In the chronic phase, the parasite adopts an intracellular behavior, and is no longer continuously found in the bloodstream, but in the tissues. Here, there is a balance between the host immune response and parasite replication. Hosts will develop potent B and T cell immunity, being able to control the acute circulating and tissue parasite burden, and will remain asymptomatic for years in the most of the cases. However, the loss of this fragile balance causes symptoms in some of the patients, who develop chronic CD with organ affectation years after the initial infection. The target organs of the disease are the heart, the esophagus and the colon. Approximately 30% of the chronically infected individuals will develop cardiac symptoms and 10% of the patients, digestive ones (Figure 5) (45,63–65).

Cardiomyopathy due to conduction system and/ or myocardial involvement is the most common and potentially severe organ damage identified in chronic CD. It represents an extensive remodeling of the cardiovascular system, which could manifest with congestive heart failure, arrhythmias, and or thromboembolic events. Stroke due to cardioembolism, microvascular disease, arteriosclerosis or atrial fibrillation is also an important manifestation of chronic CD cardiomyopathy (66). There are different classification systems to stratify CD, which are based on the abnormalities detected by ECG, chest X-ray, echocardiography, and clinical symptoms of heart failure. Five main classifications are used: Kushnir, Brazilian Consensus on CD, modified Los Andes classification, Latin American Guidelines, and American Heart Association Statement (67). In this work, we have used the Kushnir classification, which is described below (68):

Kuschnir Classification

0: Normal ECG findings and normal heart size (on chest X-ray)

I: Abnormal ECG findings and normal heart size (on chest X-ray)

II: Left ventricular enlargement

III: Congestive heart failure

Gastrointestinal manifestations, which are the second most common cause of organ involvement in CD, are associated with high morbidity and can seriously affect patient's quality of life. Although *T. cruzi* infection can affect all parts of the digestive tract, the esophagus and the colon are most commonly involved. Gastrointestinal dysfunction is caused by alterations of motility, secretion, and absorption in the digestive tract. Changes in motility start with slow transit and difficulty in emptying, followed by increased caliber of the organ, which characterize the presence of megaoesophagus (grade I to IV) or megacolon (69).

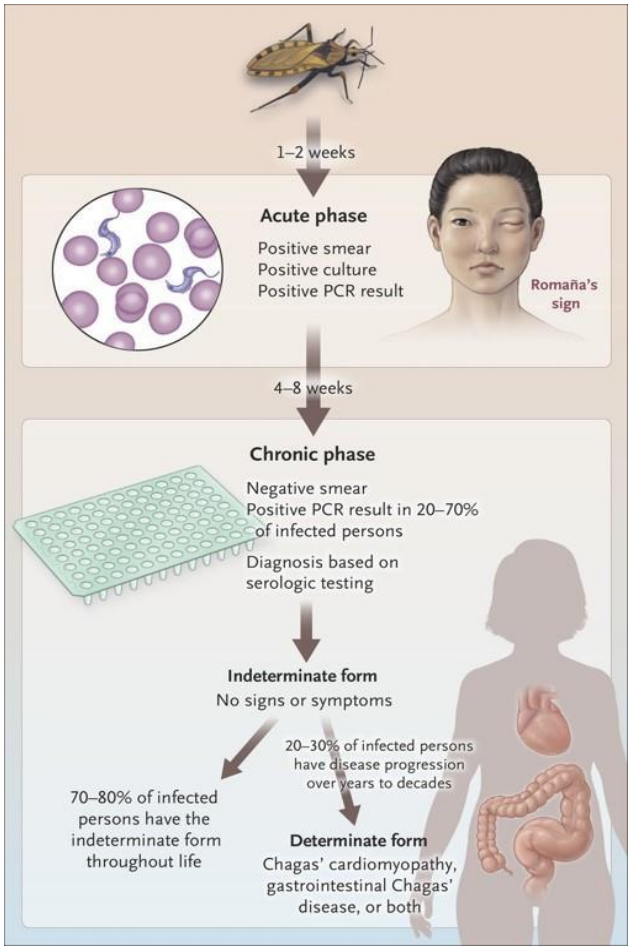


Figure 5. Phases of *T. cruzi* infection. Source of figure: (45).

1.1.5. Chagas Disease control: major challenges

Due to the zoonotic characteristics of the *T. cruzi* transmission, CD is impossible to be eradicated. Thus, improving disease control strategies is a major need (70). Many problems remain in *T. cruzi* infection management: diagnostic techniques confront several limitations, available drugs present adverse drug reactions and limited or unclear efficacy during the chronic phase of the disease, there are not vaccines, and furthermore, there are no biomarkers of therapeutic response and disease progression. Here, we describe some of these major challenges.

There are several techniques to diagnose *T. cruzi* infection, including parasitological, molecular and serological methods. The use of one or another is related to the stage of the disease (71). During the acute phase, when patients present high levels of parasitemia, the detection is performed by parasitological (microscopy based visualization of *T. cruzi* trypomastigotes in fresh blood) and molecular methods (detection of the parasite by nucleic acid amplification techniques, such PCR) (71–74). Nowadays, the acute phase of the infection and disease is mainly diagnosed in the context of mother to child transmission. Current diagnosis for

congenital transmission involves two micromethods, performed from close-to-birth up to one month after birth, followed by a serological test performed after eight months of age, when maternal transferred Immunoglobulin G (IgG) antibodies have disappeared (60). This algorithm presents two major problems: high risk of loss of pediatric follow-up, and the reduction of drug efficacies the longer the treatment is delayed (75). Moreover, molecular biology laboratories are expensive and require trained personnel, which can be difficult in the context of endemic areas, where molecular detection is not generally used beyond regional or national reference laboratories (75). During the chronic phase of the disease, characterized by low and intermittent parasitemia, serological methods are the preferred diagnostic choice (34). Due to the parasite´s antigenic diversity, the WHO criteria is to run two serological tests based on distinct antigens, and in the case both outcomes are not concurrent, to employ a third technique. Again, this algorithm is not easy to follow in endemic settings, as it requires equipment and resources, complicates the logistics, and does not facilitate patient follow-up (75). Moreover, recent studies have observed a very high level of discordance among the results obtained with several ELISA tests when using samples from a rural community in Mexico. Curiously, the seroprevalence varied from 1-1.5% with two of the commercial ELISA tests up to 31.6% with an in-house ELISA based on crude parasite extract from a local *T. cruzi* strain (76). In non-endemic areas, there is generally a better healthcare structure, which could facilitate access to diagnosis. However, in this particular setting the difficulties are related to a lack of knowledge of the disease (77). Altogether, access to diagnosis, and thus, treatment, remains an important challenge for *T. cruzi* infection control, both in endemic and non-endemic areas.

T. cruzi infection can be treated with BDZ and NFX, being BDZ the most commonly used drug. Treatment is recommended in acute, congenital cases, reactivations, women of childbearing age, and in chronic cases under the age of 19 years old. Moreover, it should also be considered for patients between 19 and 50 years of age presenting the chronic phase of the disease and no severe cardiological dysfunction (11). However, anti-trypanosomatid treatment in patients presenting advanced cardiological complications is not recommended, as do not significantly reduce cardiac clinical deterioration (78). Moreover, although treatment in the chronic phase shows adequate antiparasitic effect, correlation with clinical efficacy (measured as decrease in clinical events) is still uncertain (79). In terms of adverse drug reactions, BDZ is well tolerated by newborns and children, but most adult patients experience at least one adverse drug reaction during their treatment course (7,9,37). Adverse drug reactions are often mild to moderate; but are one of the main causes of patients abandoning treatment, resulting in therapeutic failure or ineffective treatment. Closely medical follow-up is a major need to improve

adherence and achieve therapeutic success, but can become a major limitation in remote areas of endemic countries (33,80). Currently, there are ongoing clinical trials to validate the use of alternative regimens and/or dosage of BDZ, which could facilitate logistics and decrease treatment costs (36,81,82). In conclusion, safer, well-tolerated drugs, presenting higher efficacy in the chronic phase of the disease and preventing cardiac clinical deterioration, are urgently needed.

Even though vaccines could be a very useful and cost-effective tool for prevention and control of *T. cruzi* infection and transmission, we are still far from having a beneficial vaccine for CD (83). The lack of financial support and interest from governments and the pharmaceutical industry, together with the genetic complexity of the parasite, have contributed to the slow progress in its development (84). There are two main target product profiles for developing vaccines for *T. cruzi* infection. The first one, which could be used alone or in combination with drug therapy, aims at preventing, or at least delaying, the progression of cardiac and digestive manifestations in patients presenting the indeterminate form of the disease (84). The second one aims to develop a preventive vaccine (85). Although multiples attempts have been made to develop effective and safe vaccines for CD, none of the candidates showed a complete protective immunity (85).

Finally, there are no prognosis markers neither biomarkers of therapeutic response. Availability of such biomarkers is fundamental for the clinical evaluation of new drugs or regimes in clinical trials, as well as for the clinical management of patients. Moreover, being able to predict disease progression would allow to target the patients based on their risk of progression, which would be really useful considering the limited resources available and the complexity of *T. cruzi* infection treatment. Therefore, the identification and validation of biomarkers of disease progression and/or treatment response on which to develop tests of prognosis and/or cure is a major research priority (12,86).

1.1.6. Chagas Disease biomarkers

As previously mentioned, there is a lack of validated markers to evaluate therapeutic response and disease progression in CD. Nowadays, the gold standard for evaluating treatment response is the seroconversion of conventional serological tests, which can take years to decades to assess (34). A serological study that followed-up 430 chronic *T. cruzi* infected patients showed that seroconversion was achieved in a median of 11.7 years (87,88). Thus, finding biomarkers of progression and/or treatment response would mean the greatest leap forward in the history of

CD. In this section, we will review the state of the art of research on host-derived and *T. cruzi* biomarkers.

T. cruzi biomarkers

Several parasite proteins and glycoproteins have been described as potential biomarkers for *T. cruzi* infection that could eventually be used to assess response to treatment. Moreover, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) amplification techniques have also been proposed for assessing therapeutic response. Currently, amplification of *T. cruzi* DNA is the gold standard for assessing therapeutic failure in a short period of time in patients with chronic *T. cruzi* infection. However, a negative PCR result does not guarantee parasitological cure or effective response to treatment (89). In

Table 1, we summarize the major parasite molecules described as potential biomarkers for CD.

Host biomarkers

Research on biochemical markers gathered attention in the last years due to their potentially simple analysis and low cost, which makes them easy to implement in middle- and low-income countries (89). Several biochemical markers have been proposed for CD, both for evaluating treatment response and disease progression (12,86). These include host biochemical markers related to

Table 1. List of *T. cruzi* biomarkers described up to date.

Biomarker name (acronym)	Application evaluated	References
24-kDa recombinant protein from *T. cruzi*	Therapeutic response	(90)
Recombinant *T. cruzi* complement regulatory protein	Therapeutic response	(91)
F-IV fraction from *T. cruzi* extracts and exo-antigens from trypomastigote infected mice	Therapeutic response	(92)
Trypomastigote-derived glycosylphosphatidylinositol-anchored mucin glycoproteins (GPI-mucins), recognized by lytic anti-α-Gal antibodies	Therapeutic response	(93)
Recombinant *T. cruzi* flagellar calcium-binding protein	Therapeutic response	(94,95)
Recombinant Antigen 13 and Shed Acute Phase Antigen	Therapeutic response	(96)
T. cruzi ribosomal protein	Therapeutic response	(97)
KMP11-HSP70-PFR2-3973	Therapeutic response	(98–100)
InfYnity multiplexed antigenic array	Therapeutic response	(101,102)
Short RNA ligand aptamer L44	Therapeutic response	(103)
5F2, 5A9B11 monoclonal antibodies	Prognosis marker	(104)
Flagellar recombinant repetitive antigen	Prognosis marker	(105,106)
T. cruzi DNA	Treatment failure	(107–109)

the metabolism of lipids, associated to the hypercoagulability state, markers of cardiac damage progression, inflammation, markers associated with the host immune state, and markers derived from human genetic studies. We summarize all host biochemical markers identified so far in the context of *T. cruzi* infection/ CD and their proposed application in Table 2 and in Figure 6.

Biomarkers of therapeutic response

Markers based on lipid metabolism intermediates

A study by Santamaria and co-workers with sera from 37 adult CD patients and 37 healthy subjects showed that APOA1, certain fragments of APOA1 and FBN proteins were interesting for the evaluation of treatment response. Higher levels of fragments of APOA1 and FBN were found in serum samples of *T. cruzi* seropositive patients in comparison to the control group. Contrarily, full -length APOA1 levels were lower in *T. cruzi* infected individuals compared to healthy donors (110). Such altered levels of APOA1, its specific fragments thereof, and a fragment of FBN returned to normal in 43% of the studied *T. cruzi* infected subjects three years after NFX treatment (110). These results have been recently validated in a cohort of 30 *T. cruzi* seropositive children treated with BZD (111). In the latter study, APOA1 and FBN fragments were absent at the end of BZD treatment in a significant part of the cohort (66.6% and 53.3% of the children respectively for APOA1 and FBN) (111). Also, correlation between seroconversion of the children upon treatment and absence of detection of APOA1 and FBN fragments in serum samples was observed in 100% and 96.6% of the cases, respectively (111).

Hypercoagulability state biomarkers

The presence of a hypercoagulability state in *T. cruzi* infected patients was described a few years ago (112,113), which contrasted with other report by Melo and co – workers (114). Notwithstanding, at the clinical level, the presence of a coagulation state condition could be hinted upon the description of the occurrence of thromboembolic events in *T. cruzi* infected patients who did not show any signs of cardiomyopathy (115). In 2016, a clinical study with plasma samples from 56 chronically *T. cruzi* infected adult patients reported that a high percentage of them had statistically significant altered levels of the hypercoagulability markers prothrombin fragment 1+2 (F1+2) and endogenous thrombin potential (ETP) (116). These two markers, which had not been looked upon by Melo et al., were abnormally expressed in respectively 77% and 50% of the patients (116). Moreover, after BZD treatment, both markers returned to and remained at their normal levels in respectively 76% and 96% of the patients by 36 months upon end of treatment (116). Amongst the rest of hypercoagulation markers that were evaluated in that study, plasmin -antiplasmin complexes (PAP) also showed good results in terms of percentage of patients that returned to normal levels upon treatment (94% of them) (116).

However, PAP was found altered in only 32% of the studied *T. cruzi* - infected participants before treatment and it was thus discarded from further consideration (116).

Biomarkers of disease progression

Cardiac damage is the most common clinical outcome observed in the symptomatic state of the disease (1,117). Thereby, markers of cardiac damage progression have been the most studied for CD. It is estimated that between 20% - 30% of *T. cruzi* chronically infected people will develop cardiomyopathy, and an early indicator of disease prognosis would help to prioritize treatment to those patients with a high risk of developing complications. Multiple biomarkers have been associated with CD cardiomyopathy. However, although it has been proved that their levels increase accordingly to the severity of the damage, most of them are not able to distinguish between CD cardiomyopathy and other cardiomyopathies (118). Natriuretic peptides (brain and atrial) (BNP, ANP) were among the first markers of cardiac disease progression ever considered. These are released under conditions of myocardial stress and have been shown to be increased in *T. cruzi* experimentally infected animals (119), and in CD patients with cardiomyopathy (120). Furthermore, levels of natriuretic peptides correlated to clinical prognosis (121). The N-terminal portion of brain natriuretic peptide (NT-proBNP) could be a better predictor than BNP itself, due to its high stability (122). Both peptides are strong predictors of mortality, and are some of the most well characterized markers for assessing early cardiac damage and predicting heart failure outcome. Measurement of BNP levels has also been suggested for the prognosis of patients presenting left ventricular systolic dysfunction, one of the typical signs of cardiac CD (120,123–125).

The use of natriuretic peptides in the context of CD has also been studied in combination with other host -derived molecules, such as cardiac troponin T (cTnT), which is a marker of ischemia and inflammation. A study assessing it in combination with NT-proBNP that included samples from 137 *T. cruzi* infected patients with several forms of the disease found that levels of both markers were increased in those individuals with cardiomyopathy in comparison to the asymptomatic ones (126). Moreover, their values were increased accordingly to the severity of the cardiomyopathy (126). Plasma leptin levels and their relation to different forms of the disease were also studied in combination with NT-proBNP in 52 *T. cruzi* infected patients, and those patients with heart failure had higher levels of NT-proBNP and lower levels of leptin than controls (127).

Angiotensin-converting enzyme 2 (ACE2) is another potential prognostic biomarker that has been evaluated. Wang and colleagues showed that ACE2 activity was significantly increased in those patients with signs of heart failure presenting systolic dysfunction (128). Moreover,

plasma ACE2 levels significantly correlated with clinical severity and echocardiographic parameters indicative of this (128). Similarly to cTnT and leptin, ACE2 activity was also compared to BNP levels in order to predict cardiac death and need of heart transplant, finding an additive predictive value when both markers were used in combination (128). Other enzymes that have been suggested as possible markers for early cardiac damage are glutamic oxaloacetic transaminase, glutamic –pyruvic transaminase, alkaline phosphatase, acid maltase and alpha - hydroxybutyric dehydrogenase, and their levels were found to be significantly altered in CD patients (129). Very interestingly, authors of that reference suggested that the finding of those released enzymes in patients without clinical evidence could represent good biomarkers of early myocardial damage (129). On the other side, the measurement of selenium (Se) levels has also been valued as a disease progression marker for cardiac as well as also for digestive manifestations. In a cohort of 170 *T. cruzi* infected people, Rivera and co-workers found that Se levels were lower in patients presenting the cardiac form of the disease compared with healthy donors or asymptomatic individuals (17). Moreover, such decrease of normal levels of Se was significantly correlated with malfunction of the ventricular ejection fraction. Low Se levels were also found in 6 out of the 10 *T. cruzi* infected patients with digestive mega-syndromes (17). In fact, the use of Se as dietary supplementary was suggested as a possible therapeutic strategy to preserve heart function in patients presenting the indeterminate form of the disease (130).

Finally, another biochemical biomarker that has been researched for CD is endothelin 1, although the available data about it is yet controversial. A study performed in 2001 showed that plasmatic levels of endothelin 1 were elevated in patients presenting *T. cruzi* infection related cardiomyopathy (131). However, Garcia-Alvarez and colleagues were not able to replicate those results, and reported in another study that similar plasmatic levels of endothelin 1 were observed in *T. cruzi* seropositive patients and control individuals (132). Moreover , the group of infected subjects presenting the undetermined form of the disease had even lower levels of endothelin 1 than controls (132).

Biochemical markers of inflammation.

Although the molecular mechanisms of CD pathogenesis are yet largely unknown, the presence of an inflammatory environment is a common feature to both cardiac and/or digestive tissue disruptions. Such continuous inflammation would lead to the occurrence of organ mega - syndromes, severe indicators of symptomatic chronic *T. cruzi* infection. Thereby, the prognostic value of host biochemical markers with inflammatory mediator function has also been evaluated. This is the case, for example, of the C-reactive protein (CRP) , which is liberated during the acute phase of inflammation and its serum levels are associated to vascular inflammation and

development of cardiovascular events (133). Several studies have evaluated this protein as a marker for the progression of CD, showing an association between chronic inflammation, cardiac manifestations and CRP levels (134,135).

Other interesting marker under research is the enzyme adenosine deaminase (ADA). ADA regulates adenosine levels and its activity increases as a consequence of hypoxia and inflammation associated with immunologic events (136). In the same study, which included serum samples from 28 healthy individuals and 82 *T. cruzi* infected people presenting asymptomatic and symptomatic (cardiac) forms of the disease, it was shown that CRP and ADA levels linearly increased in connection to disease severity, and further correlated with the observed echocardiographic and ECG parameters indicative of this state (136).

Galectin-1 (GAL-1), found in human heart tissue, is also involved in immunological and inflammatory processes. A study with serum samples from healthy donors and patients in the acute and chronic phase of CD showed that anti-GAL-1 IgG auto-antibodies specifically correlated with cardiac damage severity caused by *T. cruzi* infection as they were shown to be absent in non-related cardiomyopathies (137). Moreover, levels of GAL-1 were upregulated in cardiac tissue from patients presenting chronic *T. cruzi* infection, compared to cardiac tissue from healthy individuals (137).

In the presence of excessive oxidative and nitric oxide (NO) stress, a post-translational modification of cysteine residues of host proteins can occur. This modification, called S-nitrosylation (SNO), can affect cellular homeostasis and contribute to disease development. Recently, Zago and co-workers have shown that SNO modifications found in peripheral blood mononuclear cells from 53 CD patients were differentially abundant according to disease state, having the potential to identify disease severity (138).

Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) have also been investigated in the context of *T. cruzi* infection, but their participation in the disease progression is yet subject to controversy. A cross -sectional study with plasma samples from 144 patients at different stages of CD and 44 samples from healthy donors showed that patients had increased levels of MMP-2 and MMP-9 (139). This work also reported that patients presenting ECG abnormalities had a significant increase of both enzymes compared to patients presenting the indeterminate form (139). In contrast, another study with serum samples from 193 *T. cruzi* infected individuals observed an increase of the MMP-2/MMP-9 ratio that was associated with severity of the cardiac form of the disease (118). The study authors claimed that this ratio could be useful for assessing the progression from early inflammation to late fibrosis (118). *T. cruzi*

infected subjects presented progressively higher levels of MMP-2, tissue inhibitor of metalloproteinase 1 (TIMP-1, inhibitor of MMP-9) and tissue inhibitor of metalloproteinase 2 (TIMP-2, inhibitor of MMP-2) that paralleled cardiac severity (118). In line with that, Clark and colleagues have shown that serum levels of MMP-2 and TIMP-1 increased progressively in 85 individuals presenting cardiac structural changes, either in early or late stages of the disease (140). In comparison, other studies performed using serum and whole blood samples suggest that MMPs are differentially involved in CD cardiomyopathy: while MMP-2 would be associated to regulatory cytokines, MMP-9 would be correlated with inflammatory ones (141,142). Thus, MMP-2 would present a cardiac-protective and regulatory function, favoring the indeterminate form of the infection , and MMP-9 would promote an inflammatory atmosphere, favoring the development of the cardiac form (141,142).

Similarly to what occurs with BNP and NT-proBNP, high levels of creatine kinase–MB (CKMB) indicate extensive damage and worse prognosis in patients suffering heart failure. Thus, this compound is commonly used as a measure of heart failure severity, and has been suggested as a possible biomarker for CD (118,143). A first study by Okamoto and colleagues assessing serum levels of BNP, NTproBNP, CKMB, troponin I, MMP-2, MMP-9, TIMP-1, TIMP-2, transforming growth factor beta 1 (TGF-β1), and transforming growth factor beta 2 (TGF-β2), showed no differences in biomarkers levels when stratifying patients by cardiac stage and *T. cruzi* infection status (118). This was the first time that CKMB was examined as a prognostic marker in Chagas cardiomyopathy. In that study, Okamoto and co-workers saw that troponin I levels (among others) rose in relation with an increasing severity of the disease stage, but unfortunately it did not distinguish between CD cardiomyopathy and other cardiomyopathies (118). However, Sherbuk and colleagues showed that CKMB, together with BNP, NTproBNP and MMP-2, were significantly associated with mortality among patients presenting severe Chagas cardiomyopathy (143). Similarly, the conclusions from the cross -sectional case control retrospective study by Keating and co -workers, which measured CKMB, troponin, myoglobin, vascular cell adhesion molecule (VCAM), NTproBNP as well as the cytokines IFN-γ, IL-6, IL-10 and TNF-α pointed out that a clear pattern of inflammatory biomarkers was solely observed in those subjects presenting with the more severe cardiac symptomatic stages (133).

As it was mentioned above, measuring serum levels of cTnT was analyzed as prognosis test of cardiac damage progression. Determining cTnT levels with a highly sensitive assay in serum samples from 26 healthy subjects and 179 chronically infected subjects concluded that those were significantly higher in patients suffering cardiomyopathy compared to the rest of groups (118). Moreover, authors of that work indicated that cTnT value correlated with the severity of

that cardiomyopathy (118). Notably, CRP and IL-6 levels followed the same trend as cTnT changes (118).

Finally, NO levels have been also reported to be significantly higher in patients with cardiomyopathy compared with asymptomatic patients and healthy donors (144–147). This increase of NO levels in serum correlated with an increase of TNF-α, and a reduction in GPx and superoxide dismutase (144). Alterations in the oxidant/antioxidant balance had been previously reported in a cohort of 80 *T. cruzi* infected patients, 50 healthy individuals, and 20 non-chagasic cardiomyopathy subjects (145). That study showed that *T. cruzi* infected patients presented higher levels of malonylaldehyde and lower levels of glutathione, glutathione peroxidase, superoxide dismutase and manganese superoxide dismutase than healthy individuals. Patients presenting cardiomyopathy but negative for *T. cruzi* infection presented insignificant higher plasma malonylaldehyde levels compared to healthy patients, and their plasma antioxidant defense capacity was not compromised (145). Years later, the same group of researchers studied the role of inflammatory mediators such as myeloperoxidase, inducible nitric oxide synthase and xanthine oxidase, in the stimulation and sustenance of oxidative and nitrosative stress response in plasma samples of *T. cruzi* seropositive and seronegative patients (146). Those infected presented a significant increase in myeloperoxidase activity and protein level, advanced oxidation protein products, and 3-nitrotyrosine levels compared to healthy donors. However, plasma levels of xanthine oxidase and nitrate/nitrite contents were not altered. A correlation between increased myeloperoxidase activity and protein 3-nitrotyrosine formation was found, suggesting that myeloperoxidase could contribute to protein nitration and thus to oxidative and nitrosative damage in CD patients (146). More recently, the role of other markers of inflammation and oxidant/antioxidant status was studied in a cohort of 116 *T. cruzi* seropositive patients characterized as clinically-symptomatic or asymptomatic, 45 seronegative healthy individuals, and 102 *T. cruzi* seronegative patients presenting cardiac problems. Consistent with previous findings, seropositive subjects showed an increase in sera or plasma levels of myeloperoxidase, advanced oxidation protein products, nitrite, lipid peroxides and malonylaldehyde, and a decrease in superoxide dismutase and glutathione compared to healthy controls. Interestingly, myeloperoxidase and lipid peroxides levels correlated with clinical disease state, being potential biomarkers candidates for evaluating CD clinical severity (147).

Markers associated with the host immune state

The study of cytokines has also been suggested as a relevant tool for assessing cardiac disease progression (133). In 2014, a study by Sousa et al. involving plasma samples of 176 *T. cruzi* infected people and 24 healthy individuals showed that the expression of plasma inflammatory

cytokines, such as IFN-γ, TNF-α, IL-6, and IL-1β, was higher in those with cardiac form of the disease (148). These results were consistent with previous findings where high levels of TNF-α were found in patients suffering CD cardiomyopathy (144,149). Poveda and colleagues also found a higher expression of IFN-γ in serum samples from CD patients with cardiomyopathy compared to those with the indeterminate form (150). By contrast, indeterminate *T. cruzi* infected patients had a higher expression of IL-10 when compared with that of individuals with cardiac damage (150). Interestingly, IL-10 expression was associated with better cardiac function as determined by left ventricular ejection fraction and left ventricular diastolic diameter values (150). These results confirmed previous findings from Costa and co -workers, which suggested a cardiac-protective role for IL-10 in *T. cruzi* infection (151). In line with the former, other work has shown that high levels of IL-17 could correlate with better cardiac function, although this is considered a pro-inflammatory cytokine (152). Nonetheless, the role of IL-17 is still open to discussion because a recent study assessing plasma samples from 57 children showed that IL-17A levels were significantly higher in *T. cruzi* infected in comparison to seronegative individuals. Interestingly, those higher IL-17A levels decreased to normal one year after treatment (153).

At present, despite the identification of cytokine signatures indicative of a pattern of pathogenesis progression has been pursued, the only tests that would independently have value for clinical decision would be the aforementioned NTproBNP and the detection of *T. cruzi* DNA by PCR, as far as they are accompanied with electrocardiographic and echocardiographic clinical assessments (133).

Other studies have focused on the potential role of adaptive immune response mediators as biomarkers. In relation to humoral immunity, the possible impact of anti-troponin T and myosin autoantibodies has been suggested (154). Serum samples from 131 patients presenting different clinical forms of CD, healthy donors, and patients with ischemic cardiomyopathy were included in a study whose results showed that specific anti-*T. cruzi* antibodies and autoantibodies against myosin and troponin T are frequently co-detected in high levels in patients with chronic CD (154). Even though anti-troponin T autoantibodies levels were very similar in patients presenting the indeterminate form of the disease and in patients presenting cardiac symptoms, the study found a correlation between cardiac CD, the production of anti-troponin T and anti–myosin autoantibodies, and a diminished left ventricular ejection fraction, which is an important indicative of systolic dysfunction (154). Anti-*T. cruzi* specific antibodies and their correlation with disease progression and cardiac damage have also been evaluated. In a cohort of 55 *T. cruzi* infected patients (20 presenting the indeterminate form of the disease and 35 suffering cardiac damage) an inverse correlation between anti-*T. cruzi* IgG1

titers and left ventricular ejection fraction was found in patients presenting the cardiac form of the disease, indicating a worse prognosis for those *T. cruzi* infected patients presenting high titers of anti-*T. cruzi* IgG1 (155). Thus, anti-*T. cruzi* IgG1 levels could be an interesting biomarker to predict the severity of chronic CD cardiomyopathy.

In terms of T cell immune responses, it has been recently described that Chagas patients with cardiac tissue damage present higher expression levels of T-cell inhibitory receptors and lower antigen-specific capacity compared with that of asymptomatic patients (156). These features were partially reversed by BZD treatment in both asymptomatic and symptomatic patients: the co-expression of inhibitory molecules was reduced, the multifunctional antigen-specific response of CD8 + T cells was enhanced, and an increase in the subset of cells with cytotoxic proprieties and production of IFN-γ was observed. These results point at a potential application of the analysis of those immunological signatures as biomarkers of disease progression and treatment response.

Markers derived from human genetic studies

CD has a multifactorial etiology that involves complex host-parasite interactions governed by parasite and host genetics, which can be as well influenced by environmental factors. As it was mentioned before, the mechanisms of pathogenesis of chronic *T. cruzi* infection are yet largely unknown. However, immune system mediators have been described to participate in driving heart and/or gut tissues inflammation, either through response to the parasite presence and, to a certain level, by autoimmune reactions (157). Thus, most of the genetic studies performed so far have searched for sequence variations that could be associated to chronic Chagas cardiomyopathy susceptibility in immune system related genes. These searches followed a hypothesis -driven approach to find single nucleotide polymorphisms (SNPs) in genes known or suspected to play a role in those inflammatory phenomena (158). Amongst the genes studied there are: human leukocyte antigen (HLA) class I and class II alleles, cytokines (e.g.: IL - 1 β, IL -10, TNF -α, IL -17, IL -18) and chemokines and their receptors (reviewed in (158), as well as inflammasome genes (159). Some variations were associated to chronic Chagas cardiomyopathy, although controversy is open mainly due to the limited sample size analyzed and the ample genetic heterogeneity of the studied cohorts. These features have also limited the only genome-wide association study performed so far in CD (160). With the power of its “hypothesis-free” and “hypothesis-generating” nature, unfortunately this study did not report any significant associations with chronic cardiomyopathy at the genomic level. However, it interestingly suggested that SNPs in the solute carrier family gene SLCO1B1 could be associated to a cardiomyopathy phenotype (160).

Other kind of genetic studies, such as transcriptomics and epigenetics works, will be also required in order to functionally expand and integrate the aforementioned genomics data, as well as to comprehend the impact of environmental factors in the susceptibility to the disease. In this regard, a whole-blood transcriptome of *T. cruzi* infected subjects and uninfected controls identified a signature of 27 genes, mainly related to natural killer and CD8 + T cells, which would mark a disease progression pattern (161). Whereas, Frade et al. analyzed the whole-transcriptome of heart biopsies from chronic Chagas cardiomyopathy patients and identified a long non-coding RNA molecule that had been associated to heart failure (162). Long non-coding RNAs are>200 nucleotides long RNA transcripts that have been described to have a broad functionality in the regulation of gene expression at transcriptional, post-transcriptional and epigenetics levels (162).

Other series of studies analyzed the differential expression of miRNAs in chronic cardiomyopathy Chagas patients, either versus those suffering from idiopathic dilated cardiomyopathy (163) or comparing them to *T. cruzi* infected subjects at the indeterminate stage and a group of non infected subjects (164,165). These miRNAs are small non-coding RNA molecules with a cell and tissue specific expression pattern that are involved in post-transcriptional regulation and might target up to 60% of the human genes (166). A work integrating miRNA and gene expression profiles of *T. cruzi* acutely infected mice heart tissues suggested a correlation between those miRNAs and the observed pathobiology (167). Such miRNAs can be epigenetic regulators and be regulated epigenetically at the same time (168). One of the most common epigenetic modifications is DNA methylation, and the results from a whole-genome cardiac fingerprinting study revealed that up to 399 genes were differentially methylated and expressed in chronic Chagas cardiomyopathy patients in comparison to healthy controls (169)

The above mentioned studies are very necessary to achieve a deeper understanding of CD complex pathogenesis events. However, so-called genomic medicine yet lies far from being applicable for CD. The usefulness of those genetic markers in the field as potential markers of disease prognosis will largely depend on a much awaited generalization of molecular-based diagnostics, or the development of easier-to-use molecular based detection methods such as loop -mediated isothermal amplification or recombinase polymerase amplification assays based on them.

To sum up, many candidate molecules, parasite-and host-derived, have been evaluated so far. However, we are still far from having a licensed test for the early assessment of treatment efficacy or to accurately anticipate CD progression. Due to the complexity of the chronic phase of

the disease, the option of using a battery of biomarkers rather than relying on a single one to assess treatment response and/or anticipate pathological progression of the infection appears more likely (89). Moreover, new approaches for biomarkers discovery in CD are needed.

Table 2. List of *T. cruzi* infection / CD host biomarkers described up to date.

Biomarker name (acronym)	Biological classification	Application evaluated	References
Apolipoprotein A1 (APOA1), Fibronectin (FBN)	Markers based on lipid metabolism intermediates	Therapeutic response	(110,111)
Fragment 1 + 2, Endogenous thrombin potential	Hypercoagulability state biomarkers	Therapeutic response	(116)
Brain natriuretic peptide	Markers of cardiac damage progression	Cardiac damage progression	(118,120,121,123–125,140)
N-terminal portion brain natriuretic peptide	Markers of cardiac damage progression	Cardiac damage progression	(118)
Cardiac troponin T	Markers of cardiac damage progression	Cardiac damage progression	(126,170)
Leptin	Markers of cardiac damage progression	Cardiac damage progression	(127)
Angiotensin-converting enzyme 2	Markers of cardiac damage progression	Cardiac damage progression	(128)
Glutamic oxaloacetic transaminase, glutamic–pyruvic transaminase, alkaline phosphatase, acid maltase, alpha-hydroxybutyric dehydrogenase	Markers of cardiac damage progression	Cardiac damage progression	(129)
Selenium	Markers of cardiac and digestive damage progression	Cardiac and digestive disease progression	(17)
Endothelin	Markers of cardiac damage progression	Cardiac damage progression (controversial)	(131,132)
C-reactive protein (CRP)	Markers of inflammation	Cardiac damage progression	(134–136,170)
Adenosine deaminase	Markers of inflammation	Cardiac damage progression	(136)
Galectin-1 (GAL-1), anti-GAL-1 auto-antibodies	Markers of inflammation	Cardiac damage progression	(137)
Galectin-3	Markers of inflammation	Damage progression	(171)
Nitric oxide (NO) and S-nitrosylation	Markers of inflammation	Cardiac damage progression	(138,144)
Matrix metalloproteinase 2, matrix metalloproteinase 9	Markers of inflammation	Cardiac damage progression (controversial)	(118,139–143)
Tissue inhibitor of metalloproteinase 1, Tissue inhibitor of metalloproteinase 2	Markers of inflammation	Cardiac damage progression	(118,140,143)
Creatine kinase-MB, troponin I, transforming growth factor beta 1, transforming growth factor beta 2	Markers of inflammation	Cardiac damage progression	(118,133,140,170)
Glutathione peroxidase (GPx), superoxide dismutase	Markers of inflammation	Cardiac damage progression	(144)
Malonylaldehyde, glutathione, glutathione peroxidase, superoxide dismultase, manganese superoxide dismutase	Markers of inflammation	Cardiac damage progression	(145,147)
Myeloperoxidase, NO synthase, xanthine oxidase, oxidation protein products, 3-nitrotyrosine, nitrate/nitrite	Markers of inflammation	Cardiac damage progression	(146,147)
Lipid peroxides	Markers of inflammation	Cardiac damage progression	(147)
Tumor necrosis factor alpha (TNF-α)	Markers associated with the host immune state	Progression	(144,148,149)
Chemokine C-C ligands 2 and 3 (CCL-2, CCL-3)	Markers associated with the host immune state	Progression	(149)
Interleukin 6 (IL-6)	Markers associated with the host immune state	Progression	(134,170)
Interleukin 10 (IL-10)	Markers associated with the host immune state	Protection	(151)
Anti-troponin T antibodies, myosin autoantibodies	Markers associated with the host immune state	Progression	(154)

Interferon gamma (IFN-γ), interleukin 1 beta (IL-1β)	Markers associated with the host immune state	Progression	(148)
Interleukin 2 (IL-2), interleukin 4 (IL-4), *interleukin 5* (*IL-5*), interleukin 9 (IL-9), interleukin 12p70 (IL-12p70), interleukin 13 (IL-13), interleukin 22 (IL-22)	Markers associated with the host immune state	Progression/*protection*	(150)
Interleukin 17 (IL-17), interleukin 17-A (IL-17A)	Markers associated with the host immune state	Controversial	(152,153)
$CD8^+$ T cell inhibitory receptors and antigen-specific capacity	Markers associated with the host immune state	Cardiac damage progression and therapeutic response	(156)
Anti-*T. cruzi* IgG1 levels	Markers associated with the host immune state	Cardiac damage progression	(155)
Single nucleotide polymorphisms (SNPs) in genes associated to inflammation	Markers derived from human genetic studies	Cardiac damage progression	(159)
SNPs in the solute carrier family gene SLCO1B1	Markers derived from human genetic studies	Cardiac damage progression	(160)
Specific signature of 27 genes (mainly related to natural killers and CD8+ T cells)	Markers derived from human genetic studies	Disease progression	(161)
Long RNA	Markers derived from human genetic studies	Disease progression	(162)
Micro RNAs (miRNAs)	Markers derived from human genetic studies	Disease progression	(163–165)
Specific methylation signature in 399 genes	Markers derived from human genetic studies	Disease progression	(169)

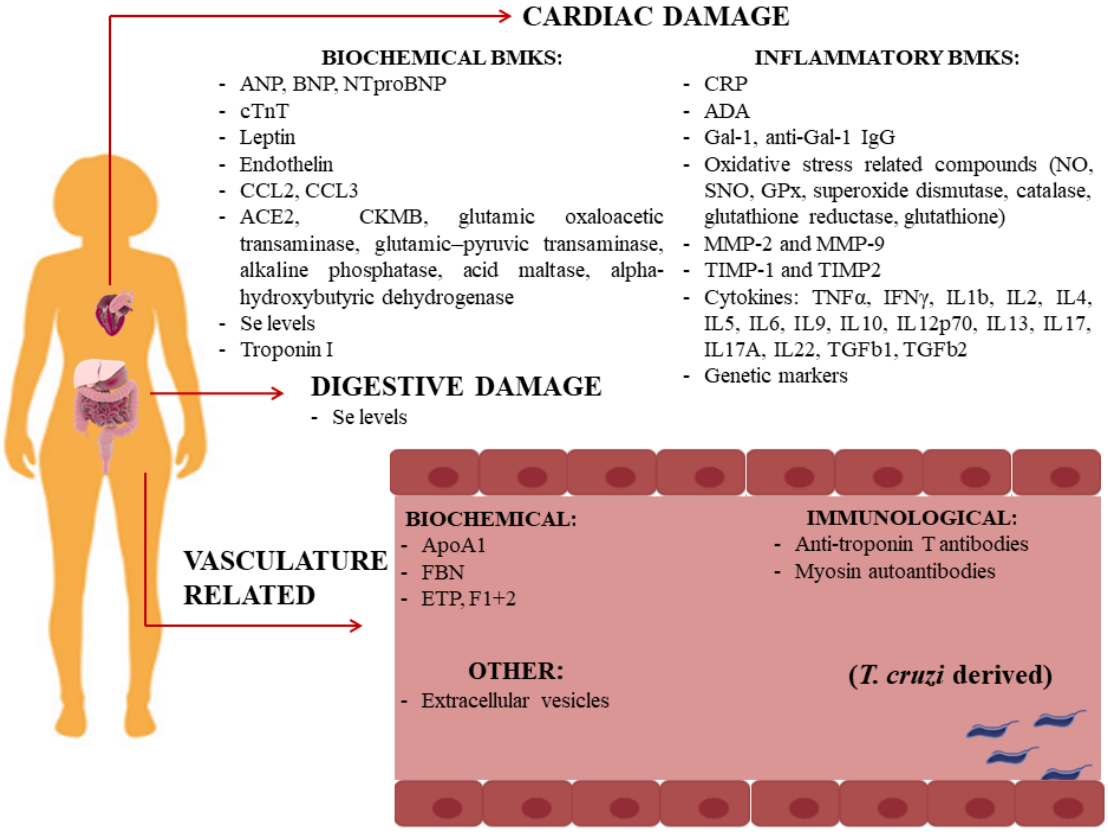


Figure 6. Summary of *T. cruzi* infection / CD host biomarkers described up to date.

1.2. EXTRACELLULAR VESICLES

EVs are heterogeneous group of double membrane nanoparticles secreted by almost all cells and present in all body fluids (13,14). First described by Peter Wolf in 1967 as "platelets dust", in the last years EVs have become essential molecules with multiple functions, participating in processes such as coagulation, cellular signaling, vascular injury, and homeostasis (14,172).

Even though EVs are highly heterogeneous, particles can be divided into several subtypes, according to their size, biogenesis and molecular composition (13). Exosomes, the smaller ones (30-100 nm), are intraluminal vesicles contained in multivesicular bodies, and are released to the circulation upon fusion of multivescular bodies with the plasma membrane (173). Microvesicles bleb directly from the plasma membrane and have a larger size (100 nm-1000 nm); and apoptotic bodies bleb directly from apoptotic cells and are very heterogeneous in size, ranging from 50 nm to five µm (Figure 7) (13). Particular characteristics have been proposed to distinguish these subgroups of EVs, but currently there is still a lack of widely accepted specific markers, and isolation procedures do not purify specific types of vesicles, but a complex mix of them (14,174).

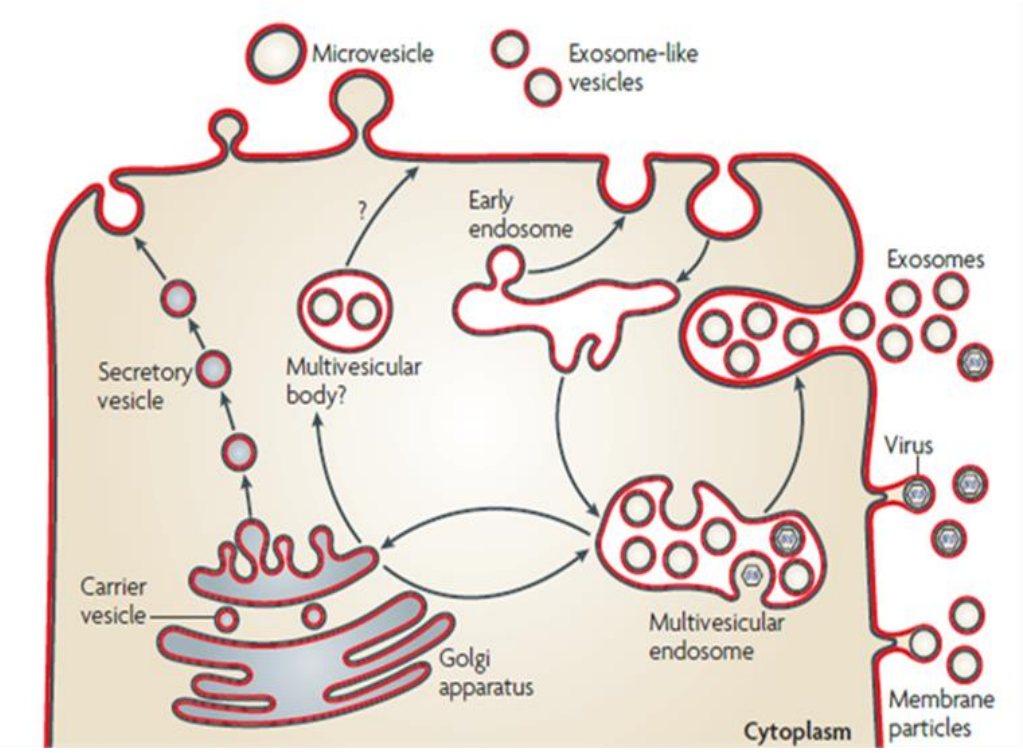


Figure 7. Biogenesis and release of EVs. Source of figure: (174)

Several key functions have been attributed to EVs during homeostasis and in pathological conditions. First, EVs mediate intercellular communication, acting as messenger entities that

deliver specific cargo to recipient cells, which alters the cell physiological status (175,176). Moreover, multiple studies have shown that EVs have a role on antigen presentation, highlighting their participation on the promotion of immune responses (177). As intercellular mediators, EVs are known to be involved in many physiological and pathological conditions, such as cardiovascular diseases, neurodegenerative diseases, and infectious diseases (178–180).

1.2.1. Extracellular Vesicles: biogenesis and content

Even though the generic term EVs is currently used to refer to all secreted membrane vesicles, these are in fact highly heterogeneous. Currently, there is not a gold-standard method to identify and isolate the different subtypes of vesicles. Therefore, we normally deal with heterogeneous EV populations, presenting different origin and content (181).

Regarding the biogenesis of exosomes, plasma invagination generates endosomes, which fuse to late endosomes, forming intraluminal vesicles. Intraluminal vesicles are included in multivesicular bodies, and are released to the extracellular milieu once those are fused to the plasma membrane. Then, intraluminal vesicles are recognized as exosomes. On the other hand, microvesicles are generated as an outward budding of the plasma membrane. Processes involved in the formation of exosomes and microvesicles include differential elements and cargo: exosomes carry elements from multivesicular bodies, while microvesicles contain elements from the plasma membrane. However, despite the differences, several intracellular mechanisms and machineries are commonly involved in the biogenesis of both EV types (181).

One of the most important breakthrough in the biogenesis of exosomes was the discovery of the endosomal sorting complex required for transport (ESCRT) machinery, involved in membrane shaping and scission for the generation of multivesicular bodies and intraluminal vesicles (182). Accessory proteins, such as Alix or syntenin, are usually involved (183). However, ESCRT-independent mechanisms have also been described during EV biogenesis. In the case of exosomes, ceramide-enriched domains have been related to the spontaneous curvature on the membranes (184). Additionally, several members of the tetraspanin family have been associated with the generation of dynamic domains and the direct sorting of cargo to exosomes (185).

The machinery of biogenesis of microvesicles has not been studied enough. However, as previously described, many mechanisms are known to be common to the ones involved in exosome biogenesis. Still, some unique flipping processes on certain lipids from the budding membrane that will originate microvesicles have been reported (186). Components of the

cytoskeleton and their regulators have been shown to be important for microvesicles biogenesis as well (187).

EV content includes cytosolic and cell-surface proteins, nucleic acids, lipids and metabolites, and again, it is highly heterogeneous and dynamic depending on the cellular source, state and environmental conditions. Thus, the molecules contained in EVs reflect the cellular origin and conditions (14,175).

Regarding the protein content, EVs are highly abundant in cytoskeletal, cytosolic, heat shock and plasma membrane proteins, as well as in proteins involved in vesicle trafficking. Intracellular organelle proteins are less abundant. Even though the EVs proteomic profiles obtained have been found to be highly dependent on the EVs isolation method and the cell source, there are several EV proteins that are considered to be pan-EV markers: markers common for most EVs. These include tetraspanins (CD9, CD63, CD81, CD82), 14-3-3 proteins, major histocompatibility complex (MHC) molecules and cytosolic proteins such as specific stress proteins (heat shock proteins), Tsg101 and the ESCRT-3 binding protein Alix (Figure 8) (175). A complete list of the EV markers used in our proteomic studies can be found in Annex 1.

The protein glycosylation pattern found in EVs has been found to be distinct from that of the parent cell membrane. EVs proteins are enriched in highly mannosylated epitopes, including complex N-glycans, N-acetyl lactosamine, sialylated and fucosylated epitopes (14). Moreover, lectin-binding patterns are also conserved in all the EVs. Several glycan-binding proteins have also been found in EVs, as well as soluble lectins such as galectins (14).

The nucleic acids carried in EVs include RNA, messenger RNA (mRNA), non-coding RNA (including microRNAs) and DNA (188,189). Extracellular RNA can be found enclosed in EVs, bound in protein complexes, or as a circulating form. While cellular mRNA varies in size from 400 to 12000 nucleotides, RNA detected in EVs has a predominant size of less than 700 nucleotides. The mechanisms of RNA loading into EVs are still unclear (190). In contrast to RNA, the presence of DNA in EVs has been less explored so far.

Although it is a new area of study, lipids are emerging as very important players for the physiological functions of these vesicles. Several specific lipids have been suggested to play a role in the formation and function of EVs. Differences in the lipid composition of EVs derived from different sources have already been found, but EVs are generally enriched in sphingomyelin, cholesterol, phosphatidylserine and glycosphingolipids compared to their parental cells (191). The characteristic lipid composition of the EVs, forming the lipid bilayer, probably contributes to

its stability, and it is a feature that should be studied to improve liposomal drug delivery systems (192).

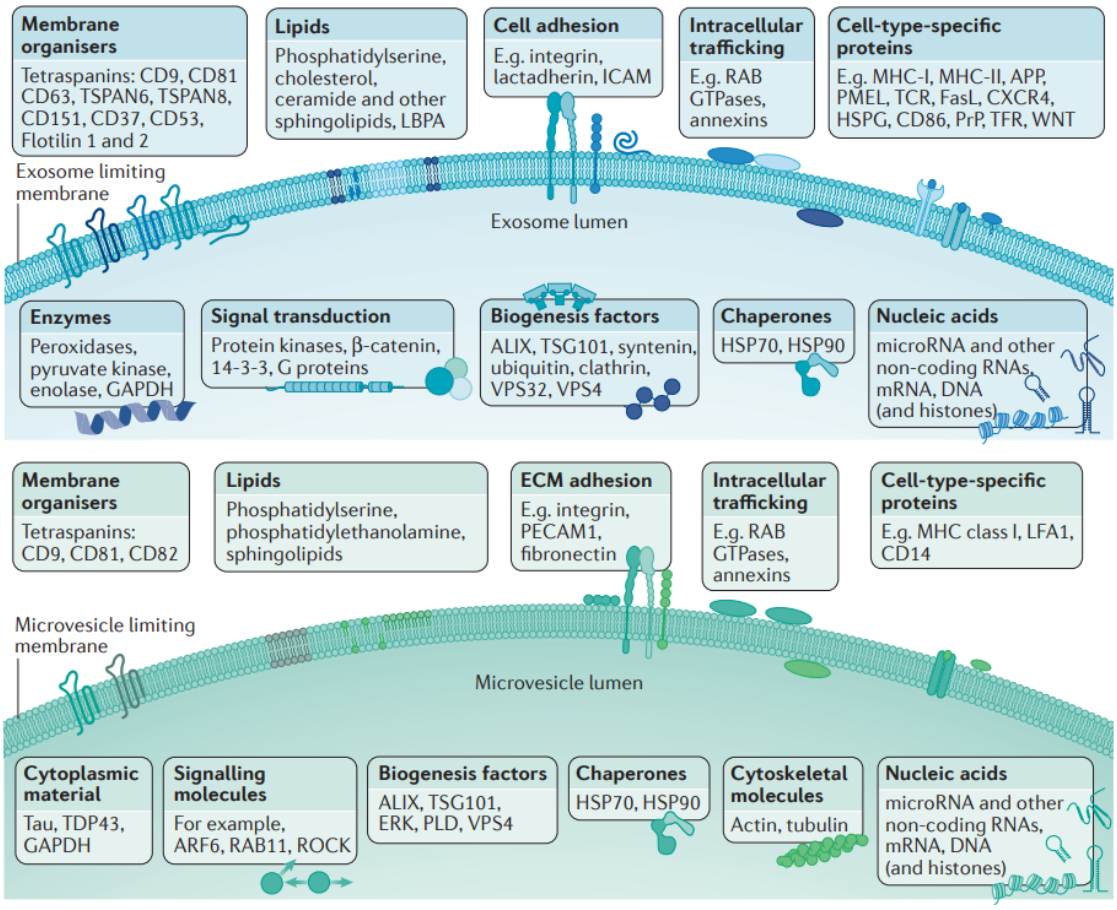


Figure 8. EVs protein composition. Upper part of the figure represents the typical components of an exosomes and the bottom part the ones of a microvesicle. Source of figure: (181).

EVs are released by all cell types, and can be found in most, if not all, body fluids. Body fluid-derived EVs are a mixture of vesicles originated from different source, presenting specific components of their cell of origin, reflecting its composition. In plasma derived EVs, the protein and RNA content and the vesicles concentration have been found to be altered in pathological states. These features, together with the fact that the EVs lipid membrane protects its content from degradation, and that EVs can be easily isolated from most body fluids, make them a valuable source of physiological and pathological information. Thus, EVs are very attractive as a new, inexpensive and minimally invasive method for the diagnostic and screening of diseases, biomarkers discovery, drug and gene therapy, as well as for developing new vaccines platforms against infectious diseases (15).

1.2.2. Extracellular Vesicles isolation and characterization

There are multiple methods available to isolate EVs from biofluids, each of them with different characteristics in terms of specificity and efficiency, affecting the results obtained in further experiments (193). Thus, the methodology used for EVs isolation is crucial. As all methods present advantages and limitations, it is not possible to establish a "gold standard" technique. Therefore, there is no single optimal separation method and the selection of the methodology should be based on downstream analysis requirements (193,194). However, it is increasingly recommended to use more than one purification method, especially if we start from biofluids (195). A summary of the most commonly used methods to isolate EVs can be found in Table 3 and Figure 9.

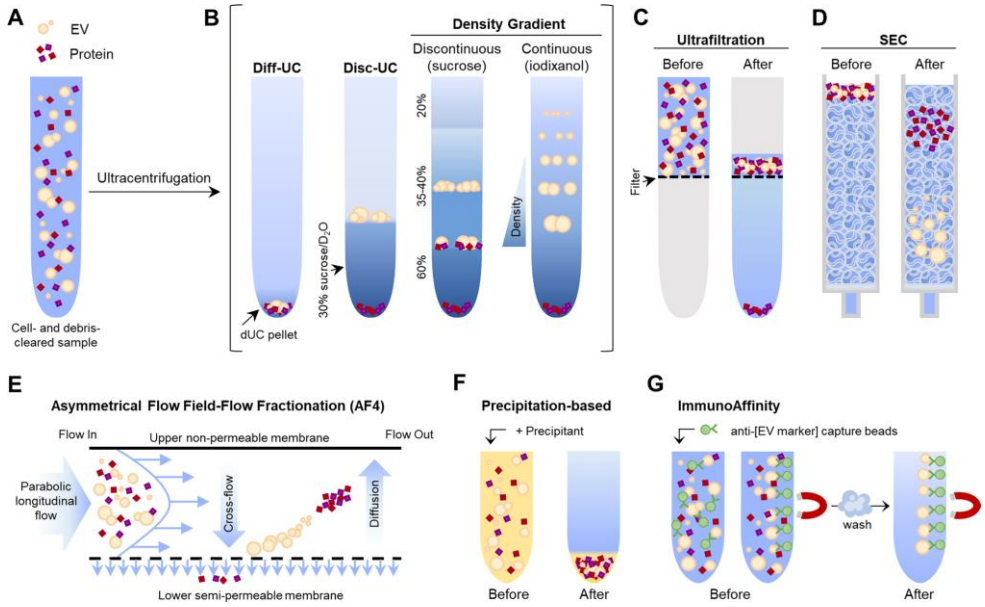


Figure 9. Summary of the main methods used for EVs isolation. Source of figure: (196).

EVs characterization is crucial in order to determine the nature and integrity of isolated EVs, avoid misinterpretation due to the presence of potential contaminants, and to ensure reproducibility. Several characterization methods have been developed, including both biochemical and physical technologies. According to the International Society for EVs, the use of two different and complementary techniques for EVs characterization it is highly recommended (207). Here, we summarize the most commonly used methods to characterize EVs (Table 4).

Table 3. Main methods for EVs isolation.

PROCEDURE	CHARACTERISTICS	REFERENCES
Differential centrifugation	First method developed and still the most widely used, it is based on sedimentation of solutes at a high centrifugation force. Unfortunately, some contaminants, as soluble proteins, might co-precipitate.	(177,197)
Density gradient ultracentrifugation (UC)	It increases the efficiency of particle separation, allowing to separate EVs from non-vesicular components, but also to further characterize EV subset. This methodology results in EVs of high purity, but it is complex, laborious and time-consuming, and produces very scarce material.	(198,199)
Precipitation-based protocols	The majority are based on the polyethylene glycol based volume exclusion precipitation, and it basically precipitates all soluble particles. User friendly, cheap and easy to implement, recovery can be high, but with low purity, as some contaminants (such as protein aggregates) co-precipitate. Several commercial isolation kits using this technique are available.	(200–202)
Size exclusion chromatography (SEC)	The isolation of EVs by SEC is based on the differential elution profiles of particles of different sizes. The column used in SEC contains a stationary matrix made of sepharose. Inside the column, big particles elute faster than smaller ones, which are slowed down by entering the pores of the polymer. Over the last years, SEC technique has gained popularity in the field of EVs as it is user friendly and provides high purity of EVs, removing most of the soluble plasma proteins. Also, SEC highly maintains the major EVs' characteristics, including vesicular structure and content.	(203,204)
Immunoaffinity based isolation methods	These methods, which have been gaining popularity in the last years, are based on antibodies against EV markers, usually of the tetraspanin family. The isolation results in high specificity and purity for a particular EV subtype, and could be useful to explore specific EV populations with biomarker or therapeutic potential. However, comparative proteomic analysis immunocapturing with different EV markers have shown that each isolated population has a different composition, and none of them represents the whole population of EVs. Moreover, without cleaning the EV sample from antibodies and their carriers, functional assays are not possible to be performed.	(205,206)

Table 4. Main techniques for EVs characterization.

PROCEDURE	CHARACTERISTICS	REFERENCES
Nanoparticle tracking analysis (NTA)	NTA determines the size and concentration of particles in a liquid suspension by tracking individual particles scattering light under laser illumination and undergoing brownian motion. However, it does not distinguish EVs from non-EV particles.	(208,209)
Electron microscopy	Electron microscopy is the gold standard method for imaging EVS. It assesses the size, morphology and integrity of EVs. However, it is an expensive technique that requires of specialized equipment and personnel.	(210)
Bicinchoninic acid (BCA) or bradford assays	The protein concentration of EVs can be determined using standard colorimetric assays, such as BCA or bradford assays.	(197,211)
Western blot or enzyme-linked immunosorbent assay (ELISA)	The most widely used methods to demonstrate the presence of a particular protein in EVs are Western blot and ELISA. In western blot, proteins are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a membrane for further immunoblotting of specific EV-related proteins, such as tetraspanins. Similarly, in the ELISA technique, EV samples are tested using antibodies against classical EV markers. Unfortunately, these techniques require a large sample volume, extensive processing and specialized instrumentation.	(212)
Flow cytometry	Flow cytometry is a powerful method to analyze EVs in biofluids, although its potential has not yet been fully realized. The small size and low abundance of surface antigens in EVs challenges conventional flow cytometry approaches. To overcome these limitations, highly sensitive flow cytometers able to discriminate particles up to 100 nm are being developed. However, most of the laboratories do not have this specialized equipment. An alternative approach is the bead-based assay flow cytometry (BBA). In BBA, EVs are captured by larger size microbeads. The bead-coupled EVs can be recognized by antibodies against specific markers, and analyzed by conventional cytometry instruments. This method it is fast and robust, and allows to characterize the surface components of EV populations.	(197,213,214)
Mass spectrometry based proteomics	Liquid chromatography–tandem mass spectrometry (LC-MS/MS) based proteomics has become a popular technique in the analysis of EVs, as it is capable to detect hundreds and thousands of proteins associated to EV samples from specific cell types or body fluids. Unfortunately, this technique requires extensive processing, highly specialized personnel and instrumentation, and it is very expensive.	(215,216)

1.2.3. Extracellular Vesicles in human diseases

EVs are involved in several physiological processes, such as inflammatory and immune responses, development, reproduction and pregnancy, tissue repair, and blood coagulation. Likewise, EVs are also implicated in pathological events. In recent years, EVs have been studied in the context of cancer (tumor development and progression, establishment of metastasis) (217), neurological diseases (218,219), liver diseases (220), autoimmune diseases (221) and cardiovascular diseases (222). Notably, EVs have also a major role in parasitic diseases as intercellular communicators (180).

1.2.4. Extracellular Vesicles in parasitic diseases

EVs have been described and are currently being studied in several protozoan parasites and helminths, such as *T. cruzi, Leishmania spp, Trypanosoma brucei, Toxoplasma gondii, Plasmodium spp, Giardia intestinalis*, *Schistosoma mansoni* and *Fasciola hepatica* (223–231). In the context of the parasitic diseases that these organisms cause, EVs are known to play a major role in intercellular communication between the parasite and the host (Figure 10).

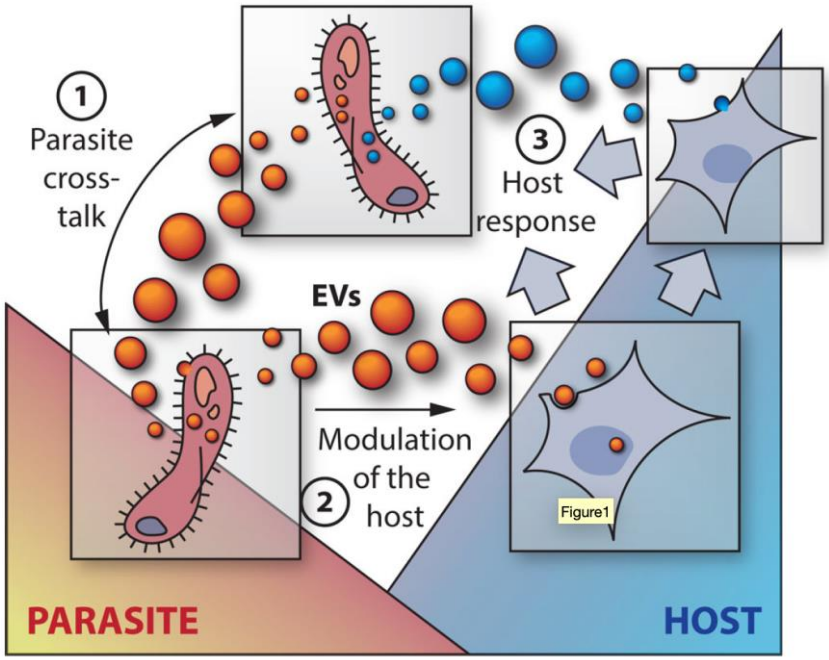


Figure 10. EVs mediated communication in parasitic diseases. Source of figure: (232).

Importantly, EVs can modulate the host immune response, increase parasite invasion, and alter the integrity and function of cells and tissues, resulting in different disease outcomes (224,233–239). The exacerbation of infection is mainly due to the EV's capacity to mediate immune evasion through a broad type of mechanisms (180). In addition, EVs can also trigger

protective responses by activating an immune cell effector mechanism that benefits the host, controlling the parasite replication and promoting host survival (240–243)

EVs released by parasites induce an immune modulatory effect and, together with their proven capacity for direct and indirect antigen presentation in adaptive immune response (244), make them promising tools for vaccine or immunotherapy development (243,245,246). Although EVs have not yet been tested as vaccines in clinical trials for parasitic diseases, several studies have demonstrated their potential. In toxoplasmosis, EVs derived from dendritic cells incubated with *Toxoplasma gondii* antigens induce a systemic immune response in mice models. The vaccinated animals demonstrate increased survival and lower cerebral parasite burden after parasite challenge (247–249). Another group has shown that EVs released by cells infected with *Toxoplasma gondii* alter cell proliferation, causing changes in neighboring cells, which is most likely a mechanism for modulating the host's immune system (250). In addition, using *Plasmodium yoelii* as a murine model of malaria, it has been shown that EVs generated from infected reticulocytes when administered in the presence of CpG as adjuvant elicited a potent host humoral immune response, decreased parasitemia, and protected mice against a challenge with a lethal strain of *Plasmodium yoelii* (227,240) (Figure 11).

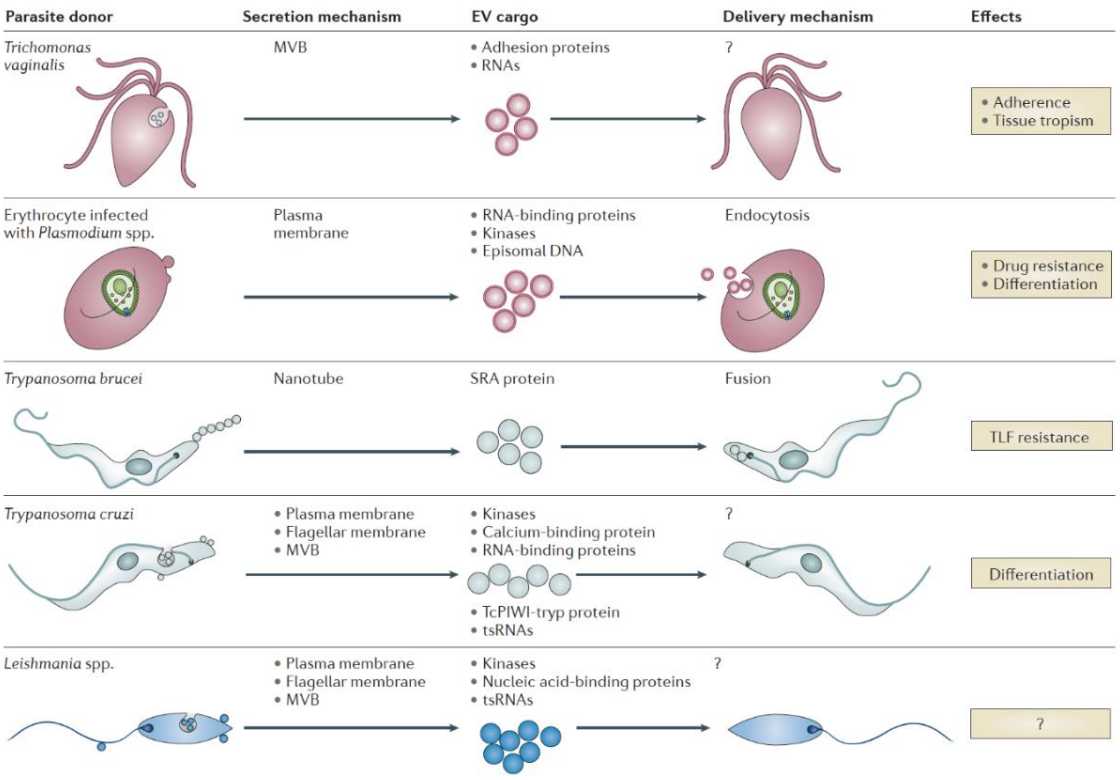


Figure 11. Interactions of EVs in the parasite population. Source of figure: (224).

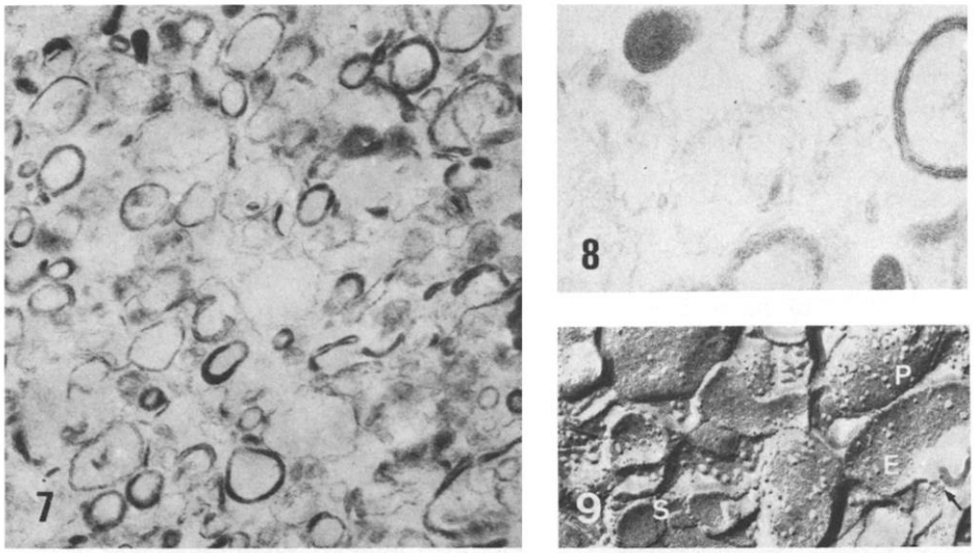
EVs mediate the transfer of parasite cargo, including proteins, nucleic acids, glycoconjugates and lipids, to host cells (236,251–254). This characteristic, together with the fact

that EVs are found in all biological fluids and present a specific molecular signature, dependent on the cell of origin, makes them interesting tools for biomarker discovery (255–257). In *Plasmodium falciparum* and *Plasmodium vivax (P. vivax)*, high circulating levels of EVs have been associated with the clinical symptoms and severity of the disease, showing that EV concentration may have applications as biomarkers of malaria severity (258–260). In schistosomiasis, schistosomal miRNAs were detected in EVs isolated from patients before treatment. These levels decreased after treatment, indicating that EVs could be used as new diagnostic tools for patients presenting low parasitic burden, and as new biomarkers for therapeutic response (261).

1.2.5. Extracellular Vesicles shed by *T. cruzi:* molecular composition and virulent factors associated

The shedding of EVs by *T. cruzi* was first described in epimastigote forms in 1979 (262) (Figure 12). Membrane vesicles were obtained by incubating the epimastigotes with either cross-linking reagents or acid pH, and were purified by sucrose density centrifugation (262). Later, Colli's group demonstrated that infective trypomastigote forms from four different *T. cruzi* strains (Y, YuYu, CA1 and RA) released surface antigens bound to particles by a spontaneous process temperature and time-dependent (263). In this work, a shedding assay was performed, followed by a chromatography of the parasite supernatant (SN) on Sepharose 4B column. The profile of the chromatography together with the results obtained by electron microscopy showed the presence of plasma membrane vesicles (263). Importantly, they also showed the presence of parasite antigens associated to the vesicles (263).

However, it was not until 2013 that the first proteomic analysis of the *T. cruzi* secretome, including soluble and EVs-associated proteins, was performed (264). In this study, EVs released by noninfective epimastigotes and infective metacyclic trypomastigotes were isolated by UC, and two sub-type of EVs (larger and smaller vesicles), as well as vesicle-free fractions, were analyzed by LC-MS/MS. The results showed a rich collection of proteins involved in metabolism, host-parasite interaction, signaling, nucleic acid binding, parasite survival and virulence (264). From then on, other proteomic studies emerged to



Figs. 7 and 8. Vesicle pellet depicting single membrane and multilamellated vesicles. Fig. 7, 28 500×; Fig. 8, 50 000×.

Figure 12. First image of purified EVs secreted by *T. cruzi* epimastigotes. Source of figure: (262)

characterize the exoproteome of trypomastigote forms (265) and to detect antigens associated with vesicles secreted by *T. cruzi* trypomastigotes (266). Later on, another proteomic analysis of trypomastigotes EVs was performed using two different strains (Y and YuYu), known to modulate the host immune responses differentially (252). The analysis confirmed previous proteins identification, and showed quantitative and qualitative differences in the EV cargo of the two strains, which correlated with differences in their infection profile (252). A timeline with the main contributions in the field of EVs in *T. cruzi* is illustrated in Figure 13.

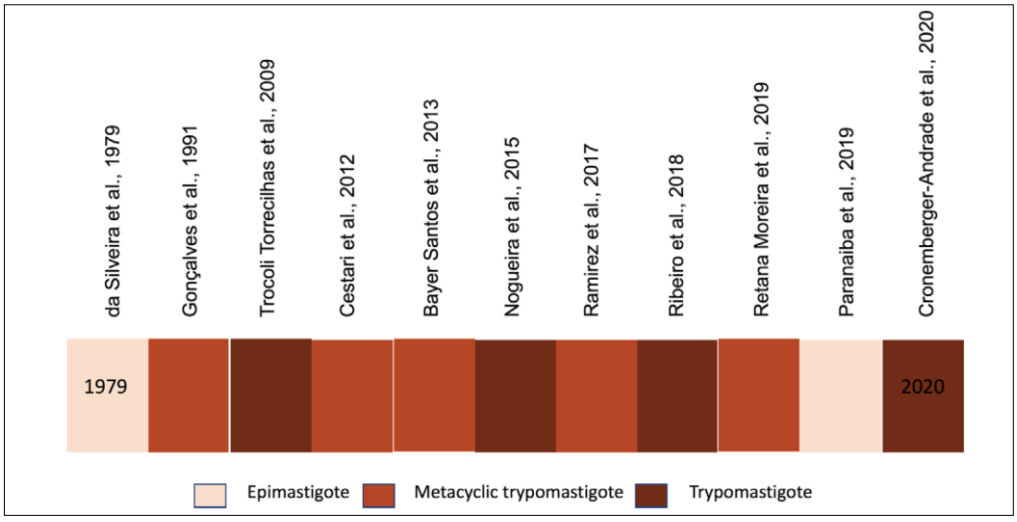


Figure 13. Timeline of *T. cruzi* Evs studies. Source of figure: (223)

Recently, another study characterized the proteome and the nanomechanical properties of EVs released from trypomastigotes and epimastigotes, finding marked differences in the EV cargo between both stages (267). The first proteomic characterization of plasma-derived EVs purified directly from a heart transplant patient with chronic CD was performed recently, identifying both human and parasite proteins in circulating EVs (for more details, see results chapter 5.1). The molecular cargo released in EVs by the different stages of *T. cruzi* parasites cultured *in vitro* is summarized in Figure 14.

Among the proteins identified in EVs released to the conditioned medium by different *T. cruzi* stages, the presence of virulent factors is worth highlighting, as their expression in the parasite is fundamental for disease establishment and the progression of the infection (Table 5). This is the case for trans-sialidases, mucin, mucin-associated surface protein, cruzipain and phosphatases, among others (268). These molecules, whose functions have been studied for years in the context of the infection and the parasite, are involved in attachment and invasion to the host cells, protecting the parasite from complement-mediated lysis system, and may act as proinflammatory agents (269–274). However, the function of these molecules in the vesicles is not yet well known and requires further investigations.

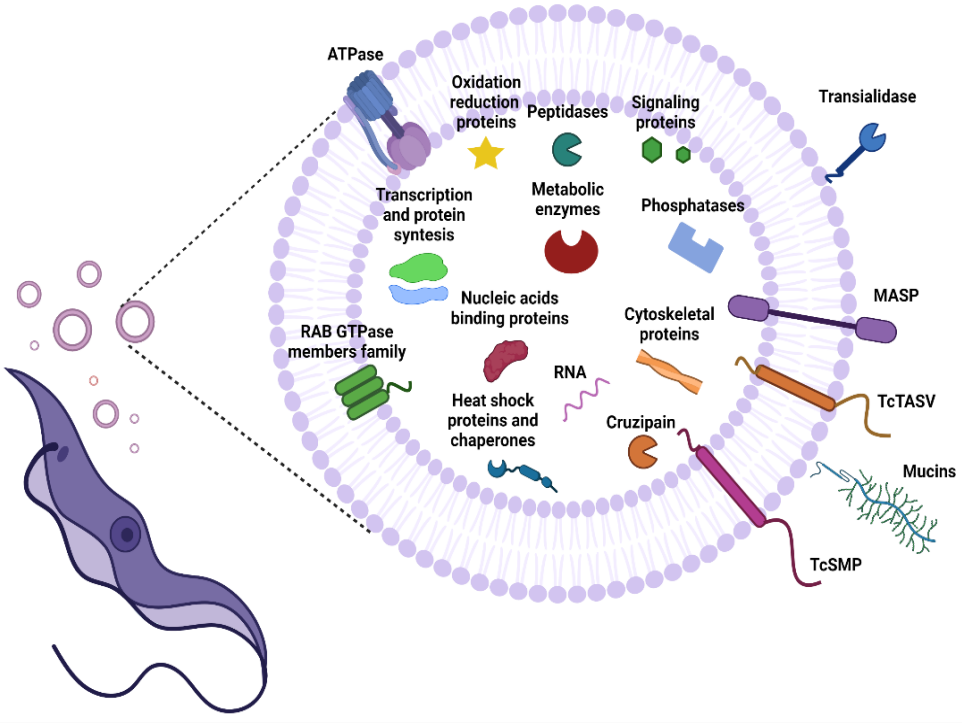


Figure 14. Schematic illustration summarizing the molecules identified in EVs secreted by *T. cruzi* trypomastigotes. Created with BioRender.com.

Table 5. Main virulence factors associated to EVs shedding by *T. cruzi* parasites. H: Healthy; T: Trypomastigotes, E: Epimastigotes, MT: Metacyclic trypomastigotes, A: Amastigotes

T. cruzi virulence factors	Description	EVs source	Reference
Trans-sialidase	*T. cruzi* is unable to synthesize sialic acid de novo. Trans-sialidase transfers α2-3-linked sialic acid from host glycoproteins and glycolipids to acceptors containing terminal β-galactosyl residues present on the parasite surface, avoiding lysis by serum factors and increasing invasion in the mammalian host.	T	(264–267)
Mucins Glycoproteins: trypomastigote-GPI-mucins or epimastigote-GPI-mucins or Metacyclic trypomastigote-GPI-mucin	Trypomastigotes - Mucins are the main acceptors of sialic acid in the parasite's surface. - Activation of the host innate immune system - Induction of the production of TNF-α, IL-12, and NO Epimastigotes - Similar cell surface glycoprotein complex, called GP24, GP31, and GP37. - Molecules maybe affect parasite migration in the vector. Metacyclic trypomastigotes - Reported originally as the 35/50-kDa antigens - Mucins from metacyclic trypomastigotes increased infectivity and the ability of the parasite to shed the mucins upon invasion of the host cell	T E MT	(264,266,275,276)
Mucin-Associated Surface Proteins (MASP)	MASPs proteins, considered one of the most antigenic *T. cruzi* proteins, are a very diverse protein family, with members involved in host-cell invasion and survival and multiplication of intracellular amastigotes	E T A	(264–267)
Phosphatases	In *T. cruzi*, phosphatases present multiple roles, such as providing a source of inorganic phosphate, facilitating epimastigotes differentiation, and promoting infection.	E MT T	(264–267)
TcSMP family	TcSMP induce calcium signaling and lysosome mobilization, facilitating the formation of the parasitophorous vacuole and parasite invasion.	E MT	(264)
T. cruzi Trypomasigote Alanine, Valine and Serine family (TcTASV)	Still unknown function, this family has been suggested as a potential target for intervention against *T. cruzi,* mainly due to the observation that some host- molecules trigger TcTASV-C expression *in vivo* during the infection.	T A	(269)
Cruzipain	The major cysteine peptidase involved in host immune evasion, cell invasion and intracellular development.	E T	(264,266,275,276)

1.2.6. Immunomodulatory role of Extracellular Vesicles derived from trypomastigote forms and *T. cruzi* infected cells

The early events of *T. cruzi* infection are crucial for the establishment of the disease. The parasites contain molecules that induce host innate immune response fundamental for parasite control (274). As macrophages and other mononuclear cells are among the host's first line of defense, several research groups have focused on the study of these cells and their interaction with EVs secreted by the parasite. EVs are among the mechanisms used by the parasite in order to escape the immune system. It has been shown that microvesicles released by THP-1 cells, after interacting with trypomastigotes in the early stages of the infection, are able to inhibit the C3 convertase, protecting the parasite against the complement system and increasing its chances of survival (277). These authors also demonstrated that the subpopulation of microvesicles carrying TGF-β, after incubation with Vero cells, increased *T. cruzi* invasion (277) (Figure 15A). Previous studies have also shown that *T. cruzi* infection requires the activation of the TGF-β signaling pathway to increase parasite invasion in epithelial and cardiac cells, and that TGF-β is also

involved in the development of chronic CD cardiomyopathy, being crucial for the formation of cardiac fibrosis (278,279).

It has been observed that EVs released by several *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) modulate the inflammatory response of macrophages via the Toll-Like Receptor 2 (TLR2)-dependent pathway, involving signaling pathways of mitogen-activated protein kinases (MAPK), and triggering an inflammatory response mediated by proinflammatory TNF-α and IL-12, IL-6, and NO (280) (Figure 15B). In the same line, other studies exploring the EV's contribution to the proinflammatory response of THP-1 macrophages showed that vesicles isolated from the plasma of chronic *T. cruzi* infected patients and experimentally infected mice also triggered the synthesis of proinflammatory cytokines and oxidative and nitrosative products (281). Interestingly, the expression levels of proinflammatory genes observed in this study were dependent on the patients´ disease stage, being higher in chronic CD patients presenting symptoms than in individuals suffering the indeterminate form of the disease (281) (Figure 15B). Notably, an unbalanced immune response favoring a proinflammatory environment is one of the main features responsible for disease progression. In this scenario, therapies capable of preventing tissue damage or reprogramming the macrophages to increase its microbicide and effector functions could be a useful tool for CD treatment. More recently, Vasconcelos and collaborators showed that the viability and / or integrity of the parasite are necessary factors for the release of EVs, which trigger a proinflammatory response in the host cell *in vitro*, and may be a strategy developed by the parasite that aims to create a more favorable environment for establishing infection in the host (282). However, other studies have shown that bone marrow-derived macrophages treated with *T. cruzi*-derived EVs (strain Y) induced lipid body and prostaglandin E2 (PGE2) formation prior to infection. Twenty-four hours after *T. cruzi* infection, these EV-treated macrophages decreased the production of PGE2, TNF-α, and IL-6, decreasing the production of proinflammatory cytokines and oxidative and nitrosative products, which favored parasite infection and persistence (Figure 15C) (283). The therapeutic potential of EVs is variable in different models and needs to be addressed carefully. Infected macrophages can also modulate the activation of other THP-1 cells, promoting an inflammatory response (280). Using a NF-kB activation reporter CHO cell line, the authors showed that EVs secreted from infected cells induced the translocation of NF-kB after interacting with TLR2 in this model. Moreover, both EVs from trypomastigotes and from infected macrophages altered the gene expression of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, and the signal transducer molecules, such as STAT-1 and STAT-3 in THP-1 macrophages (Figure 15B) (280). In another report, the mechanism of NF-κB-mediated proinflammatory cytokine response was studied further and

identified two proteins involved in sensing DNA damage, cGAS and PARP1 (a DNA repair enzyme), as factors responsible for the proinflammatory phenotype induced by parasite and infected cells-derived EVs. Oxidized DNA was detected in EVs secreted by infected macrophages, and in EVs from plasma of chronically infected mice. Interestingly, the inhibition of PARP1 decreased the overall proinflammatory response and heart inflammation of chronically infected mice, suggesting that chemical inhibitors of this enzyme could become potential therapeutic targets for CD (284). Notably, in some of the described *in vitro* models, *T. cruzi* infected cells released higher levels of EVs compared to uninfected cells, and these differs largely in their protein cargo (280,285).

Little is known about the mechanisms by which *T. cruzi* EVs alter non-immune host cells. A recent *in vitro* study showed that epithelial cells (Vero) and cardiac muscle cells (HL-1), incubated with parasite EVs, altered cell permeability and intracellular levels of calcium, which modified the dynamics of the actin cytoskeleton and arrested the cell cycle (Figure 15D). All together, these changes could explain the increased host-cell invasion observed in this study (286).

The role of EVs in the immune response of the chronic phase of *T. cruzi* infection has been less studied. Nogueira and collaborators studied the *ex vivo* effect of EVs secreted by different *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) when used to stimulate splenocytes from chronically infected mice. Interestingly, the immunomodulatory responses caused by the EV stimulus were dependent on the parasite strain. As previously reported, using other cell types, splenic cells also produced NO, TNF-α, IL-6 and IFN-γ upon stimulation with parasites EVs. However, an increase in the production of anti-inflammatory cytokine IL-10 by T and B cells was also observed, which is in contrast with the proinflammatory profile found in other studies, and reinforces the importance of IL-10 in modulating the balance between inflammatory and anti-inflammatory responses, avoiding tissue damage (Figure 15E) (276).

Several *in vivo* studies addressing the EVs effect in the pathological features of *T. cruzi* infection have also been performed, all of which used well-established mice models. Some of these studies have shown that animals treated with parasite-derived EVs prior to *T. cruzi* infection are distinguished by increased circulating EVs in plasma, parasitemia, cardiac tropism and inflammation (Figure 15F) (275,283,287). Moreover, some studies found a reduction of NO and TNF-α levels in plasma, and a decreased production of TNF-α and IL-6 in spleen cells from infected animals (283). However, some discrepancies have been observed in the mortality rates linked to EVs immunization, with some studies reporting increased mortality (275) while no differences were found in others (283).

There is growing evidence that the immunomodulatory properties of EVs are dependent on the *T. cruzi* strain and the parasite stage (276,285,288). *T. cruzi*-derived EVs from different strains present different protein cargo which correlates with differences in the sensitivity to complement-mediated lysis, parasite invasion, infectivity, virulence and immunomodulatory responses (252,276). In relation to the effect of the EVs released by host cells after interacting with different parasite developmental stages, it has been found that all *T. cruzi* stages are able to induce the release of EVs by host cells, with mammalian infective forms causing the highest release (285).

1.2.7. Extracellular Vesicles as potential source of new biomarkers in Chagas Disease

The use of EVs as a new platform to identify biomarkers has been described in the last years for different pathologies, including parasitic diseases (289). Taking into consideration that one of the biggest challenges for *T. cruzi* infection is the lack of validated biomarkers to indicate therapeutic response and disease outcome (12,33), EVs could become a promising source for developing new biomarkers in infectious diseases.

As previously mentioned, the MASP multigene family is one of the major virulence factors of *T. cruzi*. It plays a fundamental role in cell invasion and has an associated humoral immune response in *T. cruzi* infected patients. Interestingly, this response is different depending on the clinical stage of the individuals, being lower in the sera of patients presenting cardiac affections compared to sera from those suffering from the gastrointestinal form of the disease (273). Further research showed that the EVs released by the parasite containing MASP proteins are targeted by the immune system, triggering the formation of circulating immune complexes containing anti-MASP IgGs. The EVs forming the immune complexes inhibit the complement system. Interestingly, the highest percentage of inhibition appeared in the digestive group, compared to the asymptomatic and cardiac patients. Taking advantage of this particularity, these immune complexes could be used as biomarkers for the differential diagnosis or prognosis of CD, in particular in patients with digestive manifestations (270,273). In the same line, microvesicles also have potential as differential diagnosis or prognosis biomarkers during the course of the *T. cruzi* infection. The antibodies contained in the sera from *T. cruzi* infected patients detected antigens from EVs released by host cells after interacting with the infective forms of the parasite. Interestingly, these molecules were recognized differently by patients presenting the cardiac or indeterminate phase of the disease, indicating the existence of specific markers associated with a differential diagnosis depending on the organ involved (285).

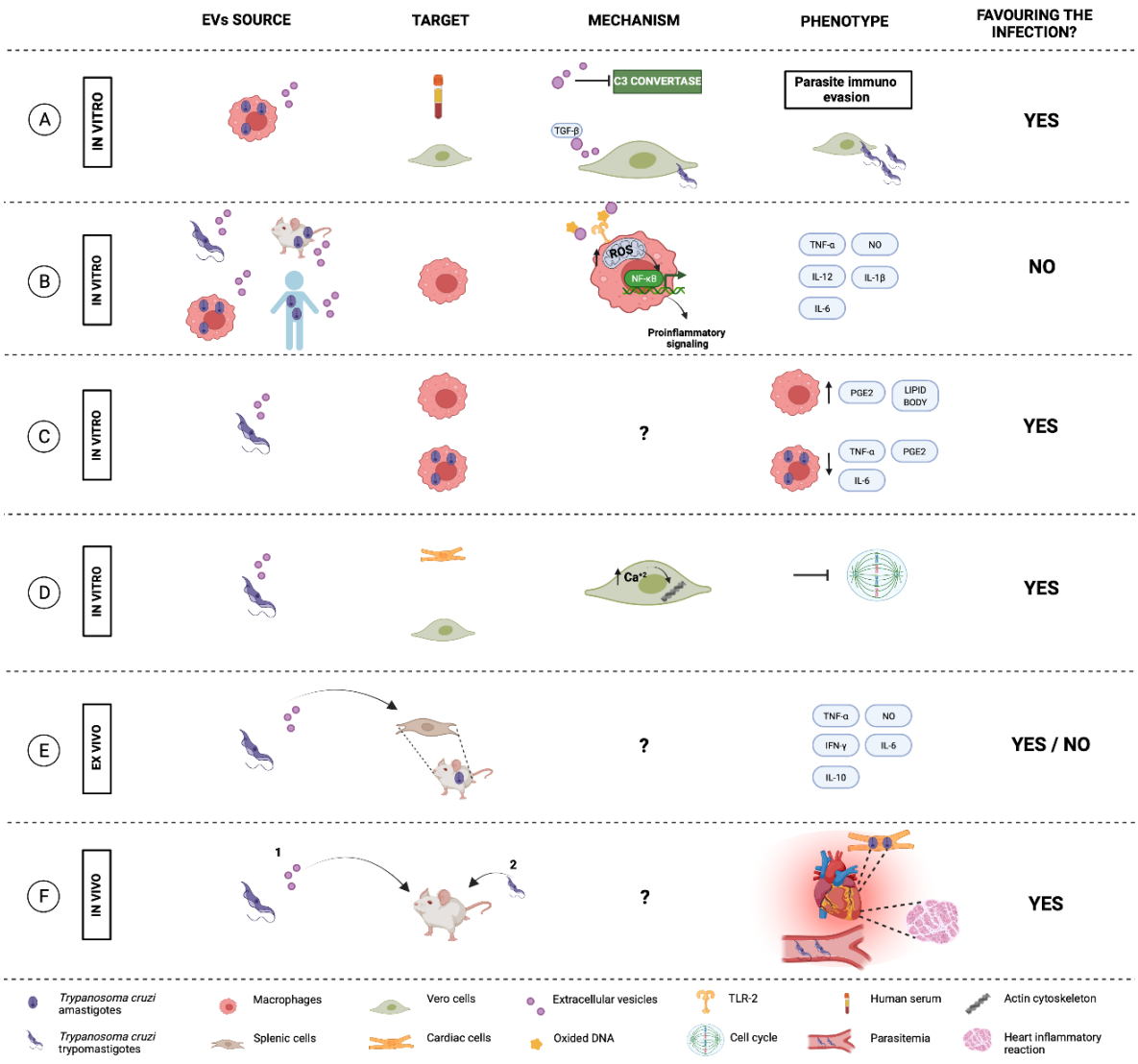


Figure 15. Immunomodulatory role of EVs derived from *T. cruzi* and *T. cruzi* infected cells. Summary of the main studies targeting the immunomodulatory effect of EVs in *T. cruzi* infection: EV source, target cell or body fluid, mechanism of action (if known), phenotype, and final effect on the infection process. Created with BioRender.com.

EVs concentration in body fluids of healthy individuals and patients presenting several forms of the disease has also been studied as a potential biomarker for differential diagnosis, with no clear results. While some studies did not find any statistical differences in the number of vesicles in *T. cruzi* infected patients compared to healthy controls (285), others did find differences in terms of concentration, showing that treated patients presented lower concentration of circulating EVs than healthy donors (256). In this study, human THP-1 cells were incubated with circulating EVs, followed by ELISA to measure cytokines and determine whether the concentration of circulating EVs was associated with differential activation of the immune

system. IFN-γ and IL-17 showed a differential profile when compared to chronic *T. cruzi* infected patients and healthy controls, finding that patient samples induced a higher production of IFN-*γ*, and lower production of IL-17, a profile that could contribute to parasite persistence and tissue damage due to continuous inflammatory signaling (256).

Two of the features of *T. cruzi* infection are chronic inflammation and oxidative stress, which are specially exacerbated in individuals suffering the cardiac form of the disease. It has been shown that microparticles generated during *T. cruzi* infection carry the host's signature of the oxidative, nitrosative and inflammatory states. Thus, EVs provide information about the disease's progression, and could be useful for evaluating disease severity (281).

In a different study, a group of human and parasite proteins were identified in plasma-derived EVs from a heart transplant patient with chronic CD, while being absent in EVs from plasma of healthy individuals. Interestingly, several human proteins, and one parasite protein (pyruvate phosphate dikinase, PPDK), were found to be present or upregulated before treatment, and were absent or downregulated after treatment. Although these results should be interpreted with caution, as they represent a single clinical case and needs validation in a larger cohort, they represent a proof-of-principle of the potential of this approach to discovering new biomarkers of therapeutic response (for more details, see results chapter 5.1) (253).

Finally, EVs from *T. cruzi* are also attractive candidates for use in the serological diagnosis of CD. In an attempt to identify antigens, present in trypomastigote excreted-secreted EVs, Bautista-López NL and collaborators incubated trypomastigotes-excreted antigens associated with EVs with affinity columns containing IgG antibodies from healthy donors, or Chagas patients with clinical symptoms. Chagasic IgG affinity resin was highly enriched in trans-sialidases, and also showed a significant enrichment in mitochondrial proteins, retrotransposon hot spot (RHS) proteins, paraflagellar rod proteins, proteases, and multiple uncharacterized proteins (266). RHS and the *T. cruzi* paraflagellar rod-3 proteins were further explored for their potential as serological antigens for the diagnosis of *T. cruzi* infections, showing robust cross-reactivity with sera from patients presenting all clinical forms of *T. cruzi* infection. Interestingly, no cross-reactivity with RHS was detected when using sera from patients with other parasitic diseases, which could be relevant for the development of a new diagnostic test with high specificity (266).

1.2.8. Chagas Disease prevention: potential of Extracellular Vesicles as new vaccine antigens against *T. cruzi* infection

As previously mentioned, even though multiples attempts have been made to develop effective and safe vaccines for CD, none of them were able to elicit complete protective immunity.

In this scenario, new approaches and ideas are needed to develop a protective vaccine for *T. cruzi* infection. Immunization with molecules delivered into EVs is an interesting possibility to explore.

Interestingly, one of the protein families present in *T. cruzi*-derived EVs that has been tested as a potential vaccine antigen is the MASPs. Taking into consideration that MASPs play a major role in host-cell invasion, are one of the most important *T. cruzi* virulence factors, and that several MASP family members have predicted MHC-I and MHC-II epitopes, a synthetic MASP-derived peptide was tested as a vaccine candidate in a murine model of CD (273,290,291). Mice immunized with the synthetic MASP peptide conjugated to keyhole limpet hemocyanin showed 86% survival rate after being infected with trypomastigotes, and had a much lower parasite load in the heart, liver and spleen compared to untreated animals. Moreover, vaccinated animals produced neutralizing antibodies, and developed a protective cytokine response against parasite infection. Interestingly, the vaccine engaged both humoral and cellular responses, indicating that MASP proteins are promising targets for the development of a CD vaccine (291).

Another well-known *T. cruzi* virulence factor essential for the invasion process and present in EVs is the trans-sialidase family. Several investigators have tested immunization with multiple genes encoding members of the trans-sialidase family, in different vaccine platforms (bacterial and viral vectors, or as a recombinant protein) and formulations (alone, together with other *T. cruzi* glycoconjugates and associated with adjuvants) (85,292). Although the results obtained showed some limitations, some vaccine formulations induced immunity in mice models challenged with *T. cruzi*, producing antibodies, preventing the development of tissue damage, and having an impact on the mortality of infected animals (292). In that context, trans-sialidase antigens conjugated to EVs could be a different approach to further develop vaccines for CD.

Another *T. cruzi* protein family secreted in EVs that has been considered for immunization is the TcTASV-C. To evaluate the performance of TcTASV-C as a vaccine antigen, mice were vaccinated following a DNA-prime protein-boost schedule of immunization. However, when animals were challenged with a highly virulent *T. cruzi* strain two weeks after the last dose, the results obtained were not very promising. Although TcTASV-C vaccinated mice showed a strong humoral response, there was a delay in the appearance of circulating trypomastigotes, and they presented lower parasitemia, mice exhibited only a 30% higher survival rate than controls (269).

Finally, some preliminary results have shown that mice immunization with three doses of EVs derived from trypomastigotes forms of *T. cruzi* (Y strain), administered in the presence of $Al(OH)_3$ as adjuvant, could induce some level of protection against experimental CD. Preliminary results showed that vaccinated mice presented lower parasitemia than non-vaccinated animals.

However, no significant changes were observed in the survival of all animal's groups. Further investigation needs to be carried out to understand which molecules are responsible for this potential protection. Moreover, experimental assays using EVs isolated from trypomastigote forms from different DTUs are needed to verify the influence of virulence factors in vaccination against experimental CD (Torrecilhas, unpublished data).

The use of different experimental models, cell types, adjuvants, doses and vaccination regimens also determine the development of the protective response. The key questions remaining for the development of new vaccine tools for CD are: further characterization of the immune responses; development of highly efficient antigen-delivery systems; animal models mimicking the chronic phase of the disease; assessment of parasite diversity and antigenic variation; study of co-infections; use of adjuvants and new vaccination regimens, together with more studies focusing on parasite tissue distribution (83,85).

The potential control strategies that could be associated with EVs secreted by *T. cruzi* or *T. cruzi* infected cells, such as biomarker discovery and / or vaccine development, are summarized in Figure 16.

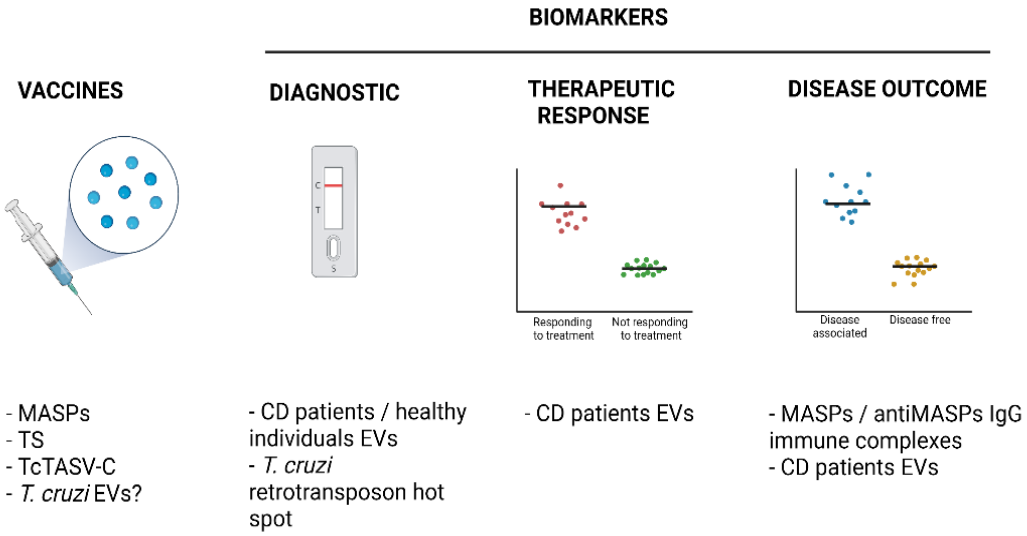


Figure 16. Potential role of EVs as new tools for CD prevention and control. Created with BioRender.com.

The identification and validation of biomarkers of disease progression and/or treatment response is one of the main challenges in *T. cruzi* infection, and a major research priority. All together, these results show the potential of EVs to identify new biomarkers in CD, and encourage further studies in this direction.

2. HYPOTHESIS

Our hypothesis is that circulating EVs from *T. cruzi* infection contain human and parasite proteins that are present or upregulated before treatment and disappear or are downregulated post-treatment. Moreover, we propose that circulating EVs present a particular signature specific of the disease stage. Therefore, EVs isolated from *T. cruzi* infected patients biofluids could be used as potential biomarkers for therapeutic response and disease outcome (Figure 17).

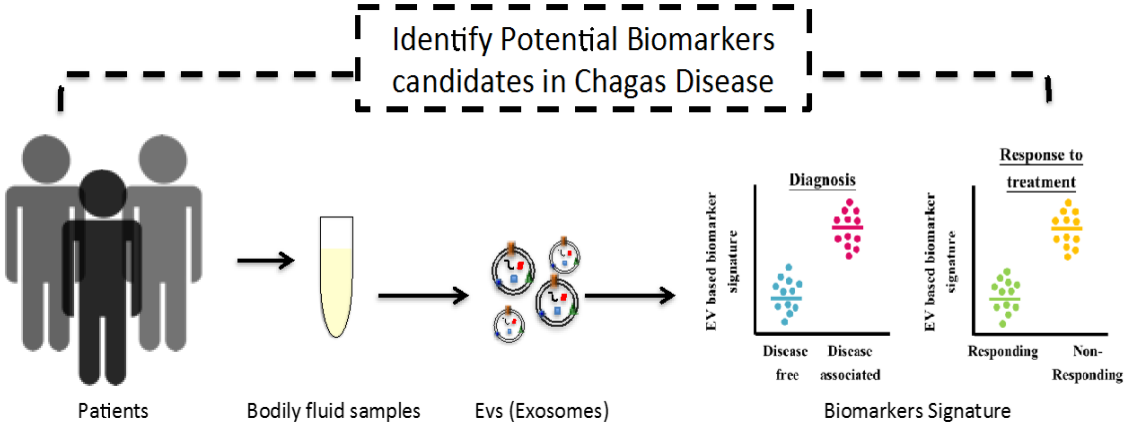


Figure 17. Schematic representation of the hypothesis of this thesis. Modified from Boukouris and Mathivanan, 2015 (293).

3. OBJECTIVES

The main objective of this study is to use EVs for identifying novel biomarkers in chronic CD, specifically in the context of therapeutic response and disease prognosis during the chronic infection.

Specific objectives:

- To identify EVs biomarkers in plasma of different clinical groups of chronic *T. cruzi* infected patients.

- To validate such biomarkers in the context of therapeutic response, and its possible correlation with the progression to cardiological clinical manifestations.

- To determine the role of circulating EVs in CD pathology.

4. MATERIALS AND METHODS

This chapter describes the materials and methods used in the investigations performed in the context of this thesis in order to address the hypothesis.

4.1. ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registered with the number Reg.HCB/2015/0616) (Annex 2) and the Ethics Committee of Fundación Ciencia y Estudios Aplicados para el Desarrollo En Salud y medioambiente (CEADES). Individual written informed consent was obtained from all study participants before the collection of samples (Annex 3).

The study was designed to evaluate the potential of EVs as biomarkers for therapeutic response and disease outcome for CD. The first approach was based in a pilot study to detect EVs in this context.

Inclusion criteria: Adult subjects (older than 15 and less than 60 years old), of both sexes, with serologic tests confirming or excluding *T. cruzi* infection were included in the study. Patients presenting positive *T. cruzi* serology and included in the study must not have been previously treated with antiparasitic treatment for *T. cruzi* infection.

Exclusion criteria: Patients who do not met the inclusion criteria or who did not sign the informed consent were excluded of the study. Information regarding samples used for each assay can be found in Annex 4.

Participants were allocated in four different groups based in the serological test and cardiological complications:

A) *T. cruzi*-seropositive individuals without evidence of organ involvement (called as individuals with the indeterminate form of the disease) (G1 = *T. cruzi* infected, indeterminate)

B) *T. cruzi*-seropositive individuals presenting cardiomyopathy (G2 = *T. cruzi* infected, cardiomyopathy)

C) *T. cruzi*-seronegative individuals presenting cardiomyopathy (G3 = Non-CD cardiomyopathy)

D) *T. cruzi*-seronegative individuals without evidence of organ involvement (G4 = Healthy)

T. cruzi infection was confirmed by conventional two serological tests, following international recommendations to diagnose *T. cruzi* infection in the chronic stage (34). During the recruitment period, and based on the hospital regular methods, several diagnostic kits were used to assess serological state of individuals involved in the study: Arquitect Chagas (Abbott, reference number 2P25-25), the CHAGAS ELISA kit IgG+IgM (Vircell, S.L., reference number T1020), and the CHAGAS IFA kit IgG+IgM (Vircell, S.L., reference number PCHAG). Specific treatment with BZD (5 mg/kg/day, BID) was offered to all *T. cruzi* seropositive patients fulfilling *T. cruzi* etiological treatment criteria following international recommendations (34), and those treated were closely monitored following treatment protocol of International Health Service - Hospital Clínic (Barcelona). Quantitative PCR (qPCR) was also performed in several *T. cruzi*-seropositive individuals from G1 and G2 to assess drug response (74).

All *T. cruzi* positive patients undergo a cardiological evaluation in order to determine if *T. cruzi* infection was evolved to CD with cardiac clinical manifestations. Cardiological evaluation was made using a protocol that included a 12-lead-ECG before treatment and at 12 months after treatment. In patients from G1, a 2D- echocardiogram was also performed. Echocardiographic studies were performed with a commercially available system (General Electric E95; General Electric S70; Philips 7C).

Figure 18 shows the flow-chart of participants throughout the study.

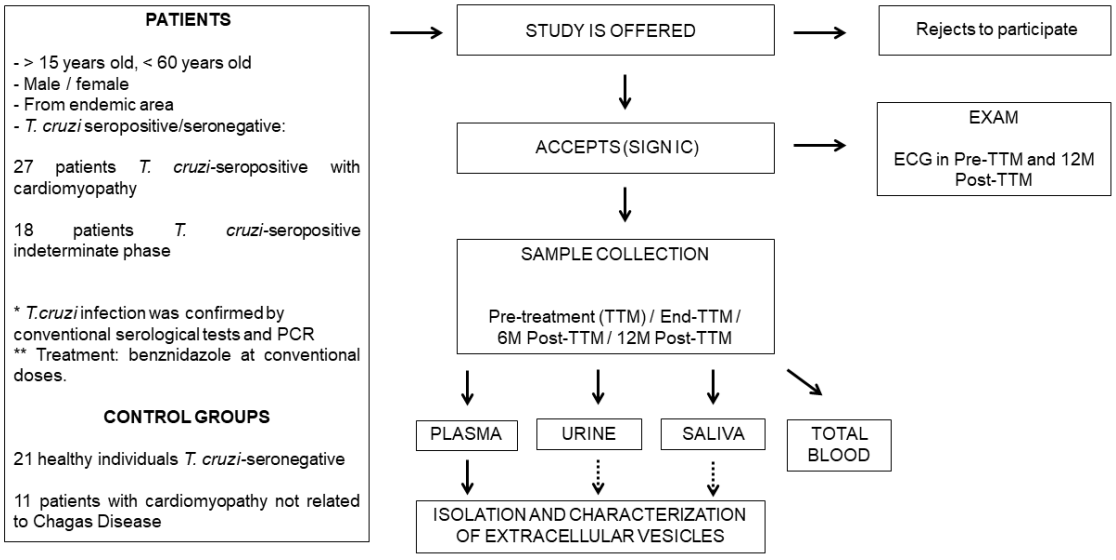


Figure 18. Flow-chart of participants throughout the study.

4.2. SELECTION OF SUBJECTS

Participants from G1, G2, G3 and G4 were recruited at the International Health Service-Hospital Clínic, Barcelona. However, two other health care facilities collaborated in the recruitment of the individuals: participants from G3 were mainly recruited at the Cardiology Department at the Hospital Clinic of Barcelona, Spain; and individuals from G2 were mainly recruited at Chagas Platform (CEADES- ISGlobal), Cochabamba, Bolivia. Recruitment of patients started on 2016, and finished on 2021.

4.3. SAMPLE COLLECTION AND PROCEDURES

Plasma, total blood, saliva and urine were collected from all study participants. Per individual, seven mL of blood were collected in citrate tubes and immediately were stored at 4°C for a few hours until they were processed. Samples were centrifuged at 500 g for 10 minutes at 4°C, and the SN was centrifuged again at 2000 g for 15 minutes at 4°C to obtain plasma. Aliquots were then frozen at -80°C until further analysis. One EDTA tube was also collected to obtain whole blood to perform qPCR. Blood was directly frozen at -80°C until further analysis.

Saliva samples were collected in 15 mL falcon tubes and centrifuged at 500 g for 10 minutes at 4°C. The resulting SN was centrifuged again at 2000 g for 15 minutes at 4°C and aliquots were frozen at -80°C until further analysis.

Urine samples (between 20-50 mL) were collected in urine collection tubes and stored in aliquots at -80°C until further analysis.

4.4. PLASMA ULTRACENTRIFUGATION (UC)

In several occasions, a pre-enrichment of the EVs before performing any isolation method was done by UC of plasma samples. Briefly, one mL of plasma was ultracentrifuged at 120.000 g for four hours at 4°C in a Thermo Sorvall WV Ultra 100 centrifuge (rotor TH-641). The pellets, enriched in EVs, were resuspended in Phosphate Buffered Saline (PBS) and kept at -80°C until further analysis. These samples were used as starting material to further isolate the EVs.

4.5. EVS ISOLATION BY SIZE-EXCLUSION CHROMATOGRAPHY

EVs were isolated from plasma samples or pre-enriched EV samples by SEC following our previous report (294), with some modifications. Two different handmade columns were used: 10 mL and one mL. Basically, sepharose CL-2B (Sigma-Aldrich, St. Louis, MO, USA) was packed in sterile conditions one day before the isolation in a syringe to a final volume of 10 mL or one mL,

sealing the syringe tip with a fragment of nylon stocking. Then, columns were equilibrated with PBS and were kept at 4°C until use. The day after, plasma or pre-enriched EV samples were thawed on ice and processed by centrifugation at 2000 g for 10 minutes at 4°C. Then, one mL or 100 µL of the plasma or pre-enriched EV samples (for 10 mL columns or one mL columns, respectively) were loaded, and 10 fractions were collected immediately and were analyzed or frozen at −80°C. For 10 mL columns, the volume of the fractions collected was 500 µL, while for one mL columns, was 100 µL. For one mL columns, four columns were used for each sample. Schematic view of the process is depicted in Figure 19.

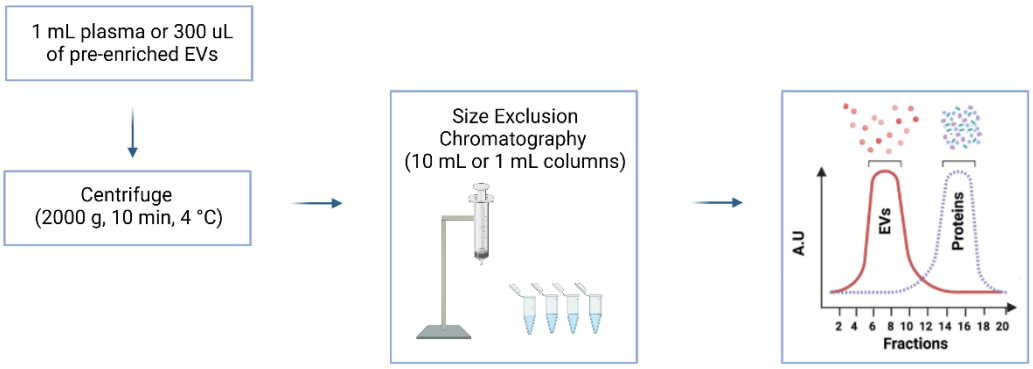


Figure 19. Purification of EVs by SEC. Created with BioRender.com.

4.6. EVS ISOLATION BY IMMUNOMAGNETIC POSITIVE SELECTION

EVs were also isolated by immunomagnetic positive selection using magnetic beads (EasySep™ Human Pan-Extracellular Vesicle Positive Selection Kit). This kit, containing tetrameric antibody complexes recognizing CD9, CD81, and CD63 and magnetic particles, allows the separation of EVs using a magnet. Briefly, 2000 µg of pre-enriched EV samples were incubated for 10 minutes at room temperature (RT) with 50 µL / mL of the selection cocktail (containing the antibody complexes). Then, 100 µL / mL of the magnetic beads were added to the samples, and were incubated for 10 minutes at RT. Samples were placed in the magneto separator, and the flow-through was collected. After performing several washes with PBS, beads coupled to the EVs were resuspended in the desired buffer (lysis buffer or PBS, according to the following procedures). A chart showing an overview of the immunoaffinity capture process can be found in Figure 20.

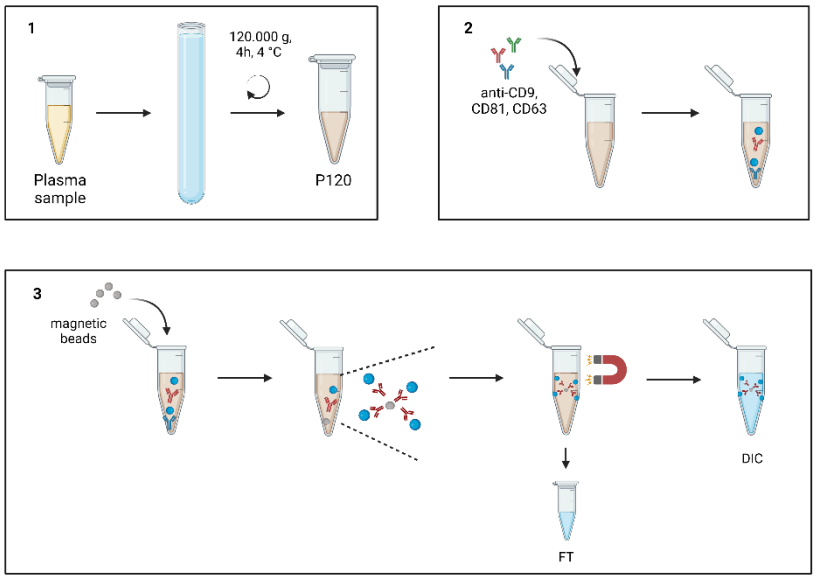


Figure 20. Purification of EVs by immunoaffinity capture using CD9, CD81, and CD63 antibodies. Created with BioRender.com. FT: flow-through

4.7. EVS QUANTIFICATION

Protein concentration was determined by BCA and microBCA protein assay kit (Pierce, Thermo Fisher Scientific) following the manufacturer instructions. The BCA assay was used to quantify samples from 20 to 2000 µg/mL, and the microBCA to quantify samples from 0.5 to 20 µg/mL. Briefly, 20 µL of each standard or unknown sample were added in a microplate (the final volume of sample should not be lower than 20 µL for BCA and to 100 µl for microBCA; when necessary, samples can be diluted), together with 200 µL (for BCA) or 100 µL (for microBCA) of working reagent. Then, the plate was incubated for 30 minutes at 37 °C (or for two hours at the same temperature, in the case of the microBCA), and the absorbance was quantified at 562 nm.

4.8. BEADS-BASED FLOW CYTOMETRY

EVs characterization was done by detection of the exosomal markers in a bead-based flow cytometry assay. Briefly, SEC fractions were coupled to Aldehyde/Sulfate Latex Beads, 4% w/v, four µm (Invitrogen) by incubation for 15 minutes with agitation. Coupled beads were then blocked by incubation overnight with one mL of BCB Buffer (PBS-BSA 0.1%) in a rotation device. Beads were further centrifuged at 2000 g for 10 minutes, SNs were removed and pelleted beads were resuspended in BCB buffer. The beads suspension was analyzed to study the presence of classical EV markers being incubated with primary antibodies diluted with BCB buffer for 30 minutes at 4 °C. After washing with BCB, EV-coated beads were incubated for 30 min at 4 °C with secondary antibodies made in rabbit or mouse. Table 6 shows the antibodies and dilutions used

to analyze the different EV samples by bead-based assay. Negative controls included SEC fractions predicted to contain a high concentration of EVs incubated with the respective secondary antibodies. Labelled EV-beads were washed twice with BCB before being finally resuspended in PBS and analyzed in a FacsVerse cytometer (BD). Flow Jo software was used to analyze the data.

Table 6. Antibodies used in bead-based flow cytometry of EVs.

ANTIBODIES	DESCRIPTION	REFERENCE	DILUTION
Rabbit-CD5L	Classical plasma EV marker	Abcam, ab45408	1:1000
Rabbit-CD71	Transferrin receptor	Abcam, ab84036	1:1000
Mouse-CD9	Tetraspanin	VJ1/20 Immunostep (9PU-01MG),	1:500
Mouse-CD63	Tetraspanin	TEA3/18 Immunostep (63PU-01MG)	1:500
Mouse-CD81	Tetraspanin	mouse monoclonal Santa Cruz Biotechnology (Sc-23962)	1:500
Goat ANTI-MOUSE IgG Human conjugated to FITC	2ary antibody	Southern Biotech, 1032-02	1:100
Goat ANTI-RABBIT IgG Human conjugated to Alexa Fluor 488	2ary antibody	Invitrogen, A11008	1:500

4.9. NANOPARTICLE TRACK ANALYSIS

Size distribution and particle concentration of total plasma and isolated vesicles was determined by NTA in a NanoSight LM10-12 instrument (Malvern Instruments Ltd, Malvern, UK) equipped with a 638 nm laser and CCD camera (model F-033). Readings were taken in single capture during 60 s at 30 frames per second, at camera level set to 680 and manual monitoring of temperature. Samples were diluted in PBS in order to obtain around 20 to 120 particles per frame. Data was analyzed using the NTA software version 3.2.

4.10. ELECTRON MICROSCOPY

SEC fractions highly enriched in EVs and distal fractions, enriched in plasma proteins contaminants, were characterized by cryo-electron microscopy (cryoEM) and by transmission electron microscopy (TEM) to estimate size and morphology of isolated vesicles as previously described (227,295). For TEM, briefly, 50 µl of SEC fractions were concentrated by speed-vac to 15 µl, were placed in a Cu-C 400 mesh grid, and were treated with uranyl acetate for one minute. Examination was performed on a Jeol JEM-1400 (Jeol Ltd, Tokyo, Japan) TEM equipped with a Gatan Orius Camera. For cryoEM, concentrated samples were laid on a HoleyCarbon TEM grid, blotted to a thin film and plunged into liquid ethane-N2 (l) in the Leica EM GP cryoworkstation (Leica, Wetzlar, Germany). Grids were transferred to a 626 Gatan cryoholder and maintained at −178°C. TEM examination was performed on a JEOL 2011 TEM (Jeol Ltd, Tokyo, Japan) equipped

with a Gatan Ultrascan ES1000 CCD Camera. Size and morphology distribution from images was assessed using the ImageJ software (NIH).

4.11. WESTERN BLOT

Magnetic beads coupled to the EVs and resuspended in lysis buffer (20Mm Tris-HCl pH 7.5, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton X-100) were sonicated for 30 seconds, incubated on ice for 30 minutes, mixed with loading buffer, heated at 70°C for 10minutes, and separated on 12% 1.5 mm SDS-PAGE gels. Proteins were then transferred to nitrocellulose membranes (Amersham Protran, catalog number 10600008) and incubated overnight in blocking buffer (1X PBS, 0.1% Tween-20, 5% milk powder). After several washes (1X PBS, 0.1% Tween-20), blots were incubated for 1hour with antibodies CD9, CD81 and CD63 (dilution 1:100). After washings, blots were incubated for 1hour with IRDye® 800CW Goat-anti-Mouse Antibody (dilution 1:15000). All antibodies incubations were performed in a buffer containing 1X PBS, 0.1% Tween-20, 1% milk powder. The signal was detected on a LICOR Odyssey Infrared Imaging System, and images were edited using the ImageJ software (NIH).

4.12. PROTEIN DIGESTION FOR LC-MS/MS

Protein digestion was performed using the PreOmics kit (P.O.00001). Basically, two-thirds of total beads samples coupled to the EVs were resuspended in 20 µL of PreOmics lysis buffer and were incubated for 10 minutes at 1000 revolutions per minute (rpm) and 70 ºC. The one-third remaining was used to characterize the EVs by western blot. Then, EVs lysates were loaded in the cartridges, and 20 µL of the PreOmics digestion solution was added to each sample. After incubating for three hours at 37 ºC, the enzyme reaction was stopped, several washes were performed, and the peptides were eluted with the elution buffer provided by the kit. Finally, peptides were completely dried in the speed-vac (45ºC).

4.13. LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS/MS) PERFORMED AT EL PASO UNIVERSITY

After protein digestion, plasma-EV samples from patients were cleaned prior to LC-MS/MS analysis by Phoenix Peptide cleanup kit (PreOmics) according to the manufacturer's instructions. Before peptide cleanup, samples were re-suspended in 50 µl of water and 50 µl of acidic stop solution from the Preomics Phoenix kit. After cleanup protocol, samples were dried in a speed vacuum concentrator (45˚C, until completely dry) and re-suspended in 10 µL (0.1% formic acid, 4% acetonitrile) and protein concentration was further measured by a NanoDrop One/One Microvolume UV-Vis Spectrophotometer (Thermo Scientific). Injection volumes were then

adjusted for a 2 µg injection on column. Peptides were loaded onto an Acclaim PepmapTM 100 C18 HPLC nano column (75µm x 25cm, nanoViper, Thermo Scientific, PN 164569) with a Thermo Scientific Dionex Ultimate 3000 RSLCnano System at 4% solvent B (100% LCMS grade acetonitrile, 0.1% formic acid), 96% solvent A (0.1% formic acid, 100% LCMS grade water). Loaded peptides were washed with 4% solvent B for 2 minutes, 5% solvent B for an additional 1 minute, and 8% solvent B for 5 minutes at 0.5 µl/min before beginning the elution gradient. The elution gradient began at 8.1 minutes at an adjusted flow rate of 0.3 µl/min and increased solvent B to 24% at 58 minutes. Solvent B was then increased to 36% at 61 minutes, then to 85% solvent B at 61.5 minutes with an adjusted flow rate at 0.5 µl/min. The 85% acetonitrile wash was maintained until 65 minutes with a sharp decrease to minute 65.1 of 4% solvent B and a flow rate maintained at 0.5 µl/min. The column was equilibrated for the next injection at 4% solvent B until the end of the run at minute 75. Eluted peptides were ionized with a nanoESI FossilIonTech emitter (customized 10cm length, 20 µm internal diameter, 365 µm outer diameter, PN 20-10) attached to a Nanospray Flex Ion Source (Thermo Scientific) and analyzed with a Q-Exactive Plus Hybrid Quadrupole Mass Spectrometer (ThermoFisher Scientific) in positive mode, as a default charge state of 2. The mass spectrometer was set to Top 10 data-dependent MS2 with Full MS parameters set to 70,000 resolution, at a 375 to 1500 m/z scan range, maximum IT at 30ms, with an auto gain control (AGC) target at 1e6. MS2 parameters were set to 17,500 resolution, maximum IT at 50ms, AGC target 5e4, with an isolation window of 2.4 m/z, and Ions were fragmented with NCE set to 27. Unassigned, 2, 6-8 and >8 charges were excluded. Column was kept at 55˚C during analysis. Two technical replicates were analyzed, and one blank sample (4% acetonitrile, 0.1% formic acid) was processed between each biological sample to prevent peptide carry-over from sample to sample.

4.14. BIOINFORMATICS

Proteome Discover (PD) 2.5.0.400 (Thermo Fisher Scientific) was utilized to process the raw files from the LC-MS/MS analyses. Database form *Trypanosoma cruzi* (78,683 sequences) and Homo sapiens (171,131 sequences) was downloaded from UniProtKB (http://www.uniprot.org/) on 24 June, 2021. A contaminant data set composed of trypsin autolysis fragments, keratins, common contaminants found in the CRAPome (296) repository and in- house contaminants were run in parallel. The following PD analysis parameters were used: 1% False Discovery Rate (FDR), higher-energy collisional dissociation (HCD), MS/MS, fully tryptic peptides only, up to 1 missed cleavage, a parent-ion mass of 10 ppm (monoisotopic); fragment mass tolerance of 0.6 Da (in Sequest) and 0.02 Da (in PD 2.1.1.21) (monoisotopic). For identification, 1-2 high confidence peptides were used per protein. Scaffold Q+ 4.8.2 (Proteome Software, Portland, OR) was used

to quantify proteins in the PD dataset. Protein validation required a protein threshold of 99%, a peptide threshold of 95%, and a minimum of two peptides.

4.15. CARDIAC CELLS CULTURE

In vitro studies using human cardiac myocytes (HCM) and human cardiac fibroblasts (HCF) have been done to study the cargo of EVs secreted by *T. cruzi* infected and uninfected cardiac cells. HCM (Promocell, C-12810) were cultured in myocyte growth culture medium supplemented with 10% Fetal Bovine Serum (FBS), 5% supplement myocyte growth media and 1% Penicillin/Streptomycin (PS). EV- depleted media was prepared by supplementing myocyte growth culture medium with 2% FBS, 5% supplement myocyte growth media and 1% PS and performing an overnight UC at 120.000 g in a Thermo Sorvall WV Ultra 100 centrifuge (rotor TH-641). HCF (Promocell, C-12375) were cultured in Dulbecco Modified Eagles Minimal Essential Medium (DMEM) F-12 culture medium supplemented with 10% FBS, 5% Smooth Muscle Growth Complement (SMGC) and 1% PS. Similarly, EV- depleted media was prepared by supplementing DMEM F-12 with 2% FBS, 5% SMGC and 1% PS and performing an overnight UC in the conditions previously described for HCM. In all cases, plates were previously coated with a solution of gelatin/water (dilution 1:10).

4.16. CARDIAC CELLS CHARACTERIZATION

Cardiac cells were characterized by flow cytometry using the following antibodies and dilutions: CD90-PE/Cy7 (Biolegend) (dilution 1:100); CD54/ICAM1-AlexaFlour488 (Biolegend) (dilution 1:400); CD9 (VJ1/20 Immunostep, 9PU-01MG) (dilution 1:500), and CD81 mouse monoclonal Santa Cruz Biotechnology (Sc-23962) (dilution 1:500). For CD9 and CD81 markers, a secondary antibody anti-mouse IgG conjugated to FITC (Southern Biotech, 1032-02) was used at 1:100 dilution. Labelled cells were acquired in a LSR-Fortessa cytometry (BD), and Flow Jo software was used to analyze the data. Unstained HCF and HCM, HCF and HCM incubated with the secondary antibody, and human spleen fibroblasts were used as a control.

4.17. *T. cruzi* PARASITES AND INFECTION OF CARDIAC CELLS

T. cruzi parasites from CL Brener strain were maintained using LLC-MK2 cells as hosts in DMEM supplemented with 2% FBS and 1% PS. Free-swimming trypomastigotes were purified as previously described to keep the parasite cycle in LLC-MK2 cells and for the infection of the cardiac cells (297). For HCF and HCM *T. cruzi* infection, parasites were added to 60% confluent cardiac cells at multiplicity of infection 1. The day after the infection, several washes with

uncompleted media were performed to eliminate remaining trypomastigotes and synchronize the cell culture, and fresh EV depleted media was added.

4.18. ISOLATION OF EVS FROM *T. cruzi* INFECTED CARDIAC CELLS

Supernatants of infected cells were collected at day four post-infection, when most *T. cruzi* parasites are found in amastigote nets. In parallel, SNs of uninfected cells were also collected at day 4. In all cases, the SNs were centrifuged at 500 g for 10 minutes at 4°, at 2000 g for 15 minutes at 4°C, and ultracentrifuged at 120.000 g for four hours at 4°C in a Thermo Sorvall WV Ultra 100 centrifuge (rotor TH-641). The pellets, enriched in EVS, were resuspended in PBS and kept at -80°C until further analysis. These samples were used as starting material to further isolate the EVs by immunoaffinity capture.

4.19. EVS ISOLATION FROM TRYPOMASTIGOTES DERIVED FROM *T. cruzi* INFECTED CARDIAC CELLS

At day 7 or 8 post-infection, when free CL Brener trypomastigote forms were detected in culture SNs, SNs were centrifuged at 2500 rpm for 15 minutes, and *T. cruzi* trypomastigotes were washed in phosphate-buffered saline and incubated for 6 hours in free media at 37°C, 5% CO2 for EVs release. The parasites were removed by centrifugation (2500 rpm for 15 minutes), and the SNs containing trypomastigotes EVs were centrifuged at 500 g for 10 minutes at 4°, at 2000 g for 15 minutes at 4°C, and ultracentrifuged at 120.000 g for four hours at 4°C in a Thermo Sorvall WV Ultra 100 centrifuge (rotor TH-641).

4.20. LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS/MS) PERFORMED AT THE CENTER FOR GENOMIC RESEARCH

The proteomic run of EVs secreted by cardiac cells (infected and uninfected HCF and HCM) and *T. cruzi* trypomastigotes was performed at the Center for Genomic Research (Barcelona, Spain). After protein digestion, data was acquired using a LTQ-Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75 µm, packed with 2 µm C18 particles spectrometer (Thermo Scientific). Chromatographic gradients started at 95% buffer A (0.1% formic acid in water) and 5% buffer B (0.1% formic acid in 80% acetonitrile) with a flow rate of 300 nl/min for 5 minutes and gradually increased to 25% buffer B and 75% A in 105 min and then to 40% buffer B and 60% A in 15 min. After each analysis, the column was washed for 10 min with 10% buffer A and 90% buffer B. The mass spectrometer was operated in

positive ionization mode with nanospray voltage set at 2.4 kV and source temperature at 305°C. The instrument was operated in data-independent acquisition mode, with a full MS scans over a mass range of m/z 400-1000 with detection in the Orbitrap at a resolution of 60,000. The AGC was set to 4e5 and a maximum injection time of 100ms was used. In each cycle of data-independent acquisition analysis, following each survey scan, 30 consecutive windows of 20 Da each were used to isolate and fragment all precursor ions from 400 to 1000 m/z. A normalized stepped collision energy of 23%, 28% and 33% was used for HCD fragmentation. MS2 scan range was set from 350 to 1850 m/z with detection in the Orbitrap at a resolution of 30,000. The AGC was set to 2E5 and a maximum injection time of 60 ms was used. Digested bovine serum albumin (New England Biolabs cat # P8108S) was analyzed between each sample to avoid sample carryover and to assure stability of the instrument and QCloud has been used to control instrument longitudinal performance during the project.

4.21. ANGIOGENIC ASSAY USING HUVEC CELLS

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Endothelial Cell Growth Medium-2 (H3CC-3162, Lonza, Cultek) supplemented with 10% FBS exosome-depleted and 1% PS. To determine angiogenic growth, HUVEC cells were incubated with 50 ng of EVs isolated from infected and uninfected HCM, 50 ng of EVs from CD patients presenting the cardiac form of the disease and 50 ng of EVs from healthy donors for 48 h. Several positive controls (total secretome of patients with a heart attack or presenting angina pectoris that showed positive results for angiogenic growth in previous experiments) were also included. 48h later, a Geltrex Matrix was prepared in µ-slide angiogenesis plates. After polymerization, 50 µL of the cell suspension were added to each well. Angiogenic growth images were taken four hours after seeding. Image capture was performed using an inverted microscope equipped with a camera (5x objective; Axiovet 200, Zeiss, Carl Zeiss Iberia, SL, Madrid). Angiogenesis capacity was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA), through the analysis of the number of cell junctions, branches and meshes.

5. RESULTS

5.1. IDENTIFICATION OF EVS BIOMARKERS IN PLASMA OF DIFFERENT CLINICAL GROUPS OF CHRONIC *T. cruzi* INFECTED PATIENTS

5.1.1. Cohort characterization

To evaluate the potential of EVs as biomarkers for CD, we collected samples from a cohort of *T. cruzi* infected patients and healthy individuals. This cohort included *T. cruzi*-seropositive individuals presenting the indeterminate form of the disease (G1, n=18), *T. cruzi*-seropositive individuals presenting cardiomyopathy (G2, n=27), and two control groups consisting of *T. cruzi*-seronegative individuals presenting cardiomyopathy (G3, n=11), and *T. cruzi*-seronegative individuals without symptoms (G4, n=21) (Table 7). G1, G2 and G4 patients were originally from Latin American countries, where CD is endemic, while G3 patients were mostly originally from Europe. Information regarding the clinical status and demographic data of the patients can be found in Annex 5.

Table 7. Summary of the clinical groups included in our study.

CLINICAL GROUP	Group	N	SEROLOGY	PCR POSITIVE	RECRUITMENT
T. cruzi infection, indeterminate	G1	18	Positive	4	International Health Service-Hospital Clínic, Barcelona
CD, cardiac form	G2	27	Positive	15	International Health Service-Hospital Clínic, Barcelona and Fundación CEADES, Bolivia
Non *T. cruzi* infection, cardiac pathology	G3	11	Negative	-	International Health Service-Hospital Clínic, Barcelona and Cardiology-Hospital Clínic
Healthy individuals	G4	21	Negative	-	International Health Service-Hospital Clínic, Barcelona

Plasma, total blood, saliva and urine were collected from all study participants. However, the first approach to validate our hypothesis has been done using EVs isolated from plasma samples. For *T. cruzi* infected individuals (G1, G2), plasma derived-EVs were purified before and at the end of the treatment, following the study protocol. In addition, samples at six and 12 months post-treatment were also collected, but due to logistical problems, they have not been processed and therefore EVs from these samples will be analysed in future studies.

The age of patients in G1 (mean 51.3) and G3 (mean 54.1) was significantly different to that of patients in G2 (mean 43.4) and healthy volunteers in G4 (mean 40.2). This difference is particularly surprising for individuals in G1 (presenting the indeterminate form of the disease)

and G2 (presenting the cardiac form): G1 patients, which have not developed the disease, are older than individuals from group G2. One potential explanation of the age distribution in G2 is that most of the individuals of this group of the cohort were recruited in Bolivia, in collaboration with CEADES Foundation. The range of people attending the Chagas platform is wider, giving that it is a healthcare center in a highly endemic area for *T. cruzi* infection. On the contrary, individuals belonging to G1 were migrant people coming mainly from Bolivia, and in this case, the range of age of this specific population is shorter and higher in average.

Regarding gender distribution, G1, G2 and G4 were mostly composed of females, whereas in G3 the distribution was very similar for both genders.

We did not observe significant differences concerning the percentage of patients presenting comorbidities between G1 (33.3%) and G2 (37%). A low number of comorbidities was observed in the cohort of healthy individuals (14.3%), while non cardiac patients suffering cardiac disease exhibited the higher percentage (81.8%).

A *T. cruzi* PCR test was offered to all *T. cruzi* infected participants involved in the study (G1 and G2). Out of the total patients presenting the indeterminate form of the disease (G1), 22.2% were PCR positive before treatment. Curiously, this figure is much higher for *T. cruzi* infected individuals presenting the cardiac form of the disease (75% of the total patients having a PCR test).

5.1.2. First proteomic characterization of plasma-derived EVs from a heart transplant patient with chronic CD

To preliminarily determine whether circulating EVs from *T. cruzi* infected patients can be used as predictive biomarkers to evaluate therapeutic response and disease outcome, we described in a case report (298) the first proteomic profiling of plasma-derived EVs purified directly from a heart transplant patient with chronic Chagas disease who exhibited reactivation after immunosuppression (patient EVCARD20). We identified human and parasite proteins present or upregulated in plasma-derived EVs from a chronic Chagas disease patient before chemotherapy that are absent or downregulated after treatment.

In 2009, a 51-year-old patient from Bolivia with a history of chronic Chagas disease, exhibiting severe organ involvement (chronic cardiomyopathy Kuschnir III and megacolon and megaesophagus degree IV) (64), was admitted to the International Health Department (Hospital Clinic, Barcelona). Serologic diagnosis for chronic Chagas disease was performed using 2 ELISA kits (Ortho-Clinical Diagnostics, https://www.orthoclinicaldiagnostics.com) and BioELISA Chagas. Together with clinical management of dysphagia and constipation, a pacemaker in the context of

third-degree atrioventricular block was implanted. In July 2015, an echocardiogram revealed iterative cardiac failure and severe ventricular dysfunction (ejection fraction 15%–20%). On November 28th, 2015, the patient underwent heart transplantation without incident, and results of follow-up endomyocardial biopsies showed no early signs of transplant rejection.

After transplantation and in the context of immunosuppression therapy (Table 8), qPCR was performed weekly to detect *T. cruzi* in the blood. First BZD treatment was started when several consecutive positive qPCRs confirmed CD reactivation with an increasing parasitemia. Three weeks after BZD treatment, the qPCR became negative. After completion of 80% of the treatment, the BZD course was interrupted due to pancytopenia and liver dysfunction. Then, the qPCR became positive and a second BZD course was initiated, completing this time the total prescribed dose without evidence of therapeutic failure based on qPCR results. Plasma samples for purification and characterization of EVs were collected before the first BZD treatment and just after the second course (Table 8). Unexpectedly, the patient died in August 2016.

Table 8. Timeline of heart transplant patient with chronic CD from initial diagnosis to last follow-up and death.

Date	Infection	Observation, treatment, outcome
2015 Aug	Cytomegalovirus, detected by serology	Diagnosed only by positive IgG serology, no active infection (no positive IgM serology). No treatment.
2015 Aug	Toxoplasmosis, detected by serology	Diagnosed only by positive IgG serology, no active infection (no positive IgM serology). No treatment.
2015 Nov		Heart transplantation on Nov. 28. Patient started with immunosuppressive therapy (tacrolimus, azathioprine, prednisone) until the end of follow-up.
2016 Jan	Chagas disease reactivation, detection by qPCR	Pretreatment sample collected on Jan 28. Patient started BZN treatment (2.5 mg/kg, twice a day, 60 d) on Feb 3.
2016 Mar	Bronchopulmonary aspergillosis, detected by serology and CT	BZN course interrupted on Mar 21. Completed 80% of the prescribed treatment.
2016 Mar	Bronchopulmonary aspergillosis	Aspergillosis treatment started on Mar 22. Initially with voriconazole and amphotericin B liposomal. Treatment was changed to posaconazole until the end of the follow-up.†
2016 Apr	Chagas disease reactivation, detected by qPCR	On Apr 14, patient started second round of BZN treatment until May 5, completing 100% of the prescribed treatment.
2016 May		Posttreatment sample collected on May 11.
2016 Aug		Late organ rejection. Patient died.

To determine whether circulating EVs from this patient could have been used as predictive biomarkers to evaluate therapeutic response and disease outcome in the Chagas disease context, we collected pre-treatment and post-treatment plasma samples, and EVs were enriched by SEC and characterized as described (Figure 21A). As negative controls, plasma samples from two healthy donors were also subjected to SEC. We characterized eluting EVs by BBA and NTA (Figure 21B and C). We pooled aliquots (100 µL) from SEC fractions 7–10 and determined protein composition using LC-MS/MS (Proteomic Unit, El Paso University, USA). Using a FDR <1% and 2 UP normalized per protein, we detected only one *T. cruzi* protein (PPDK), and 288 human proteins.

Among the 288 human proteins detected, 19 of them were only identified in pre-treatment samples (Table 9, Figure 22). Moreover, of the total proteins identified in which

statistical analysis was feasible, four were significantly upregulated in patient-derived EVs before treatment, particularly the proteins complement C1s subcomponent, isoform CRA_b, FLJ00385 protein, and cDNA FLJ75416. The complete LC-MS/MS proteomic data can be found in the PRoteomics IDEntifications Database (PRIDE, https://www.ebi.ac.uk/pride/) partner repository, with the dataset identifier PXD014668 and 10.6019/PXD014668.

The identification of one *T. cruzi* protein, together with the 23 human proteins uniquely identified or significantly upregulated in EVs from the patient with chronic CD before treatment, allowed us to hypothesize that EV proteins released by the host or parasite during infection might be potential biomarker candidates for evaluating therapeutic response and disease outcome in chronic CD.

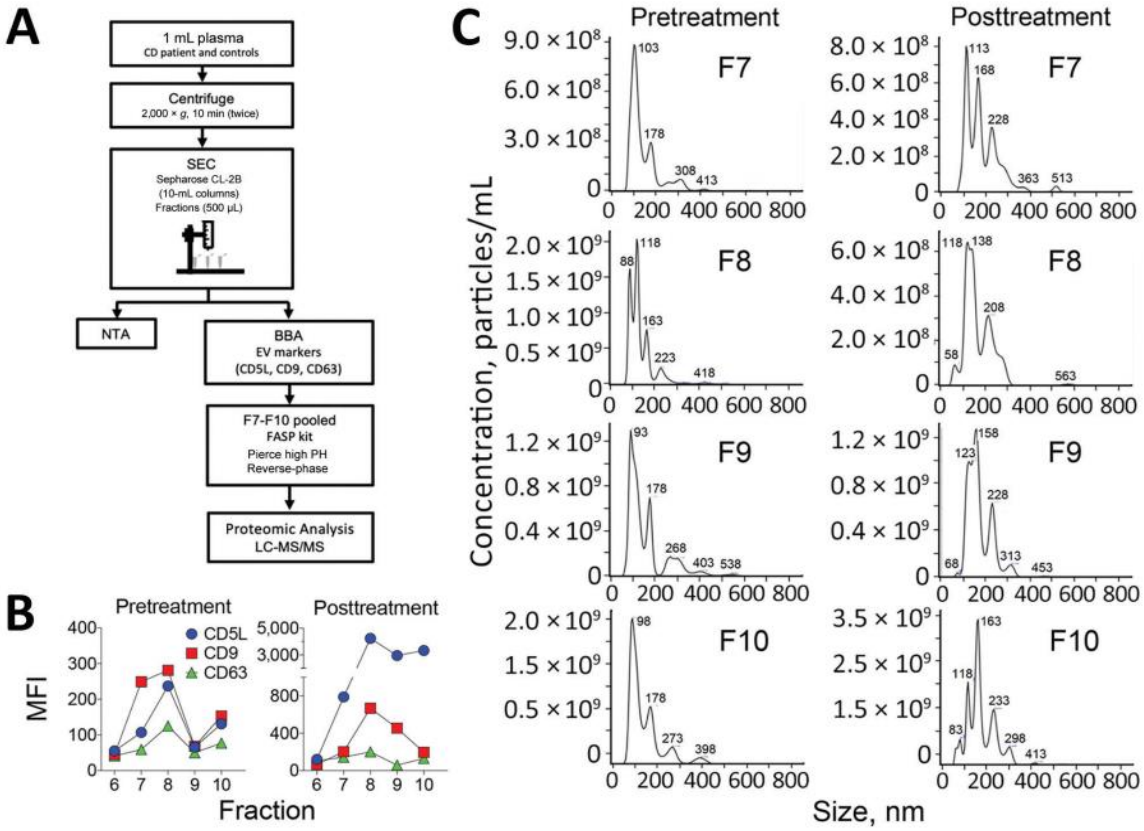


Figure 21. Isolation and characterization of plasma-derived EVs. (A) Schematic diagram of the isolation and characterization of EVs derived from plasma samples. (B) EVs were characterized by BBA using the classical EV markers CD5L, CD9, and CD63. (C) NTA of SEC fractions F7–10.

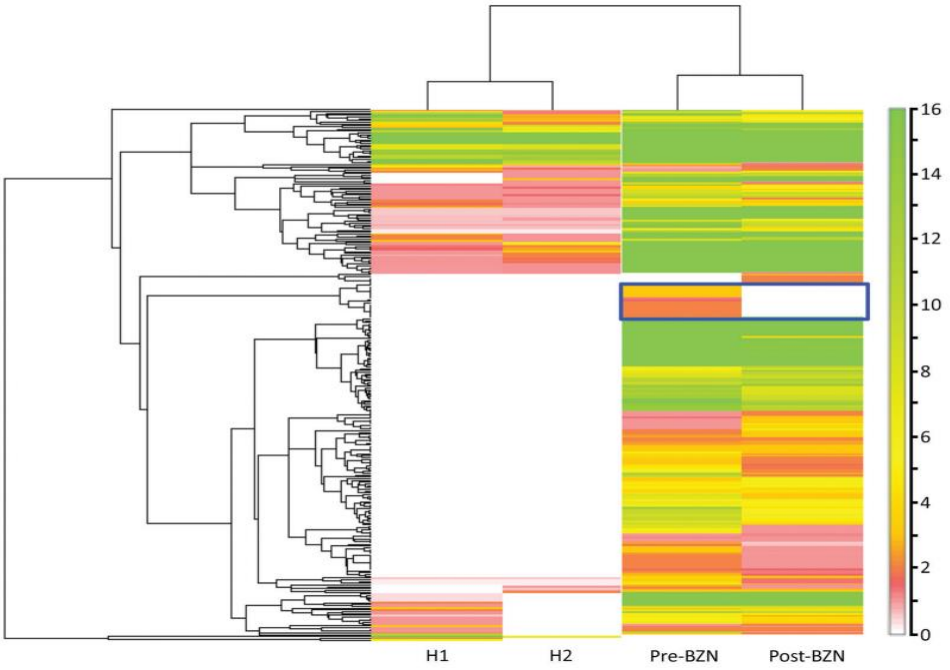


Figure 22. Human proteomic profile of plasma-derived EVs from a heart transplant patient with chronic CD before and after BZD treatment and from two healthy donors. Heatmap of the identified human EV-derived proteins. Scale bar indicates intensity.

Table 9. *T. cruzi* and human proteins identified in plasma-derived EVs from a heart transplant patient with chronic CD before BZD chemotherapy but absent after treatment and in healthy donors. All proteins were identified by at least 2 UP. ‡Normalized total spectrum count values are indicated in parenthesis. ChD: Chagas Disease; BZN: Benznidazole

		Unique peptides			
Protein name†	UniProt accession no.	ChD Pre-BZN‡	ChD Post-BZN	Healthy 1	Healthy 2
T. cruzi					
Pyruvate. phosphate dikinase OS = Trypanosoma cruzi marinkellei GN = MOQ_000480 PE = 3 SV = 1	K2MVM1_TRYCR	2 (0.96)	0	0	0
Homo sapiens					
Collagen α-1(VI) chain OS = Homo sapiens GN = COL6A1 PE = 1 SV = 3	CO6A1_HUMAN	3 (1.44)	0	0	0
Group of Angiopoietin-related protein 6 OS = Homo sapiens GN = ANGPTL6 PE = 1 SV = 1+1	ANGL6_HUMAN (+1)	3 (1.44)	0	0	0
sp|PPIA_HUMAN|	sp|PPIA_HUMAN|	3 (1.44)	0	0	0
Mannan binding lectin serine protease 2 OS = Homo sapiens GN = MASP2 PE = 1 SV = 4	MASP2_HUMAN	2 (1.92)	0	0	0
Myosin regulatory light chain 12B OS = Homo sapiens GN = MYL12B PE = 1 SV = 2	ML12B_HUMAN	2 (1.92)	0	0	0
Collagen α-2(VI) chain OS = Homo sapiens GN = COL6A2 PE = 1 SV = 4	CO6A2_HUMAN	2 (1.44)	0	0	0
Collectin subfamily member 10 (C-type lectin). isoform CRA_a OS = Homo sapiens GN = COLEC10 PE = 4 SV = 1	tr|A0A024R9J3|A0A024R9J3_HUMAN	2 (1.44)	0	0	0
Group of Coagulation factor XIII A chain OS = Homo sapiens GN = F13A1 PE = 1 SV = 4+2	F13A_HUMAN (+2)	2 (1.44)	0	0	0
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. eta polypeptide. isoform CRA_b OS = Homo sapiens GN = YWHAH PE = 3 SV = 1	tr|A0A024R1K7|A0A024R1K7_HUMAN	2 (1.44)	0	0	0
Fibrinogen-like protein 1 OS = Homo sapiens GN = FGL1 PE = 1 SV = 3	FGL1_HUMAN	2 (0.96)	0	0	0
Group of L-lactate dehydrogenase A chain OS = Homo sapiens GN = LDHA PE = 1 SV = 2+1	LDHA_HUMAN (+1)	2 (0.96)	0	0	0
Group of Laminin subunit α-2 OS = Homo sapiens GN = LAMA2 PE = 1 SV = 1+1	A0A087WX80_HUMAN (+1)	2 (0.96)	0	0	0
Group of MHC class I antigen (Fragment) OS = Homo sapiens GN = HLA-A PE = 3 SV = 1+3	tr|D2KZ27|D2KZ27_HUMAN (+3)	2 (0.96)	0	0	0
Group of Serum amyloid A protein OS = Homo sapiens GN = SAA1 PE = 1 SV = 1+2	E9PQD6_HUMAN (+2)	2 (0.96)	0	0	0
Group of Transforming growth factor β-induced 68kDa isoform 2 (Fragment) OS = Homo sapiens GN = TGFBI PE = 2 SV = 1+1	tr|A0A0S2Z4K6|A0A0S2Z4K6_HUMAN (+1)	2 (0.96)	0	0	0
Heparan sulfate proteoglycan 2 (Perlecan). isoform CRA_b OS = Homo sapiens GN = HSPG2 PE = 4 SV = 1	tr|A0A024RAB6|A0A024RAB6_HUMAN	2 (0.96)	0	0	0
Neurogenic locus notch homologue protein 3 OS = Homo sapiens GN = NOTCH3 PE = 1 SV = 2	NOTC3_HUMAN	2 (0.96)	0	0	0
V1–16 protein (Fragment) OS = Homo sapiens GN = V1–16 PE = 4 SV = 1	tr|Q5NV81|Q5NV81_HUMAN	2 (2.88)	0	0	0
Rheumatoid factor RF-ET6 (Fragment) OS = Homo sapiens PE = 2 SV = 1	tr|A2J1N5|A2J1N5_HUMAN	2 (5.29)	0	0	0

5.1.3. Molecular characterization of plasma-derived EVs isolated by size exclusion chromatography from a sub-group of Chagas disease patients

The identification of parasite and human proteins present or upregulated in EVs isolated from plasma samples of a particular CD patient before treatment that disappear or were downregulated after treatment were the first proof-of-principle of the potential of this approach to discover new biomarkers. However, these observations derived from the analysis of a single clinical case. Moreover, this patient presented particular clinical characteristics: he presented a severe cardiomyopathy, heart transplantation, megacolon, megaesophagus, and suffered a reactivation of the infection in the context of the immunosuppression. Thus, further research was needed to validate these results in a larger cohort of individuals.

To further explore the role of plasma EVs from CD patients as potential biomarkers, we applied a new strategy for EVs isolation, adapting the SEC protocol to one mL sepharose handmade columns. The use of this new approach allowed us to load a lower sample volume (100 µL per column), and to obtain a sharper profile, concentrating all EV markers in one fraction when characterizing the EVs by BBA, reducing the number of fractions containing the EVs and therefore the number of possible contaminating plasma proteins (Figure 23). For each sample, four SEC columns of one mL were performed. In this particular example, the standardization was performed using the sample of the healthy patient H16.

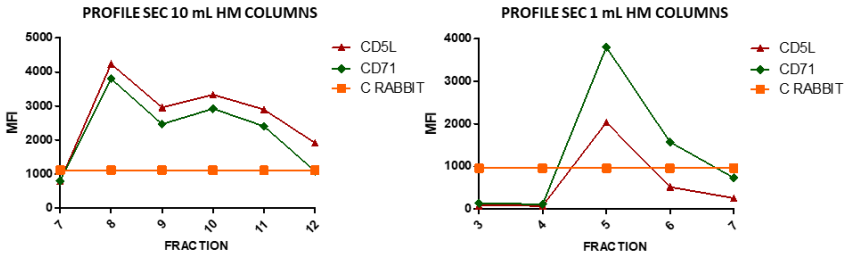


Figure 23. Isolation of EVs from plasma of a *T. cruzi* infected patient by SEC using 10 or one mL handmade columns. **Chromatographic fractions were analyzed by bead-based flow cytometry for the detection of classical EV markers CD71 and CD5L to identify/characterize EV-enriched fractions.**

Once it was determined that the use of one mL columns for SEC purification of EVs was adequate, we proceeded to apply this approach to a larger number of samples with the final goal of identifying parasite and human proteins associated with circulating EVs from natural *T. cruzi* infections. To do so, EVs derived from plasma of three different *T. cruzi* infected patients before and after treatment (G1, samples EVINDET11, EVINDET13, EVINDET16) and two healthy individuals (G4, samples H7, H11) were characterized by BBA and microscopy, and analyzed by

LC-MS/MS. All *T. cruzi* infected individuals presented the indeterminate form of the disease and were PCR negative.

EVs were isolated by SEC as previously described. Due to the expected limited amount of EVs, four different one mL columns were prepared for each sample, and the fractions containing the EVs were pooled. The BBA characterization of these specific samples can be found in

Figure 24.

We also performed cryoEM and TEM of the SEC fractions containing the EVs (F5, enriched in EVs) and the distal fractions of the SEC (F8, enriched in plasma proteins contaminants) of the samples EVINDET11 and EVINDET16 (Figure 25A). Both methodologies demonstrated the presence of highly pure vesicles free of protein aggregates in enriched EVs samples (Figure 25A). In contrast, an aggregated material can be observed in SEC distal fractions, where soluble plasma proteins are eluted (Figure 25A).

Using the cry-EM methodology, we also performed a detailed morphological analysis of the EVs present in our samples, observing a high degree of diversity. Adapting the system proposed by Zabeo and collaborators (299), the EVs were classified into seven different categories according to their size and shape: single, double, triple, tubule, oval, pleomorphic, and incomplete. Single vesicles, presenting a round shape, represented the majority of all counted vesicles (56.8%). Double vesicles, containing a smaller vesicle enclosed in a larger one, were the second ones in abundance, representing the 23.3% of the total counted EVs. Triple vesicles, presenting two or more vesicles inside a larger one, were also found (8.3% of the total population). Less abundant were oval vesicles (similar to single vesicles but elongated in shape, 6% of the total EVs), tubule vesicles (presenting a tubular shape, 3% of the EVs), and incomplete EVs (showing an interrupted membrane, 0.8% of the total population). Pleomorphic EVs, which were considered all vesicles whose shape could not be classified in any of the previous categories, represented 1.9% of the total EVs (Figure 25B).

Regarding the size of the vesicles found in our preparations, most of the EVs presented a diameter of 0 to 100 nm and 100 to 200 nm (41.2% and 42.4%, respectively). These results were consistent with previous NTA findings, which showed that the mean vesicles size was of 163.5 nm of diameter. Out of the total EVs, 9.1% presented a size from 200 to 300 nm. Larger EVs were clearly less abundant in our preparation: only 3.6% of the EVs presented a diameter from 300 to 400 nm, 1.2% 400 to 500 nm, 1.8% 500 to 600 nm, and 0.6% 700 to 800 nm (Figure 25C).

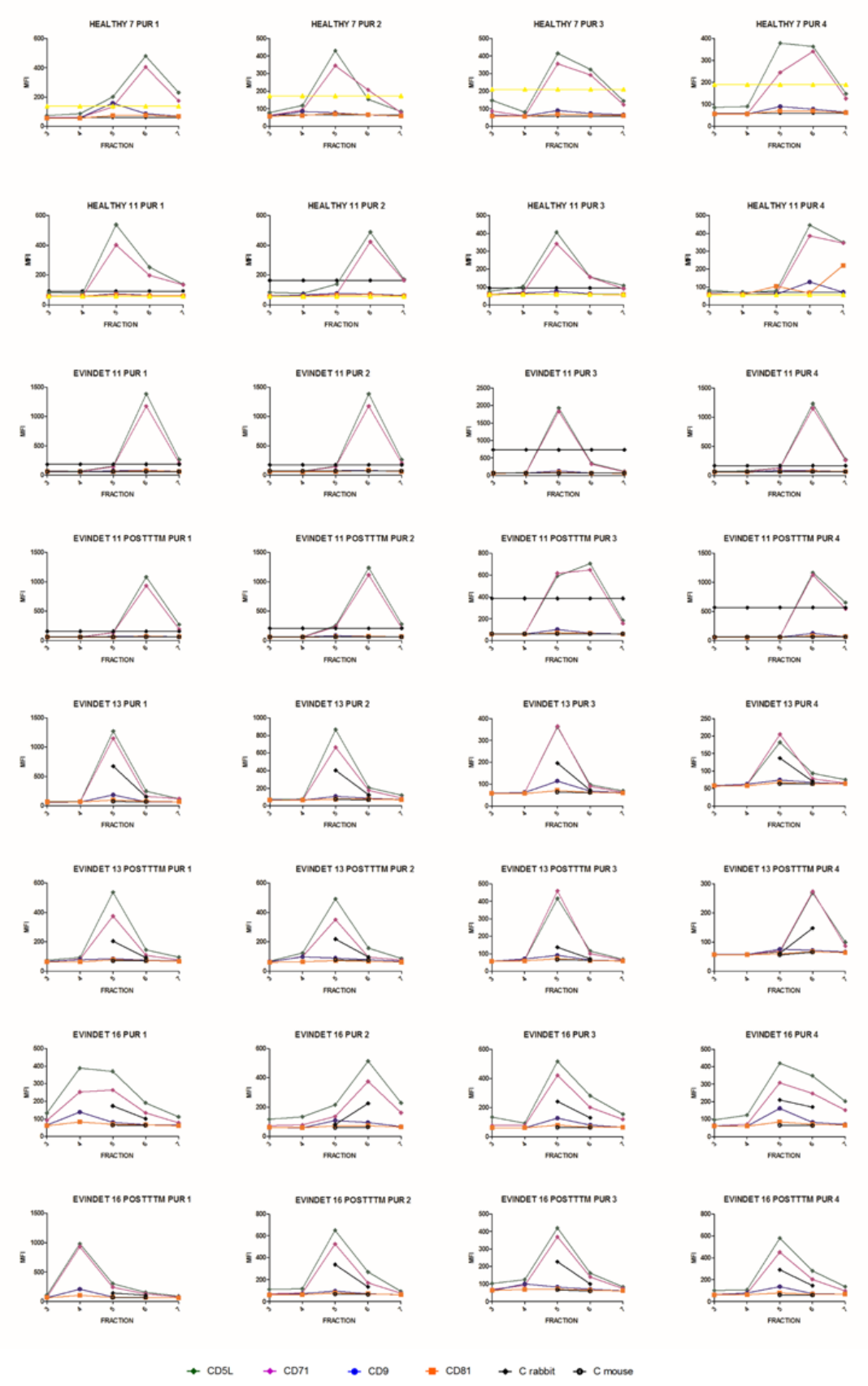


Figure 24. Characterization of EVs from healthy donors and *T. cruzi* infected patients. SEC fractions were analyzed by bead-based flow cytometry for the presence of EV-associated markers such as CD5L, CD71,

CD9, and CD81. Negative controls of EVs-beads incubated with secondary antibodies are also shown. TTM, treatment.

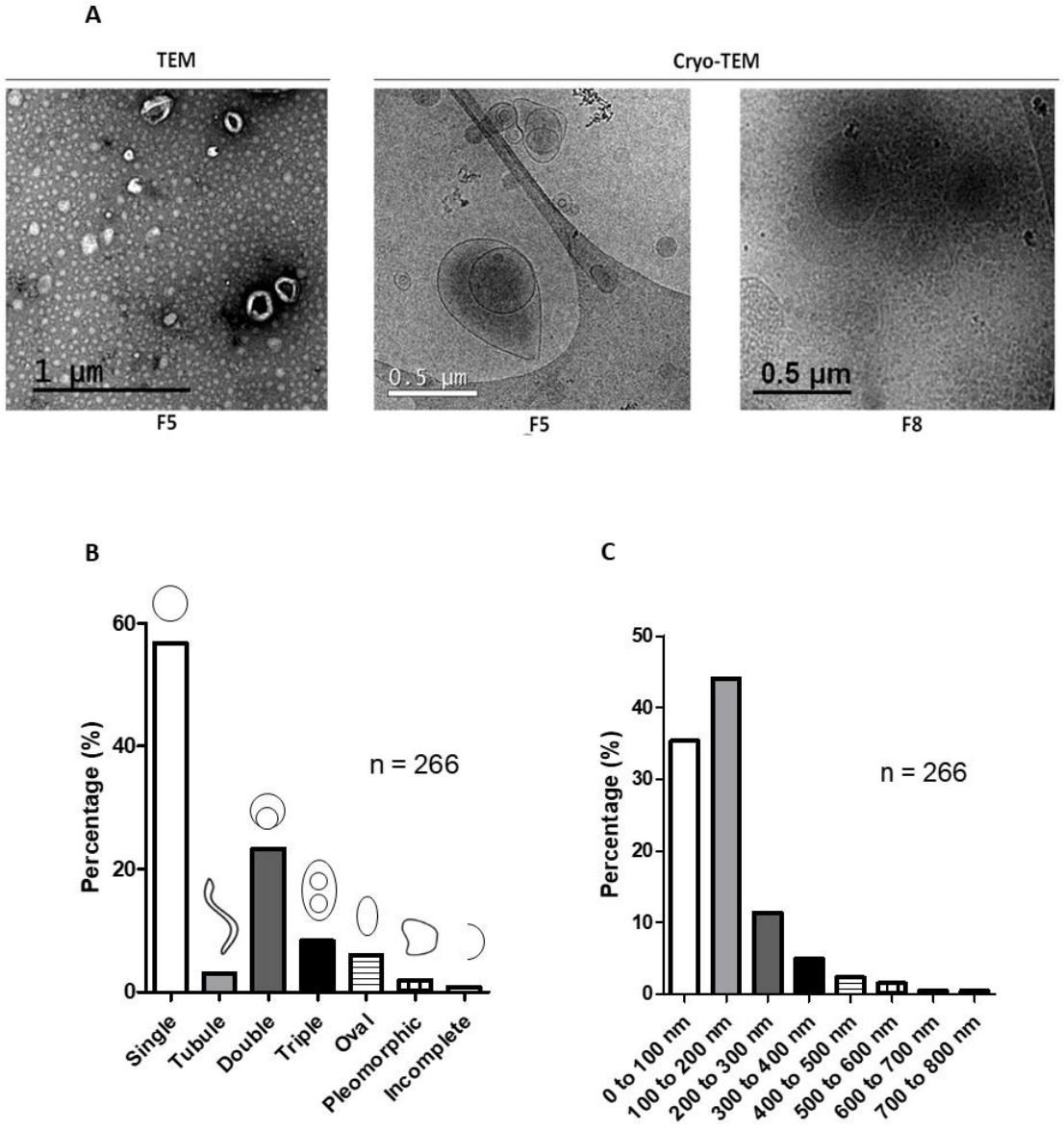


Figure 25. Morphological analysis of shape and size of the EVs isolated by SEC from plasma samples of *T. cruzi* infected individuals. A) Electron microscopy. Representative TEM and cryoEM image of isolated EVs eluted in fraction five of SEC. On the right, representative cryoEM image of the plasma contaminants eluted in fraction eight of SEC (distal fraction in one mL SEC). (B) EVs were classified into seven different categories according to their shape, representing single round vesicles the majority of all counted vesicles. (C) The large majority of the EVs (83.6%) presented a diameter of 0 to 200 nm. Shape and size distribution from cryoEM images was quantified by ImageJ.

To identify parasite and human proteins associated to circulating EVs in CD patients, 0.3 µg of protein from pooled fractions were digested and analyzed by LC-MS/MS (Proteomic Unit, El Paso University, USA). We searched for peptide spectrum matches against human protein

databases, and using a FDR <1%, we detected a total of 341 human proteins. However, when applying a more stringent criterion of at least 2 UP normalized per protein, we detected 274 human proteins. As can be observed in Table 10, the total number of proteins detected was very similar for each sample.

Among the human proteins identified, the large majority were common for the three group of samples (healthy and indeterminate pre-treatment and post-treatment). However, we identified a group of proteins exclusively detected in EV samples from *T. cruzi* infected patients presenting the indeterminate form of the disease before treatment, and *T. cruzi* infected patients presenting the indeterminate form of the disease after treatment, which could be further studied as potential biomarkers of therapeutic response (Figure 26A).

Several EV markers were also detected in EVs from *T. cruzi* infected patients and healthy donors: 18 when taking into account all proteins detected, and 15 when considering only those detected by at least two UP. No differences in the number of EV markers between samples was observed (Table 10, Figure 26B).

Table 10. Total human proteins and EV markers detected in EVs samples from *T. cruzi* infected patients and healthy donors.

	H7	H11	EVINDET11 PRE-TTM	EVINDET11 POST-TTM	EVINDET16 PRE-TTM	EVINDET16 POST-TTM	EVINDET13 PRE-TTM	EVINDET13 POST-TTM
Total human proteins	282	287	287	282	256	308	278	221
Total human proteins (≥ 2UP)	155	158	160	146	188	189	200	132
Total EV markers	15	16	13	10	16	16	15	7
Total EV markers (≥ 2UP)	10	8	4	3	9	11	10	4

Unfortunately, when we searched for peptide spectrum matches against *T. cruzi* protein databases, we were not able to detect parasite proteins in these particular samples. This feature could be explained by the clinical characteristics of the patients, which presented the indeterminate form of the disease and were PCR negative, or by the low amount of circulating EVs derived from infected cells.

To increment the chances of detecting parasite proteins, we decided to perform a new characterization by LC-MS/MS pooling the EVs isolated from plasma samples of CD individuals presenting different forms of the disease (n=8; five patients presenting the indeterminate form

of the disease and three presenting cardiac sympthomatology), and healthy donors (n=10). In this case, all *T. cruzi* infected patients but one were PCR negative. Again, even though classical plasma proteins were found in both preparations, together with several EV markers, no parasite proteins were found (results not shown).

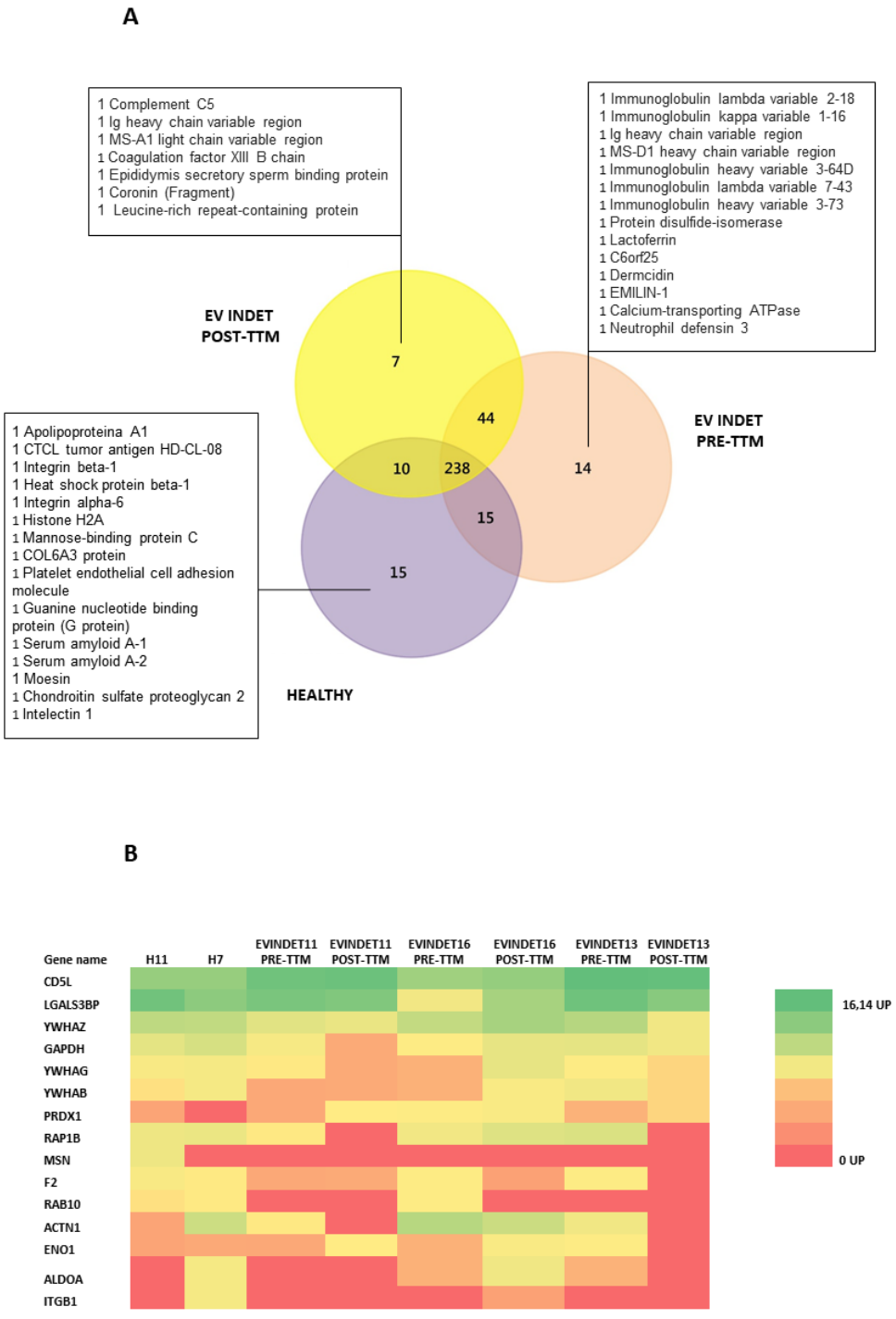


Figure 26. Human proteome of EVs from plasma samples of indeterminate *T. cruzi* infected patients and healthy donors isolated by SEC. (A) Venn diagram showing the overlap of human protein groups detected

in EVs secreted by healthy individuals, individuals presenting the indeterminate form of the disease before treatment and after treatment. (B) EV markers distribution and abundance.

Taking into consideration these results, new approaches to isolate EVs were explored to find proteins with potential as CD biomarkers in the vesicles of our cohort of individuals. In that context, previous results from our group have shown that in *P. vivax* infection, the immunoaffinity capture technique using CD71, one of the main surface markers of reticulocytes-derived EVs, which are the cells preferentially invaded by *P. vivax* parasites, allows the detection of high number of parasite proteins in plasma-derived EVs (251). Thus, the immunoaffinity capture technique could be a promising methodology to apply also in samples of *T. cruzi* infected patients. For this purpose, the identification of specific EV-markers derived from *T. cruzi* infected cells is a major need.

5.1.4. Summary

In this chapter we have presented the first molecular characterization and proteomic profiling of plasma-derived EVs purified by SEC directly from *T. cruzi* infected patients (presenting the cardiac and the indeterminate form of the disease). Although one parasite protein was detected in the pre-treatment sample of the cardiac CD patient, no parasite proteins were identified in individuals presenting the indeterminate form of the disease. Altogether this data encourages us to continue investigating the potential of circulating EVs as biomarkers for CD, exploring alternative approaches to isolate the vesicles, such as the immune-affinity capture.

5.2. IDENTIFICATION OF SURFACES MARKERS FOR IMMUNOAFFINITY CAPTURED EVS DERIVED FROM *T. cruzi* INFECTED HUMAN CARDIAC CELLS

In order to identify specific EV-markers derived from *T. cruzi* infected cells, and knowing that in natural infections one of the main cell target for *T. cruzi* parasites are cardiac cells, we performed the following approach (Figure 27). Commercial HCF and HCM were phenotypically characterized by specific surface markers and subsequently infected with *T. cruzi* (CL-Brener). SNs, containing EVs-derived from infected cells were collected and EVs characterized.

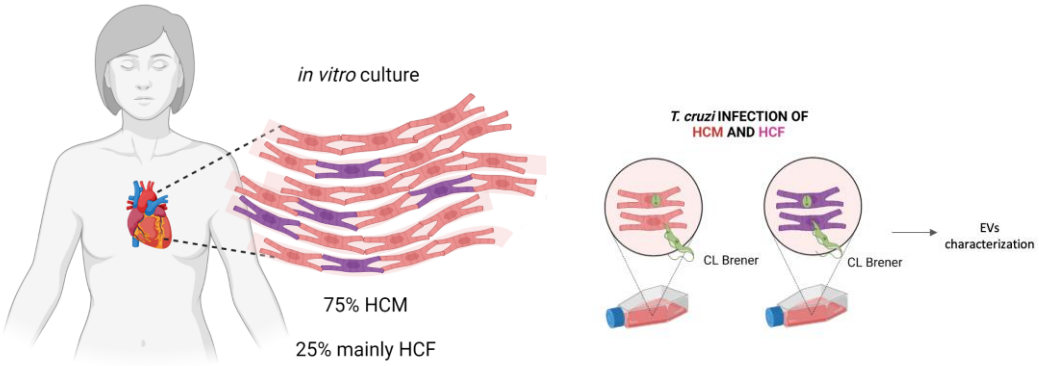


Figure 27. Schematic representation of the approach followed to identify specific EV-markers derived from *T. cruzi* infected cardiac cells. Created with BioRender.com.

5.2.1. Characterization of human cardiac fibroblasts and human cardiomyocytes

We first performed a phenotypic characterization of uninfected HCF and HCM by flow cytometry using CD90, CD54, CD81 and CD9 antibodies (Figure 28). Human spleen fibroblast (HSF) were used as control cells. CD90 and CD54 (also known as ICAM-1) are cell adhesion markers from fibroblasts and mesenchymal cells, and CD9 and CD81 are classical EV markers, present in many types of cells (300–302).

As expected, out of the total HSF, 92.6% expressed CD90, 67.9% CD54, 92.3% CD81 and 85.4% CD9. In HCF, was observed that 63.9% of the cell population expressed CD90, 81.6% CD54, 97.5% CD81, and 83.1% CD9. In HCM, the expression of CD90 and CD54 was lower compared to HCF (35.8% and 41.4%, respectively); the same as for CD81 and CD9, which expression was 74.4% and 80.8%, respectively. As seen in Figure 28, the most expressed EV marker in the cell surface of HCF was CD81, while for CD9 the expression was similar in both type of cardiac cells.

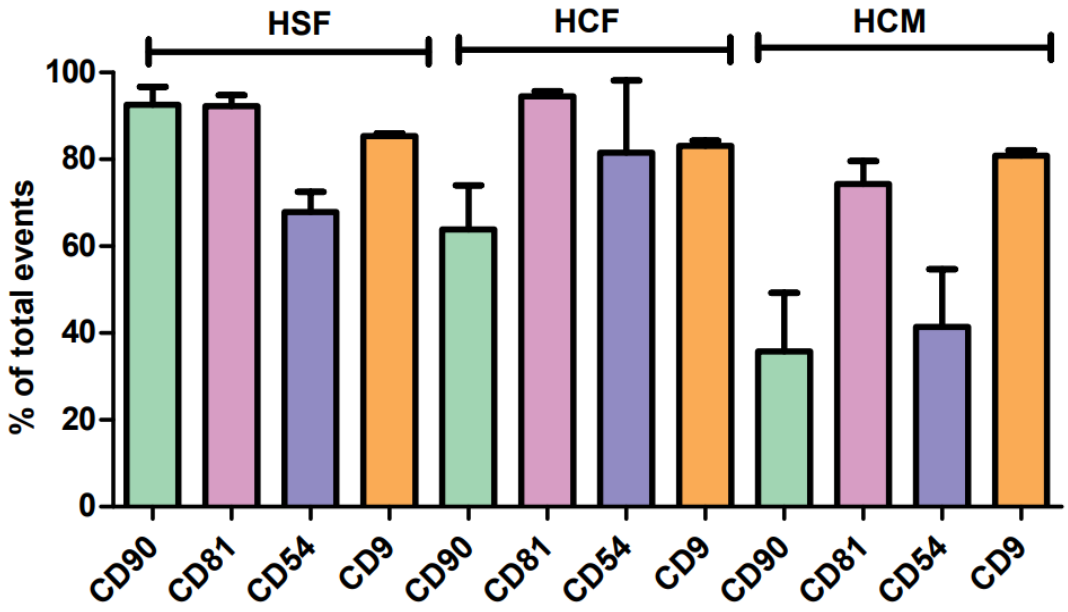


Figure 28. Phenotypic characterization of uninfected HCF and HCM. Antibodies against fibroblasts markers, such as CD90 and CD54, and classical EV markers, such as CD81 and CD9 were used. HSF were used as a positive control. Data shows the percentage of total events positive for each specific marker measured by flow cytometry. Quantification of three technical replicates is shown.

5.2.2. Isolation and characterization of EVs secreted by uninfected and infected human cardiac cells

Once it was determined that cardiac cells expressed the classical markers of EVs on their surface (CD81 and CD9), we proceed to characterize the vesicles released to the culture medium by uninfected and *T. cruzi* infected cells. To do so, this time we performed two consecutive methods for EVs isolation. First, a pre-enrichment of the vesicles present in the SN of the cardiac cells was done by UC, followed by SEC using one mL columns as previously described.

EVs were first characterized by BBA, using anti-CD81, CD9 and CD63 to detect the classical EV markers. As expected, the higher median fluorescence intensity (MFI) values was observed in fractions five or six. Interestingly, EVs secreted by HCF presented higher expression of CD81, while EVs from myocytes presented a higher expression of CD9, in concordance with the results previously described at the cell surface in both cell types (Figure 29A).

We also characterized the EVs secreted by infected and uninfected cells and isolated by SEC by NTA. For HCF, no significant differences were observed in terms of particles concentration; while a tendency of higher particle size was observed in infected cells compared to uninfected (unpaired *t*-test). For HCM, no significant differences were observed in terms of particles concentration or particle size between infected and uninfected cells (unpaired *t*-test) (Figure 29B, C, D).

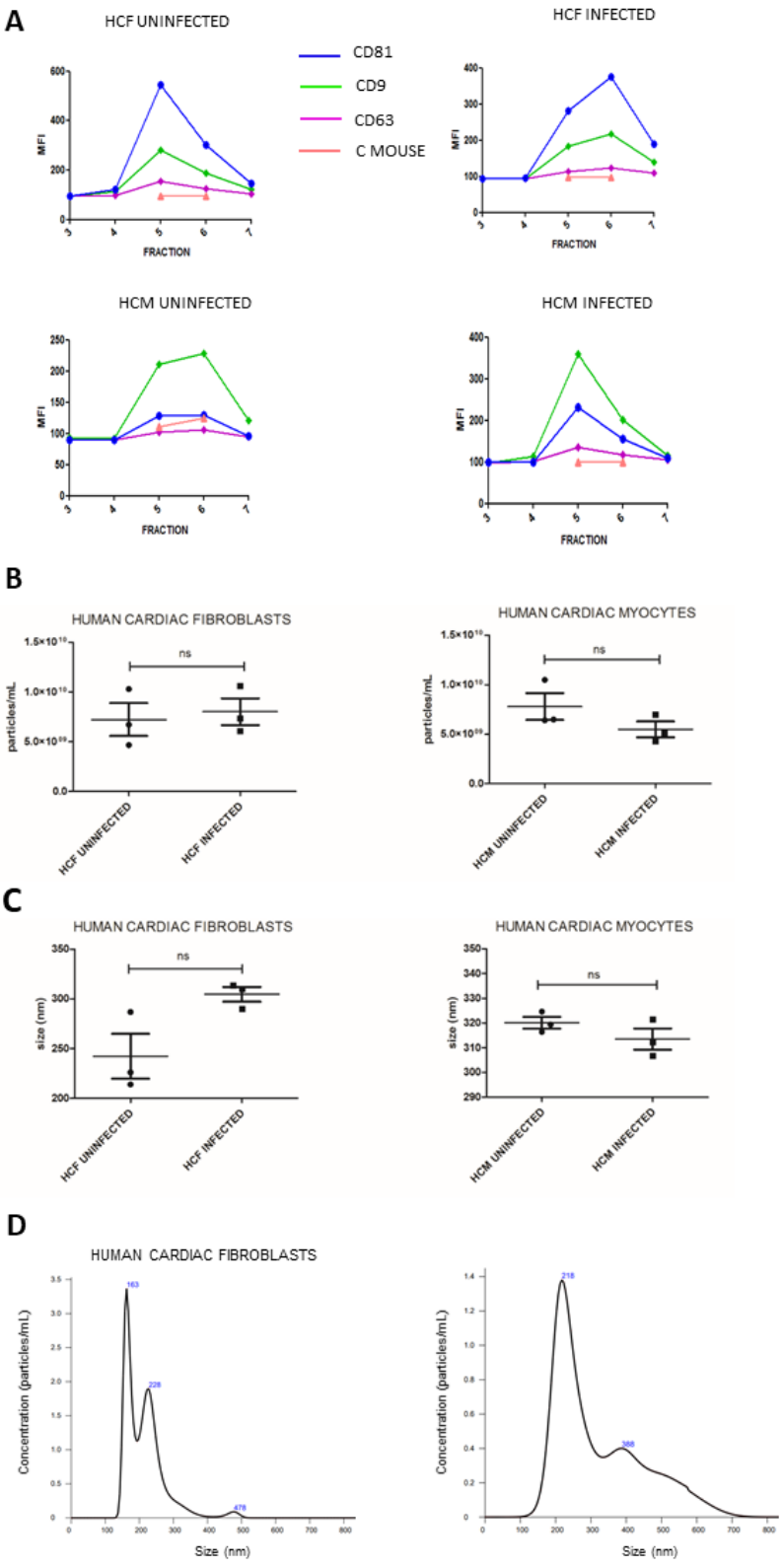


Figure 29. Isolation and characterization of EVs secreted by infected and uninfected HCF and HCM isolated by SEC. (A) SEC fractions of cardiac-cell derived EVs were analyzed in a flow cytometry bead-based assay for the presence of the classical exosomal markers CD81, CD9, and CD63. Mean fluorescence intensity of antibodies and control was assayed for each fraction. (B) Particles concentration, measured by NTA, is

shown in particles/mL. No significant differences were found between infected and uninfected cells (unpaired *t*-test). (C) Particles size, shown in nm, was also measured by NTA. No significant differences were found between infected and uninfected cells (unpaired *t*-test). (D) NTA profiles of particles found in the cell SN of infected and uninfected HCF and HCM. Data shows a representative NTA profile from each biological condition.

After demonstrating that classical EV markers such as CD81 and CD9 were present on the surface of cardiac cells, as well as in EVs derived from *T. cruzi* infected cardiac cells, we proceeded to immunoaffinity capture the EVs using the commercial kit EasySep™ Human Pan-Extracellular Vesicle Positive Selection. This kit isolates vesicles from plasma, serum and cell culture SN by immunomagnetic positive selection. EVs are labeled with antibodies recognizing CD9, CD81, and CD63 coupled to magnetic particles, and then separated using a magnet.

EVs derived from cardiac cells were then pre-enriched by UC, isolated by direct immunoaffinity capture using the EasySep kit, and characterized by western blot. Interestingly, the results showed an important increase in CD9, CD63 and CD81 signal in all conditions when compared to equivalent protein amount of the input and flow-through fractions, indicating a successful enrichment (Figure 30).

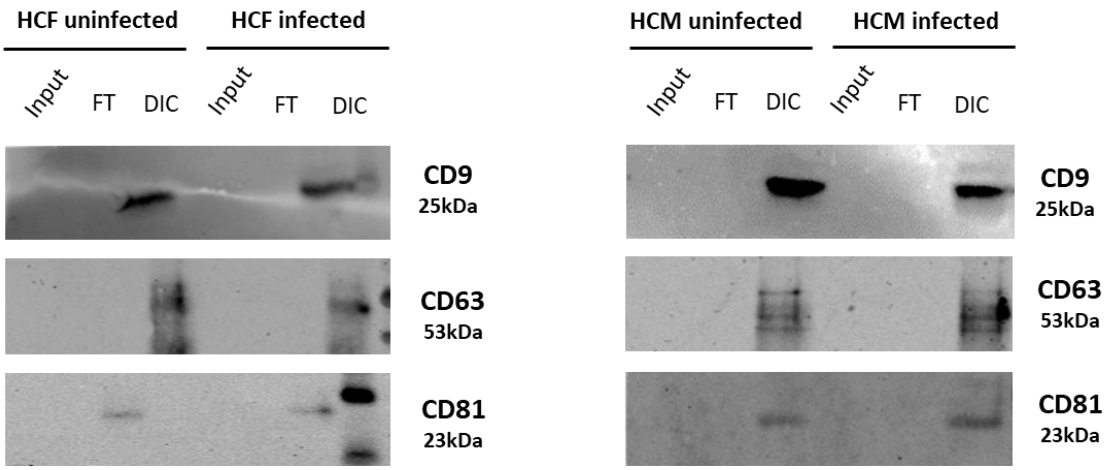


Figure 30. Isolation and characterization of EVs secreted by infected and uninfected HCF and HCM isolated by immunoaffinity capture. Western blot analysis of EVs immunocaptured from culture SN of HCF and HCM using anti-CD9, CD81 and CD63 beads. CD9, CD81, and CD63 are enriched in the immunoaffinity capture fraction as compared to equivalent protein amount loaded from the input and flow-through. FT: flow-through; DIC: direct immunoaffinity capture.

5.2.3. Proteomic characterization of EVs secreted by uninfected and *T. cruzi* infected human cardiac cells

After performing the immunoaffinity capture using CD9, CD63, and CD81, between 1 and 3 µg of isolated EVs were digested using the PreOmics kit and analyzed by LC-MS/MS (Proteomic Unit, Centre for Genomic Regulation, Spain). Three biological replicates of EVs isolated from uninfected and *T.cruzi* infected HCF and HCM were processed. In parallel, two samples of EVs

secreted by trypomastigotes (free parasites) isolated after the infection of HCF and HCM were also included.

Parasite proteins

Using a FDR <1%, we identified twelve *T. cruzi* protein groups secreted in EVs from infected HCF. Among these 12 parasite protein groups, three were found in the three biological replicates, while nine were found in two of them. Only one *T. cruzi* protein group was identified in EVs secreted by infected HCM. All *T. cruzi* protein groups identified are shown in Table 11. Of particular interest are the ones shown in red color, as can be found in the three biological replicates.

Regarding the parasite proteins, we identified one leishmanolysin-like peptidase (presenting metalloendopeptidase activity), one C2-C2_1 domain-containing protein (unknown molecular function), one chaperonin HSP60 mitocondrial (involved in protein folding), one glucose-regulated protein 78 (involved in protein folding) and one tubulin beta chain (structural molecule) secreted in EVs from *T. cruzi* infected HCF. Among the parasite proteins identified in EVs released by infected HCF, the presence of five trans-sialidases and one mucin-like glycoprotein is worth to highlight, as are well known virulence factors (223). Only one parasite protein was detected in EVs secreted by HCM: a translation initiation factor IF-2 (presenting translation initiation factor activity).

EVs released directly from free trypomastigotes obtained after infection of HCF and HCM were purified by UC and analysed by LC-MS/MS. 140 parasite protein groups were detected in EVs secreted by trypomastigotes derived from HCF, while 117 *T. cruzi* protein groups were detected in EVs secreted by trypomastigotes that were infecting HCM. Most of these protein groups (109) were common for both preparations, however, 31 protein groups were specifically found in EVs from trypomastigotes isolated after infecting HCF, while eight protein groups were specifically found in EVs from trypomastigotes isolated after infecting HCM. Interestingly, none of the parasite protein groups found in EVs secreted by infected HCF and HCM (with amastigote niches) were found in the EVs secreted by trypomastigotes, meaning that these proteins could be specific of the amastigote intracellular form of the parasite (Figure 31).

Table 11. *T. cruzi* protein groups identified in proteomic analysis of CD9, CD63 and CD81 positive EVs isolated by direct immunoaffinity capture from SN of HCF and HCM. Results are shown in total unique peptides and peptides abundance normalized.

T. cruzi Protein Name	Accession Number	Unique peptides	Abundance infected 1	Abundance infected 2	Abundance infected 3
Leishmanolysin-like peptidase	Q4CNJ0	1	7892138.6	1703596.9	11434386.2
Trans-sialidase	Q4DWV4	2	4179370.8	0	3667952.5
C2-C2_1 domain-containing protein	Q4DDE9	1	13598368.7	21470352.4	74616011.2
Trans-sialidase	Q4D110	2	7723725.2	0	13683717.1
Trans-sialidase	Q4DH24	1	6289136.3	0	6863979.2
Mucin-like glycoprotein	Q4E332	1	0	0	5559649.1
Trans-sialidase	Q4CL30	1	3766061.6	0	7121486
Chaperonin HSP60, mitochondrial	Q4DYP6	3	0	0	1867488.7
Mucin-like glycoprotein	Q4DVZ2	3	579135.5	0	639676.1
Trans-sialidase	Q4CSG0	4	16155991.1	0	16249697.9
Glucose-regulated protein 78	Q4D620	3	0	0	2423591.3
Tubulin beta chain	Q4DQP2	6	977486.5	1735512.8	13799671.1
T. cruzi Protein Name	Accession Number	Unique peptides	Abundance infected 1	Abundance infected 2	Abundance infected 3
Translation initiation factor IF-2	Q4DX02	1	43619621.08	43619621.1	43619621.1

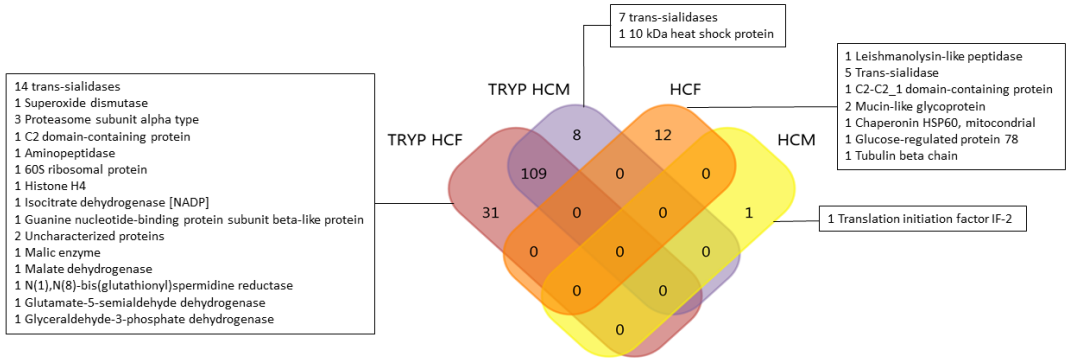


Figure 31. Venn diagram showing the overlap of *T. cruzi* protein groups detected in EVs secreted by free trypomastigotes after infecting HCF or HCM (purified by UC), and EVs secreted by infected HCF and HCM (purified by UC + SEC).

Human proteins

Focusing on human proteins detected in EVs secreted by HCF, we identified similar numbers of protein groups in each biological replicate: 1432 human protein groups in HCF uninfected 1, 1429 in HCF uninfected 2, and 1440 in HCF uninfected 3. In *T. cruzi* infected cells, the number of human protein groups identified was lower: 1395 in HCF infected 1, 1350 in HCF infected 2, and 1342 in HCF infected 3. Thus, higher number of human protein groups were detected in uninfected cells compared to infected cells (unpaired *t*-test; *p=0.0133).

Regarding human proteins detected in EVs secreted by HCM, 1277 protein groups were identified in HCM uninfected 1, 1769 in HCM uninfected 2, and 1818 in HCM uninfected 3. In *T. cruzi* infected cells, 1784 protein groups were identified in HCM infected 1, 1686 in HCM infected 2, and 1715 in HCM infected 3. Here, no significant statistical differences were found in terms of number of protein groups detected between uninfected and infected cells.

Among the human proteins, high number of EV markers were identified in both cell types. Interestingly, we could identify a particular set of protein groups present in EVs secreted by infected cells (HCF and HCM) and not present in EVs from uninfected cells (Figure 32). Notably, some of the identified proteins, such as unconventional myosin-XVIIIa, kinectin, mitochondrial 2-oxaglutarate/malate carrier protein, or cytosolic iron-sulfur assembly component 12B, are mainly associated to the cardiac tissue or to cardiomyoyctes, according to the human atlas. We have also identified specific protein markers of fibroblasts, such as Inactive rhomboid protein 1, NOXP20, PRA1 family protein 2 or COP9 signalosome complex subunit 8. The use of several of these markers in combination to further immunoaffinity capture the EVs found in circulation in plasma of *T. cruzi* infected patients could allow to capture specifically those EVs secreted by infected cardiac cells.

Functional enrichment analysis of the human proteins exclusively identified in infected cardiac cells showed a clearly enriched cellular component related to extracellular exosomes and membrane. Regarding enriched biological processes, we identified proteins related to actin cytoskeleton organization, endoplasmic reticulum to Golgi vesicle-mediated transport and protein dephosphorylation, among others. Interestingly, proteins associated to antigen presentation were also identified (Figure 33).

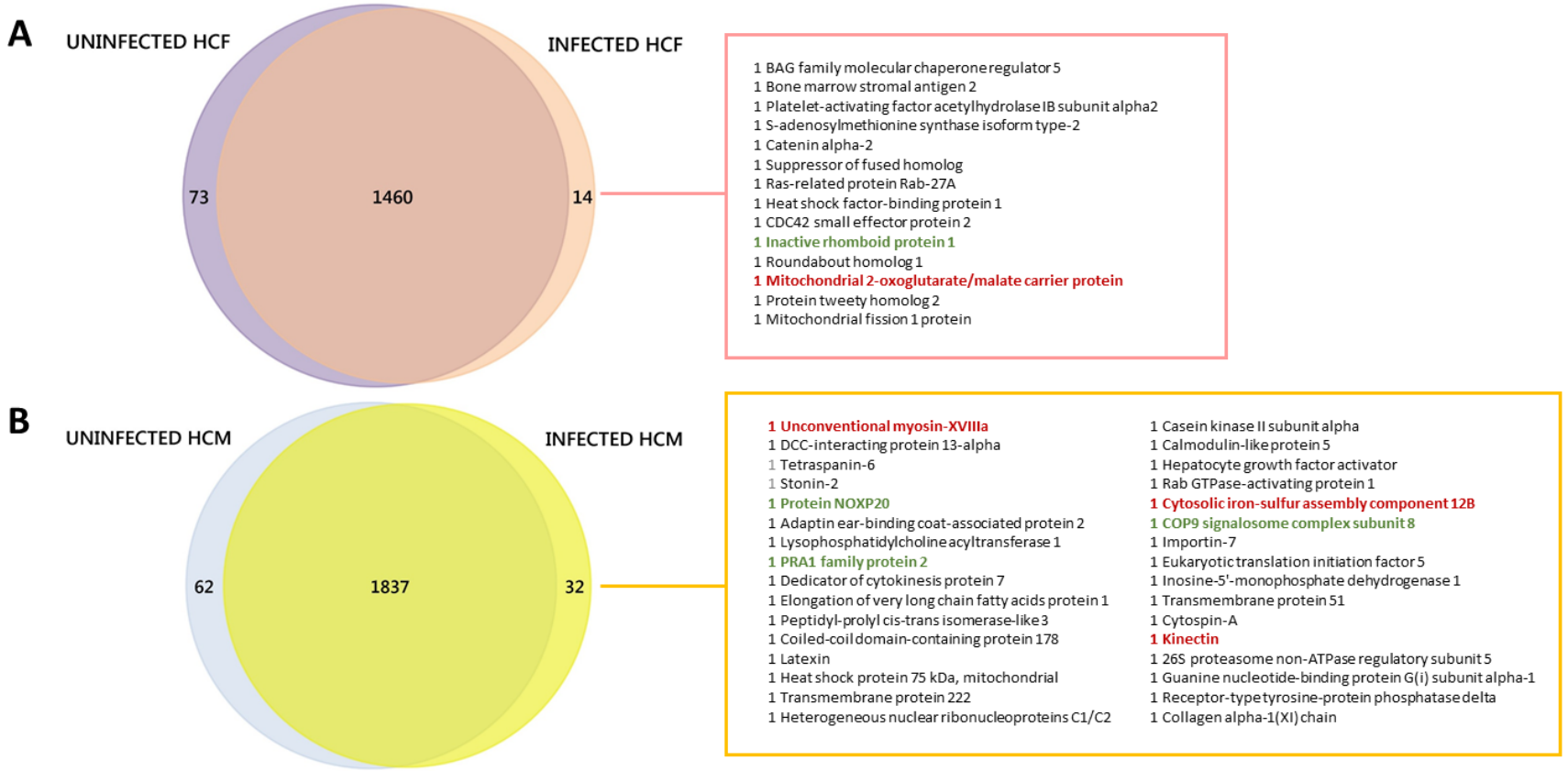


Figure 32. Human proteome of CD81, CD9, CD63EVs isolated by direct immunoaffinity capture from SN of uninfected and *T. cruzi* infected HCF and HCM. (A) Venn diagram showing the intersection between total human proteins detected in uninfected HCF and the ones detected in *T. cruzi* infected HCF. (B) Venn diagram showing the intersection between total human proteins detected in uninfected HCM and the ones detected in *T. cruzi* infected HCM. Proteins shown in red color are mainly found in cardiac tissue, and proteins shown in green are markers of fibroblasts.

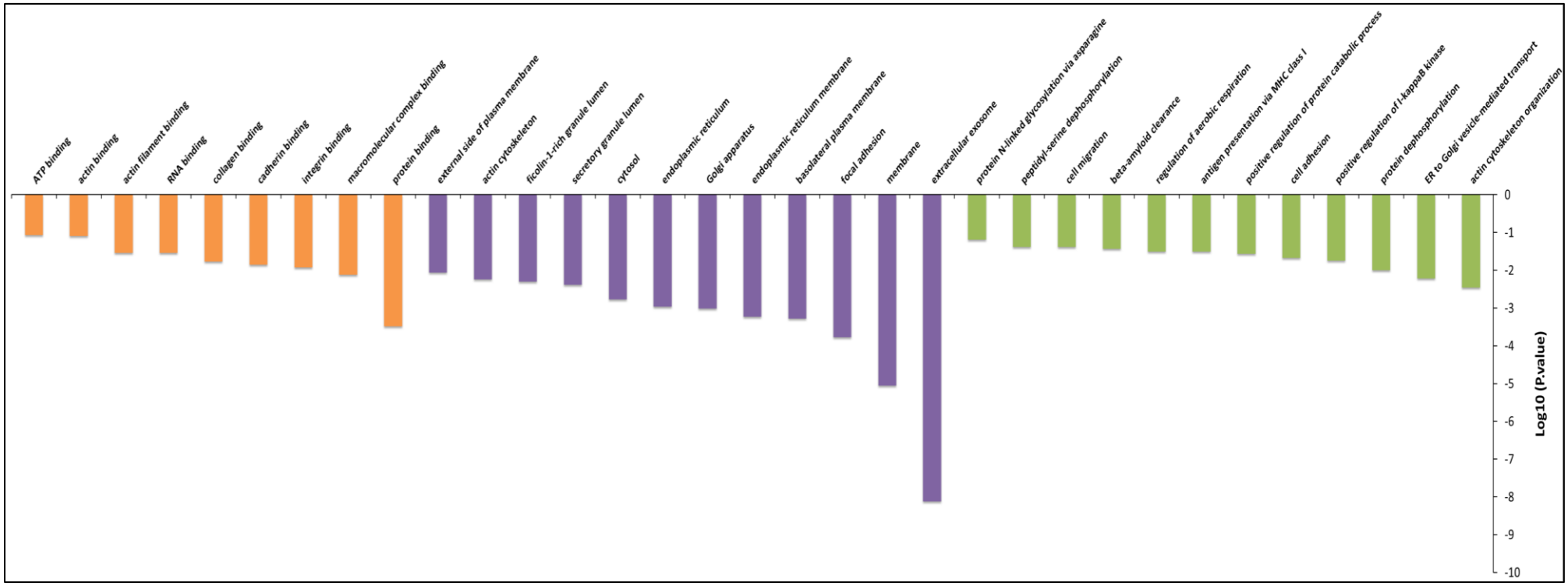


Figure 33. Enrichment analysis of human proteins exclusively identified in infected cardiac cells (HCF and HCM) by gene ontology (GO). GO enrichment analysis shows terms of Biological Process (green), Cellular Component (purple) and Molecular Function (orange).

5.2.4. Summary

In this chapter we have presented the first mass spectrometry-based proteomic analysis of EVs derived from *T. cruzi* infected and uninfected cardiac cells and purified by immunoaffinity capture using CD9, CD63, and CD81 antibodies. Noticeably, we identified a cluster of *T. cruzi* and human proteins uniquely identified in EVs secreted by infected cardiac cells, which can be used in the future to immunoaffinity capture this particular EVs sub-population. These data represent a valuable resource to identify specific EV markers from *T. cruzi* infected cells.

5.3. VALIDATION AND COMPARISON OF EVS PURIFICATION TECHNIQUES IDENTIFICATION OF EVS BIOMARKERS IN THE CONTEXT OF THERAPEUTIC RESPONSE AND DISEASE PROGRESSION IN CHRONIC CHAGAS DISEASE

After showing that uninfected and *T. cruzi* infected HCF and HCM cells express CD9, CD81 and CD63 in their surface, and more importantly, in the secreted EVs, the immunoaffinity capture methodology using these tetraspanin markers seemed a robust method to further isolate the circulating EVs from plasma of chronic CD individuals. Moreover, our proteomic results demonstrated the presence of parasite and specific human proteins in EVs secreted by cardiac infected cells isolated by immunoaffinity capture.

Therefore, we decided to apply this methodology to our cohort of individuals for EVs purification and identification of possible biomarkers in the context of therapeutic response and disease progression. To this end, and due to time and logistical constraints by to the COVID-19 pandemic, we only analysed a subset of samples from each of the experimental group and EVs were purified and characterized as described in Figure 34A.

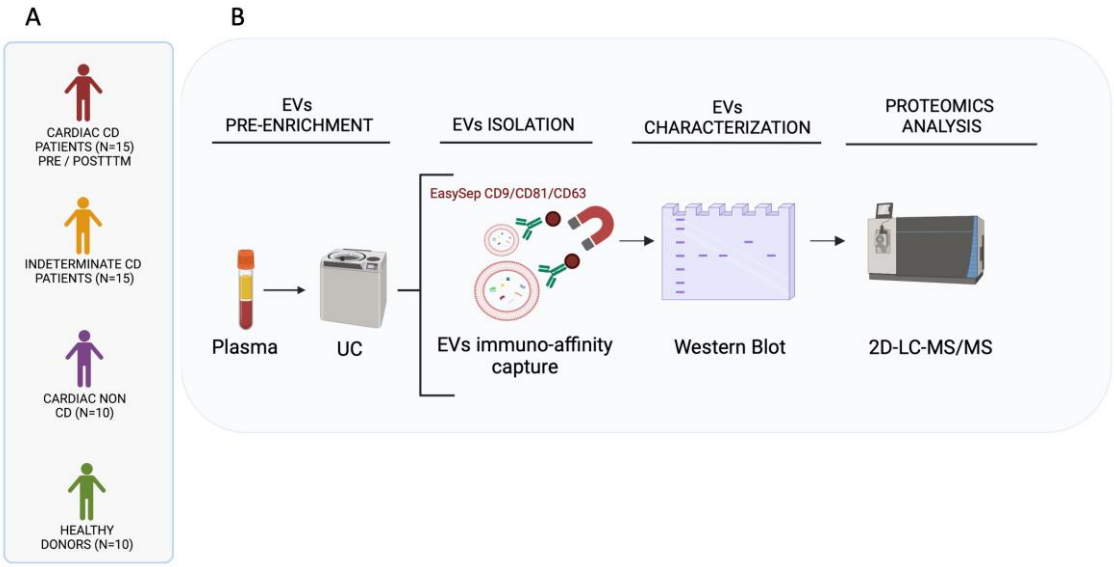


Figure 34. Isolation and characterization of EVs from plasma samples of cardiac CD patients and controls. (A) Group of participants analysed in each experimental group. (B) Schematic representation of the experimental designed applied for EVs enrichment and molecular characterization. Created with BioRender.com.

5.3.1. Description of the participants

From all the participants included in the cohort, EV samples from 15 individuals presenting the cardiac form of the disease before and after BDZ treatment (recruited in Cochabamba, Bolivia), 15 from CD patients presenting the indeterminate form of the disease (recruited at Hospital Clinic, Barcelona), 10 cardiac individuals not presenting CD (recruited at

Hospital Clinic, Barcelona) and 10 healthy individuals (recruited at Hospital Clinic, Barcelona) (Figure 35A) were isolated by UC followed by immunoaffinity capture and characterized by western blot and proteomics analysis (Figure 34B). From all the individuals presenting cardiac CD, 14 were PCR positive before BDZ treatment, while only 2 *T. cruzi* infected patients presenting the indeterminate form of the disease were PCR positive before BDZ treatment.

To first characterize these subgroups, we performed NTA directly from plasma. No differences in terms of particle concentration were found between healthy individuals and patients presenting cardiac CD in plasma. As shown in Figure 35, BZD treatment did not affect the particles concentration found in total plasma (Figure 35A). Similarly, no differences were found in terms of particle size in any of the conditions (Figure 35B).

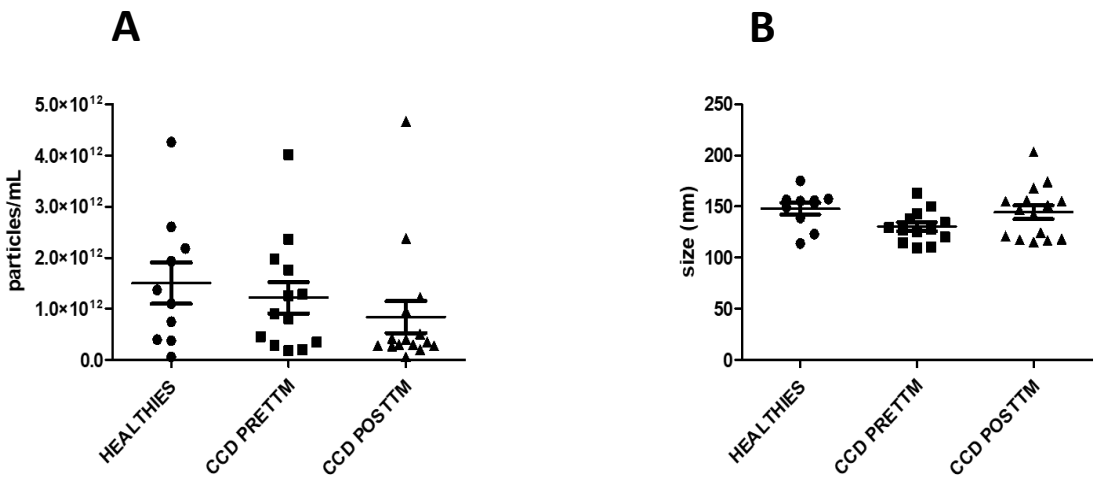


Figure 35. Characterization of EVs from *T. cruzi* infected patients and healthy donors by NTA. Total plasma of healthy individuals and *T. cruzi* infected patients before and after treatment was analyzed by NTA. No differences in terms of concentration of particles (A) or particle size (B) was found.

5.3.2. EVs isolation and characterization

Then, EVs were isolated by UC followed by immunoaffinity capture using CD9, CD81 and CD63 antibodies (Figure 36). To further characterize the EVs, a western blot was performed, showing an important increase in the three EV markers signal in all conditions when compared to equivalent protein amount of the input and flow-through fractions, indicating a successful enrichment of these particular populations of EVs (Figure 36).

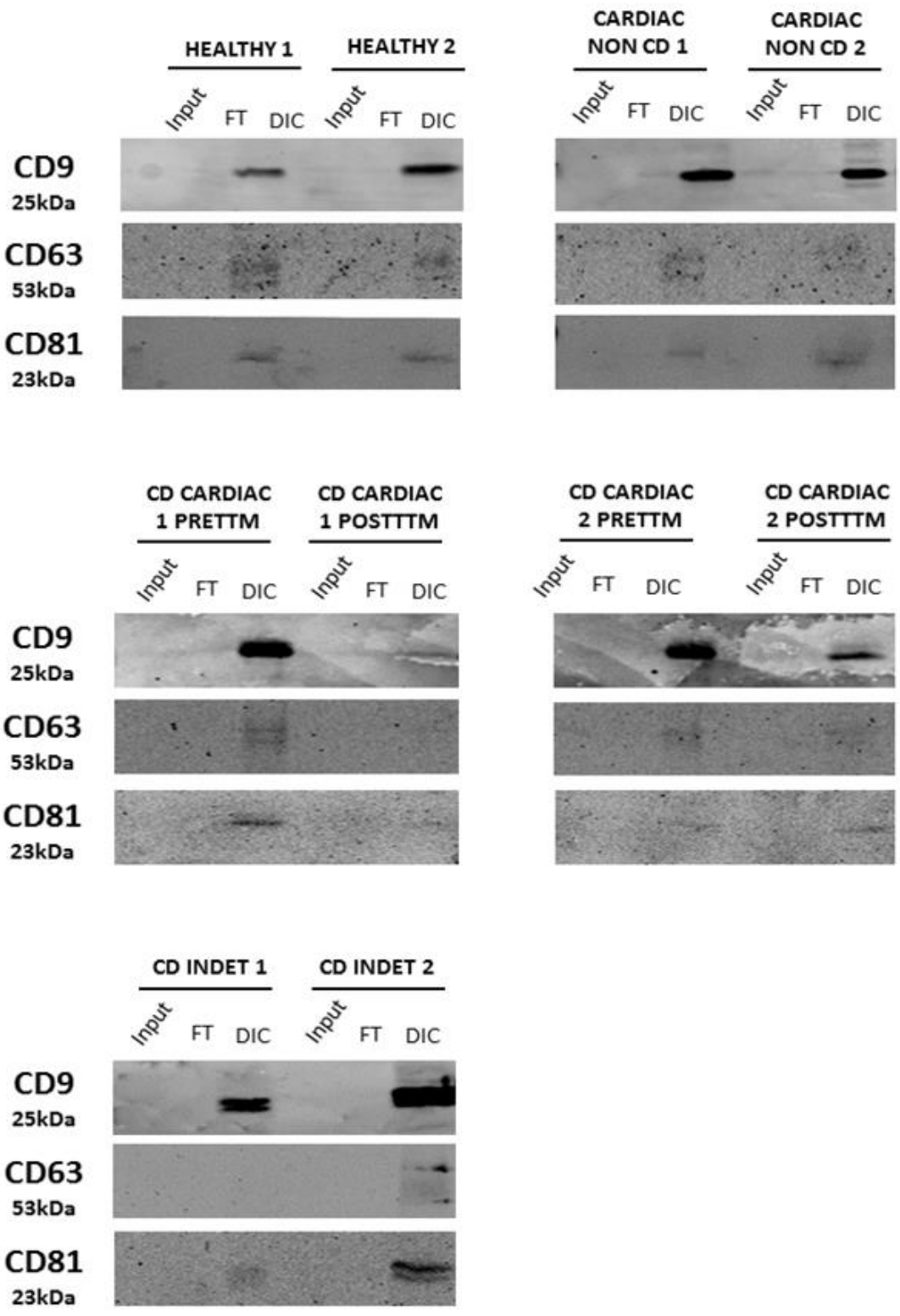


Figure 36. Characterization of EVs from *T. cruzi* infected patients and healthy donors by western blot. EVs isolated by immunoaffinity capture were characterized by western blot using anti-CD9, CD81 and CD63 antibodies. All markers are enriched in the immunoaffinity capture fraction as compared to equivalent protein amount loaded from the input and flow-through. FT: flow-through; DIC: direct immunoaffinity capture.

5.3.3. Proteomic characterization

Once the EVs were immunoaffinity purified and characterized by western blot, we proceed to perform the proteomic analysis (Proteomic Unit, El Paso University, USA). We searched for peptide spectrum matches against human and *T. cruzi* protein databases, and using a FDR <1% we identified a total of 709 human proteins and 74 parasite proteins. However, when applying a more stringent criterion of at least 2 UP normalized per protein, 375 human proteins and 62 *T. cruzi* proteins were identified among the different individuals analyzed. From here on, we will focus on these proteins detected by at least two UP.

Parasite proteins

T. cruzi proteins were detected in both groups of *T. cruzi* infected patients: CD cardiac patients pre-and post-treatment, and patients presenting the indeterminate form of the disease. Notably, parasite proteins have been identified in almost all patients (Table 18). Although this is the first time that we are working with these figures in terms of high detection of parasite proteins, many of these proteins are only found in a few number of samples of the cohort (Table 13).

Surprisingly, higher number of parasite proteins were detected in patients presenting the indeterminate form of the disease compared to the patients presenting the cardiac form, although these results were not statistically significant (Table 12). Moreover, higher levels of parasite proteins were detected in samples of cardiac CD patients not treated, compared to the same patients after treatment (although, again, the difference was non significant) (Table 12). Interestingly, we detected a cluster of parasite proteins in samples of cardiac CD patients before treatment that disappeared after treatment. These results highlight the potential of the parasite proteins identified in EVs as biomarkers of therapeutic response for CD (Figure 37).

We were able to identify a cluster of parasite proteins found in a large number of samples and in all groups of *T. cruzi* infected patients, independently of the disease form (Table 13). These parasite proteins, which could be considered markers of the disease, are the mucin-associated surface protein (V5AN53), identified in nine samples, one *T. cruzi* uncharacterized protein (K2NIL0), detected in eight samples, and finally one *T. cruzi* uncharacterized protein (A0A2V2US84), found in seven samples. Interestingly, we also identified a cluster of 17 parasite proteins found exclusively in EVs secreted by *T. cruzi* indeterminate patients, and a cluster of 24 *T. cruzi* proteins exclusively identified in EVs from cardiac CD patients, which can be considered potential biomarkers of disease outcome (62 parasite proteins were identified with at least 2 UP normalized in EVs isolated from plasma of CD patients. *T. cruzi* proteins identified exclusively in

samples of patients presenting the indeterminate form of the disease are shown in the red square. Similarly, biomarkers of the cardiac form of the disease are shown in the green square, and biomarkers of therapeutic response can be found in (Figure 37, Table 13).

Table 12. Number of *T. cruzi* proteins detected in EVs from *T. cruzi* infected patients with at least 2 UP normalized.

CD CARDIAC PRE-TTM	01	02	03	04	06	07	08	09	10	14	15	16	17	18	19
	1	0	0	3	6	2	3	7	2	7	0	1	0	1	0
CD CARDIAC POST-TTM	01	02	03	04	06	07	08	09	10	14	15	16	17	18	19
	0	0	0	0	0	0	3	3	0	0	0	0	0	5	4
CD INDETERMINATE	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
	5	4	7	0	9	3	1	5	4	3	0	4	0	0	3

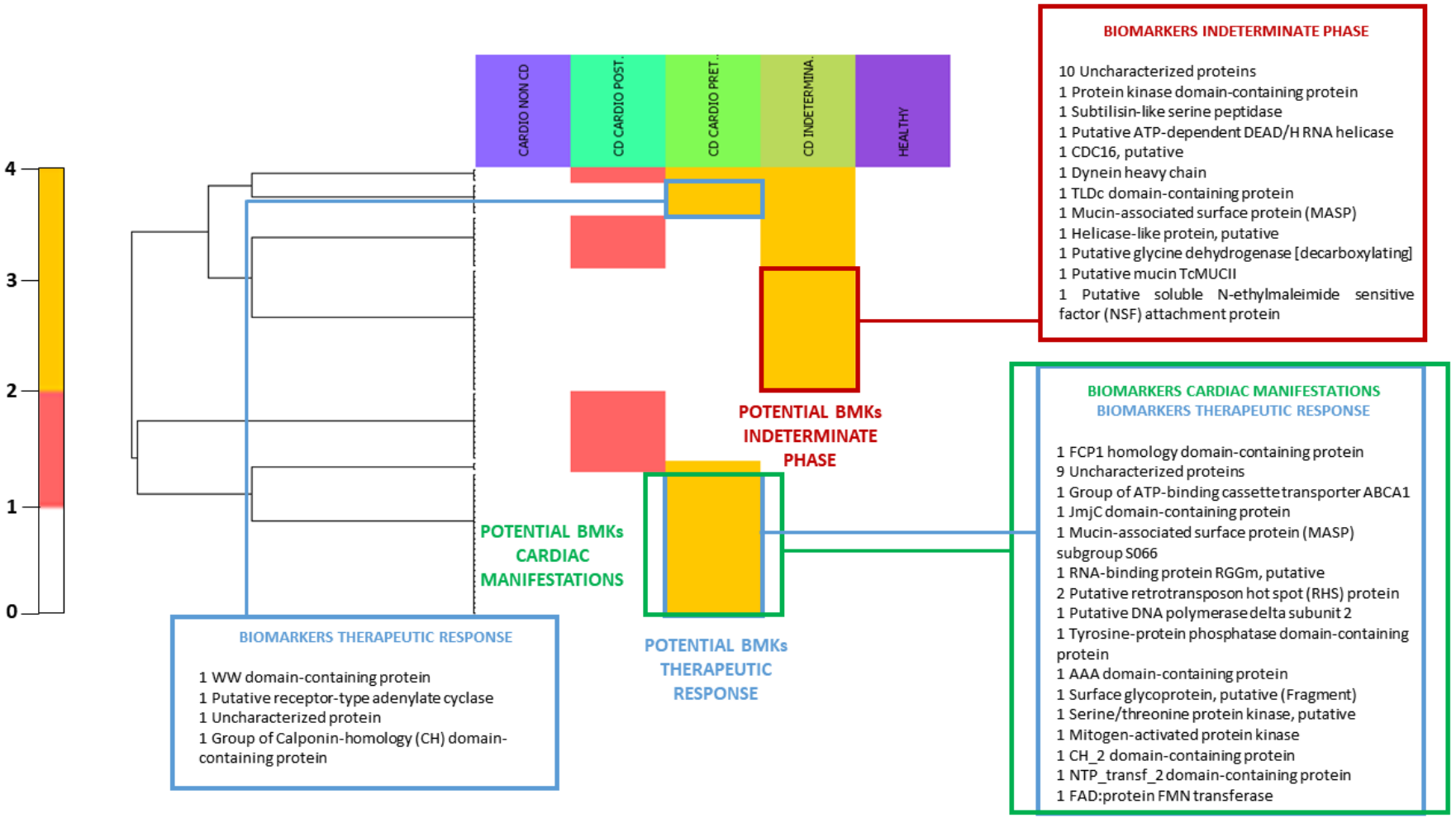


Figure 37. 62 parasite proteins were identified with at least 2 UP normalized in EVs isolated from plasma of CD patients. *T. cruzi* proteins identified exclusively in samples of patients presenting the indeterminate form of the disease are shown in the red square. Similarly, biomarkers of the cardiac form of the disease are shown in the green square, and biomarkers of therapeutic response can be found in the blue square.

Table 13. *T. cruzi* proteins identified from plasma-EVs of *T. cruzi* infected patients. Values are shown in normalized total UP. Pink color: potential biomarkers indeterminate form; green color: potential biomarkers cardiac form and therapeutic response; blue color: potential biomarkers therapeutic response.

Protein Name	Accession Number	*T. cruzi* CARDIO PRE-TTM	*T. cruzi* CARDIO POST-TTM	*T. cruzi* INDETERMINATE
AAA domain-containing protein	A0A7J6Y2H5	2.087 (N=1)	0	0
CDC16, putative	K2N759	0	0	2.475 (N=1)
CH_2 domain-containing protein	K2MH76	2.087 (N=1)	0	0
DUF4520 domain-containing protein	K2NCC3	2.087 (N=1)	1.527 (N=1)	0
Dynein heavy chain, putative	K2N5S2	0	0	2.475 (N=1)
FAD:protein FMN transferase (Fragment)	K2MAW5	2.087 (N=1)	0	0
FCP1 homology domain-containing protein	V5C1N1	2.087 (N=1)	0	0
Group of ATP-binding cassette transporter ABCA1	V5BWI9 (+1)	2.087 (N=1)	0	0
Group of Calponin-homology (CH) domain-containing protein	V5DA75 (+1)	2.087 (N=1)	0	2.475 (N=2)
Group of Guanine nucleotide-binding protein beta subunit-like protein	V5C0S8 (+3)	0	1.527 (N=1)	2.475 (N=2)
Group of TLDc domain-containing protein	Q4D4B7 (+1)	0	0	2.475 (N=1)
Helicase-like protein, putative	Q4DGU3	0	0	2.475 (N=1)
JmjC domain-containing protein	K2MA77	2.087 (N=1)	0	0
Mitogen-activated protein kinase	V5AX03	2.087 (N=1)	0	0
Mucin-associated surface protein (MASP)	V5AN53	2.087 (N=1)	1.527 (N=3)	2.475 (N=8)
Mucin-associated surface protein (MASP)	A0A2V2W9P8	0	0	2.475 (N=1)
Mucin-associated surface protein (MASP)	A0A7J6XNM8	2.087 (N=1)	0	0
NTP_transf_2 domain-containing protein	K2N6L7	2.087 (N=1)	0	0
Protein kinase domain-containing protein	A0A2V2XC89	0	0	2.475 (N=1)
Putative ATP-dependent DEAD/H RNA helicase	A0A2V2V144	0	0	2.475 (N=2)
Putative DNA polymerase delta subunit 2	A0A2V2VT77	2.087 (N=1)	0	0
Putative glycine dehydrogenase (decarboxylating)	A0A2V2WSN3	0	0	2.475 (N=1)
Putative mucin TcMUCII	A0A2V2WZK8	0	0	2.475 (N=1)
Putative receptor-type adenylate cyclase	A0A2V2WH45	2.087 (N= 2)	0	2.475 (N=1)
Putative retrotransposon hot spot (RHS) protein	A0A2V2US33	2.087 (N=1)	0	0
Putative retrotransposon hot spot (RHS) protein	A0A2V2WHM0	2.087 (N=1)	0	0
Putative soluble N-ethylmaleimide sensitive factor (NSF) attachment protein	A0A2V2UVX0	0	0	2.475 (N=1)
RNA-binding protein RGGm, putative	K2NCJ6	2.087 (N=1)	0	0
Serine/threonine protein kinase, putative	K2M3U9	2.087 (N=1)	0	0
Subtilisin-like serine peptidase	V5B875	0	0	2.475 (N=2)
Surface glycoprotein, putative (Fragment)	K2NI88	2.087 (N=1)	0	0
Trans-sialidase, putative (Fragment)	Q4CR60	0	1.527 (N=1)	0
Tyrosine-protein phosphatase domain-containing protein	A0A2V2XEN3	2.087 (N=1)	0	0
Uncharacterized protein (Fragment)	Q4CR94	2.087 (N=1)	0	0
Uncharacterized protein (Fragment)	Q4DF30	2.087 (N=1)	0	0
Uncharacterized protein (Fragment)	G0Z160	2.087 (N=1)	0	0

Uncharacterized protein	Q4D8K2	2.087 (N=1)	0	0
Uncharacterized protein	Q4DCX7	0	1.527 (N=3)	2.475 (N=1)
Uncharacterized protein	Q4D1E2	0	0	2.475 (N=1)
Uncharacterized protein	Q4CTL4	0	0	2.475 (N=1)
Uncharacterized protein	Q4DU77	2.087 (N=1)	0	0
Uncharacterized protein	V5BNY6	2.087 (N=1)	0	0
Uncharacterized protein	V5DFQ9	0	1.527 (N=1)	2.475 (N=5)
Uncharacterized protein	V5CTZ2	0	0	2.475 (N=1)
Uncharacterized protein	K2NIL0	2.087 (N=1)	1.527 (N=2)	2.475 (N=6)
Uncharacterized protein	K2NGK9	2.087 (N=1)	1.527 (N=1)	0
Uncharacterized protein	K2MZA4	0	1.527 (N=1)	2.475 (N=8)
Uncharacterized protein	K2NB52	0	0	2.475 (N=1)
Uncharacterized protein	A0A2V2WH50	0	0	2.475 (N=1)
Uncharacterized protein	A0A2V2W9Y4	2.087 (N=1)	0	2.475 (N=1)
Uncharacterized protein	A0A2V2X6A9	2.087 (N=1)	0	0
Uncharacterized protein	A0A2V2V1T4	0	1.527 (N=2)	2.475 (N=1)
Uncharacterized protein	A0A2V2US84	2.087 (N=3)	1.527 (N=3)	2.475 (N=3)
Uncharacterized protein	A0A2V2VEU6	0	0	2.475 (N=1)
Uncharacterized protein	A0A2V2V832	0	0	2.475 (N=1)
Uncharacterized protein	A0A7J6YEE9	0	0	2.475 (N=2)
Uncharacterized protein	A0A7J6YAA1	2.087 (N=1)	0	0
Uncharacterized protein	A0A7J6Y2I3	2.087 (N=1)	0	0
Uncharacterized protein	A0A7J6XTK5	0	0	2.475 (N=2)
Uncharacterized protein	A0A7J6XR22	0	0	2.475 (N=2)
WW domain-containing protein	Q4DNN9	2.087 (N=1)	0	2.475 (N=1)
Zinc-binding phosphatase, putative	K2MTF3	0	1.527 (N=1)	2.475 (N=2)

Human proteins

375 human proteins were detected with at least two UP normalized. Among those, 25 EV markers were found, showing that the preparation was clearly enriched in EVs. No differences were found in terms of number of human proteins identified among clinical groups.

The Partial least squares-discriminant analysis (PLSDA) of all samples further illustrates the differences of protein expression among different sample groups. In the 2D analysis, the samples from healthy individuals, cardiac *T. cruzi* negative individuals, and *T. cruzi* infected patients presenting the indeterminate and the cardiac form of the disease clearly show a distinct pattern. However, samples from cardiac *T. cruzi* infected patients before and after treatment presented similar patterns, and could not be separated based on their treatment status. When performing the 3D PLSDA plot, all populations could be separated based on their protein expression profile (Figure 38).

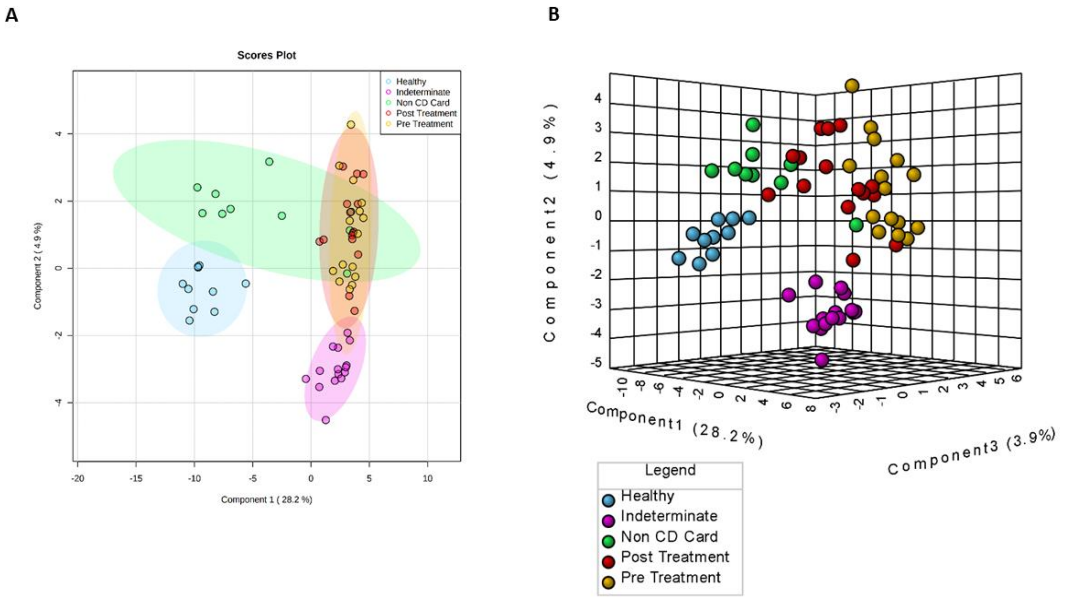


Figure 38. Partial least squares-discriminant analysis (PLSDA) plot (A) 2D and (B) 3D showing the proteomic variation among EV samples of *T. cruzi* infected patients presenting the indeterminate form of the disease, *T. cruzi* infected patients presenting the cardiac form before and after treatment, healthy individuals and cardiac patients not presenting CD. Colored symbols correspond to the five groups of patients.

Functional enrichment analysis of human proteins identified in *T. cruzi* infected patients (presenting the cardiac and the indeterminate form of the disease) and not found in the control groups showed a clearly enriched cellular component related to extracellular exosomes, blood microparticle, extracellular region and extracellular space. Enriched biological processes found in this particular set of proteins are related to regulation of endopeptidases, fibrinolysis, platelet

aggregation and blood coagulation, among others (Figure 39A). Notably, KEGG pathway analysis indicate the enrichment of proteins related to cardiomyopathy and inflammation.

Interestingly, we identified a cluster of human proteins found or upregulated in samples of *T. cruzi* infected patients, independently of the clinical status, and not found or downregulated in samples from cardiac non *T. cruzi* infected patients and heathy donors. Therefore, these proteins could be considered markers of *T. cruzi* infection (Figure 40). Moreover, we have identified a cluster of proteins specifically found in samples of *T. cruzi* infected patients presenting the indeterminate form of the disease, and a group of human proteins differentially expressed in samples from cardiac CD patients compared to the other clinical groups. Thus, these proteins can be considered specific markers of disease severity (Figure 40). Among the human proteins identified specifically in samples from cardiac CD patients, we found a cluster of proteins that appear before treatment and disappear after treatment, which should be further studied as potential biomarkers of therapeutic response (Figure 41).

Among the human proteins identified, we found APOA1, FBN and CRP, previously described as interesting proteins for CD pathology (110,111). APOA1 was identified in all samples. However, it was more abundant in EVs from *T. cruzi* infected patients (independently of the disease status) when compared to EVs from healthy individuals and non chagasic cardiac patients. Regarding FBN, we did not find any differences in the levels of among the different clinical study groups. Finally, CRP was identified in EVs from one CD patient presenting cardiopathy (Kushnir II) before BZD treatment. The protein was also found in much lower abundance in samples from healthy donors (n=3) and cardiac patients seronegative for *T. cruzi* (n=2).

One limitation of our last proteomic analysis is that the number of human proteins identified was very low. Unfortunately, there was a technical problem with the chromatography system, causing a drop in the human protein ID's throughout sample set. Thus, it is possible that several low abundant human proteins were under the detection limit, and could not be detected. Even so, the number of parasite proteins detected was higher than in any other occasion, and human proteins could also be identified, including several EV markers. Once the technical issue is solved, these samples will be run again in order to maximize the chromatography system efficiency and detect the highest number of parasite and human proteins.

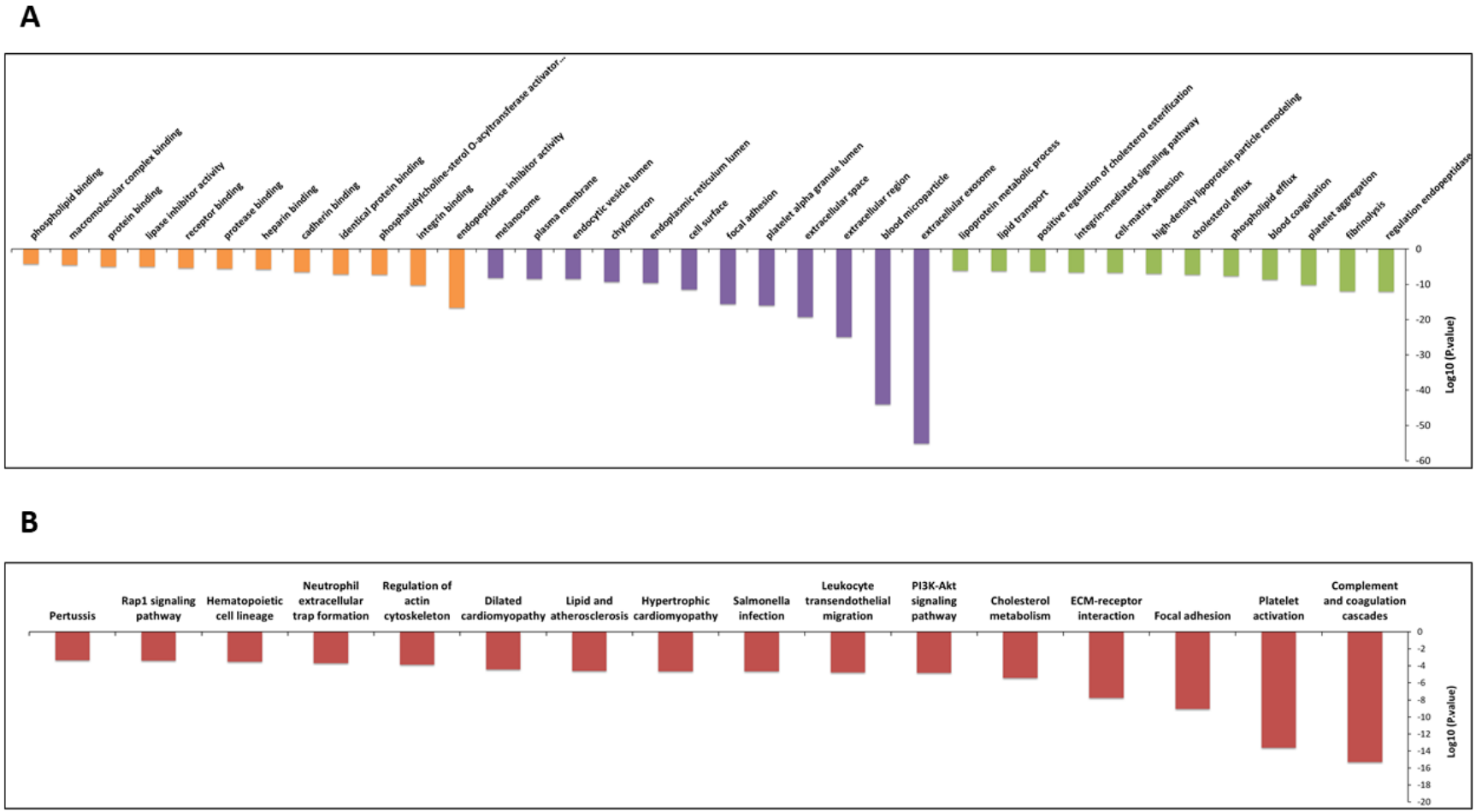


Figure 39. Enrichment analysis of human proteins identified in *T. cruzi* infected patients (presenting the cardiac and the indeterminate form of the disease) by gene ontology (A) and KEGG pathway (B). Gene Ontology enrichment analysis shows terms of Biological Process (green), Cellular Component (purple) and Molecular Function (orange).

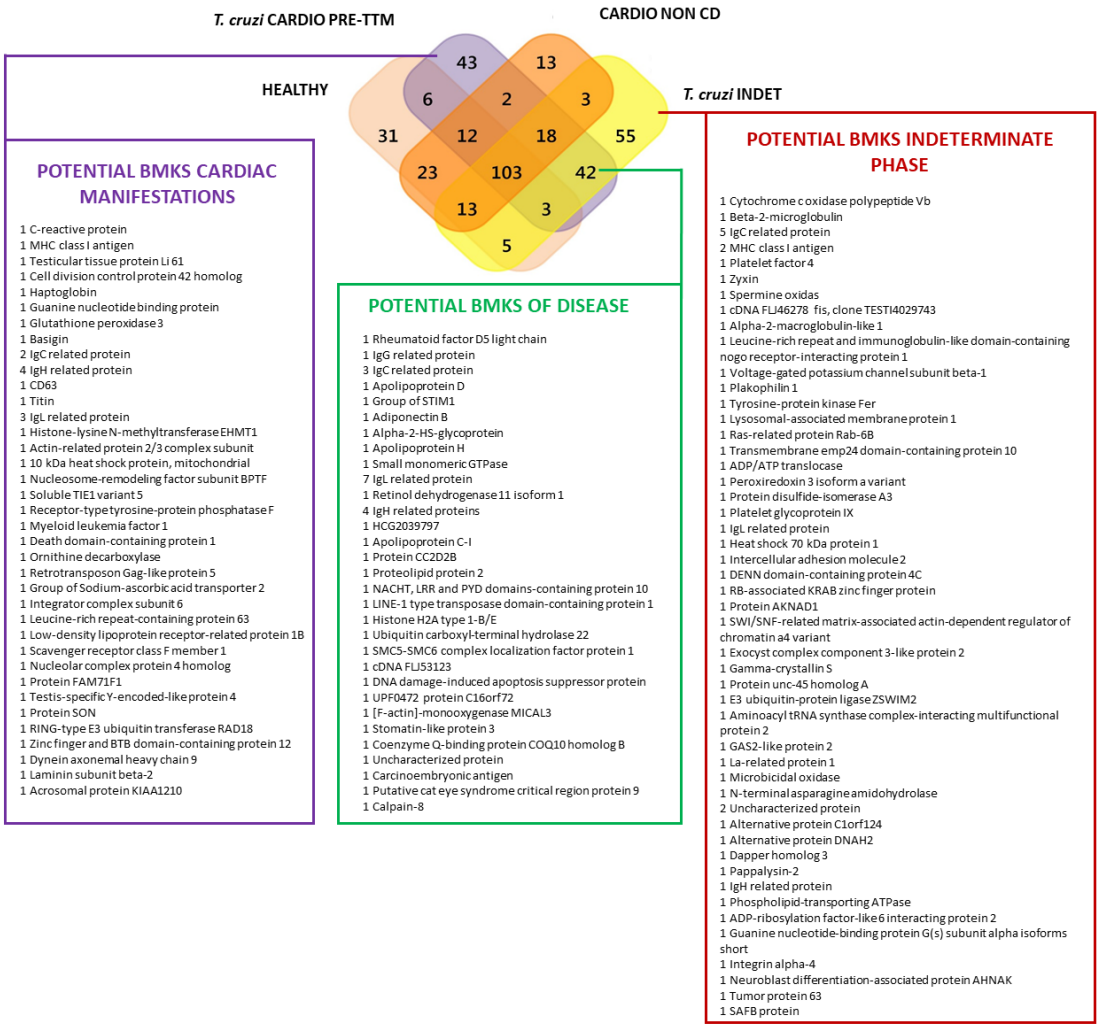


Figure 40. Venn diagram showing the intersection of human protein groups detected in EVs secreted by healthy individuals, *T. cruzi* infected individuals presenting the indeterminate form of the disease, cardiac CD patients before treatment, and non cardiac CD patients. Proteins detected exclusively in EVs from CD cardiac patients are potential biomarkers of cardiac disease and those detected only in samples of indeterminate patients are considered potential biomarkers of the indeterminate form. These proteins detected exclusively in both groups of *T. cruzi* infected patients are biomarkers of CD. Indet: indeterminate.

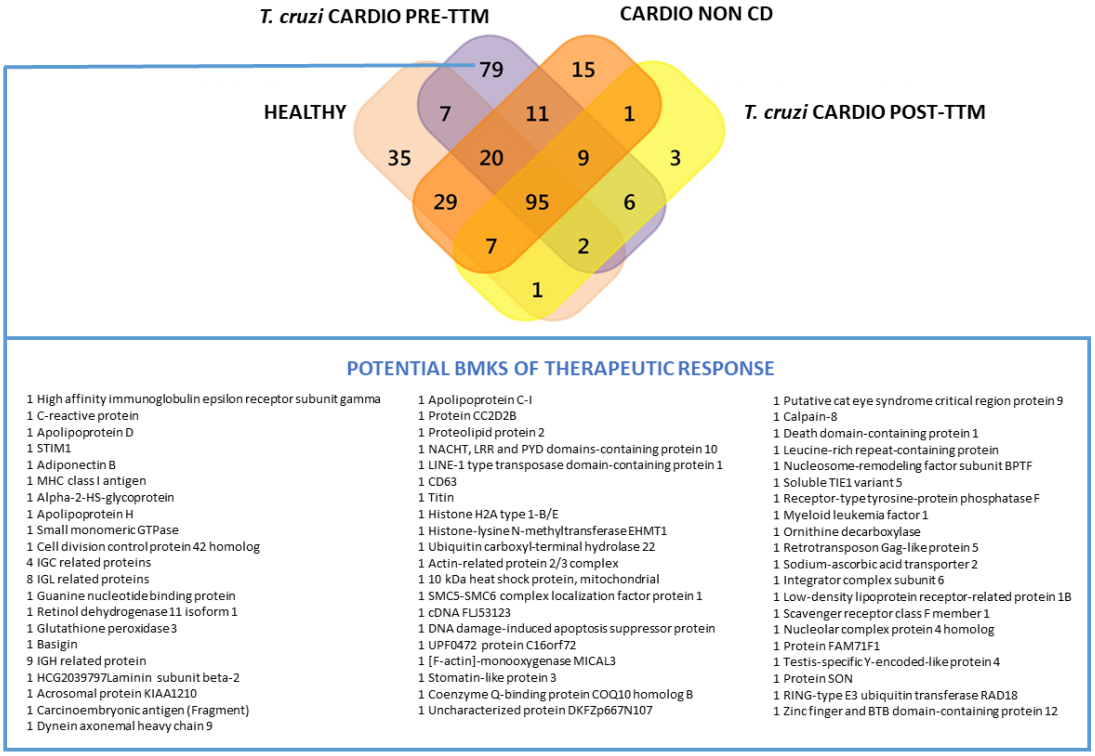


Figure 41. Venn diagram showing the overlap of human protein groups detected in EVs secreted by healthy individuals, cardiac CD patients before treatment and after treatment, and non cardiac CD patients. Proteins detected exclusively in EVs from CD cardiac patients before treatment and disappearing after treatment are potential biomarkers of therapeutic response.

5.3.4. Summary

In this chapter, we have presented a proteomic characterization of plasma-derived EV isolated by immunoaffinity capture using CD9, CD63 and CD81 markers from a cohort of individuals. EV samples from 15 *T. cruzi* infected patients presenting the cardiac form (before and after BDZ treatment), 15 *T. cruzi* infected patients presenting the indeterminate form of the disease, 10 healthy donors and 10 *T. cruzi* seronegative individuals presenting cardiac problems were included in the analysis. Interestingly, we have identified parasite proteins in the plasma-derived EVs from *T. cruzi* infected patients presenting both forms of the disease. Moreover, we have shown that circulating EVs from *T. cruzi* infected patients present an altered cargo compared to EVs from healthy donors and cardiac patients not presenting CD. Notably, we detected a cluster of parasite and human proteins which are present or upregulated in plasma-derived EVs from cardiac CD patients before treatment that are absent or downregulated following treatment, showing that EVs are potential biomarkers of therapeutic response for CD. Finally, we have identified human and parasite proteins specific from patients presenting *T. cruzi* infection, which could be further explored as potential biomarkers of disease for diagnosis purposes. Also,

human and parasite proteins specifically identified in EVs from *T. cruzi* infected patients presenting the cardiac or the indeterminate form encourage the use of circulating EVs as potential biomarkers of disease outcome.

5.4. BIOLOGICAL FUNCTION OF EXTRACELLULAR VESICLES SECRETED DURING *T. cruzi* INFECTION

EVs obtained from in vitro culture of *T. cruzi* trypomastigotes and *T. cruzi* infected cells have been shown to modulate immune cells, affecting the host immunoresponse and the inflammatory cascade (223,303,304). It is also known that inflammation and angiogenesis are closely interconnected (305,306). During inflammatory reactions, immune cells secrete pro-angiogenic factors, which promote neovascularization. On the other hand, new vessels may enhance tissue inflammation by promoting the migration of inflammatory cells (307). Nevertheless, there are not studies assessing the role of EVs derived from plasma of CD patients and *T. cruzi* infected cells in the angiogenic response. To address this issue, we performed an assay based in the experimental design described in Figure 42. This chapter presents the findings related to the investigation of the effect of EVs isolated from plasma of CD individuals and cardiac *T. cruzi* infected cells in the angiogenic process.

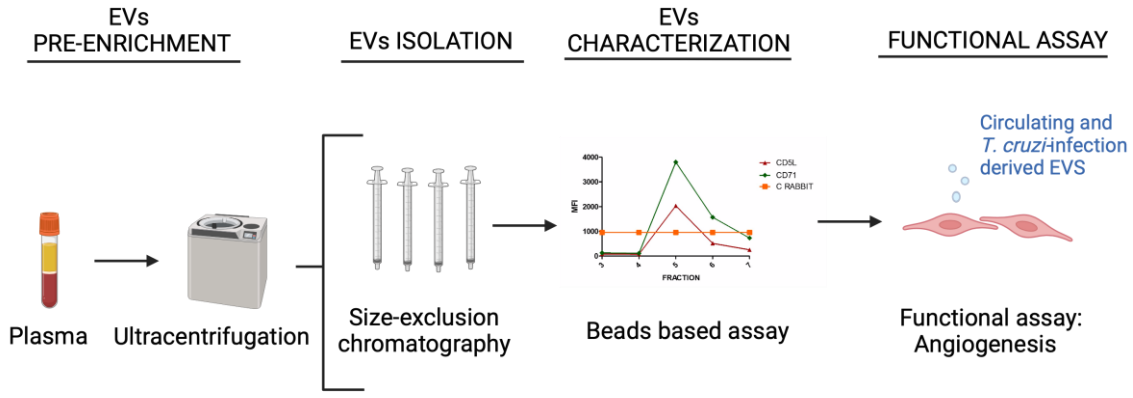


Figure 42. Schematic representation of the approach followed to assess the effect of EVs in the angiogenic response. Created with BioRender.com.

5.4.1. Is the angiogenic response affected by extracellular vesicles derived from CD patients and *T. cruzi* infected cells?

EVs from *T. cruzi* infected and uninfected HCM, cardiac CD patients and healthy donors were purified in sterile conditions by UC and SEC, as previously described, to be used in angiogenic assays. Three biological replicates were analyzed for each condition. Next, we evaluated the influence of EVs in affecting the angiogenic response of HUVEC cells. Thus, we measured the number of junctions, branches and meshes of HUVEC cells after being incubated with the EVs for 48h (Figure 43).

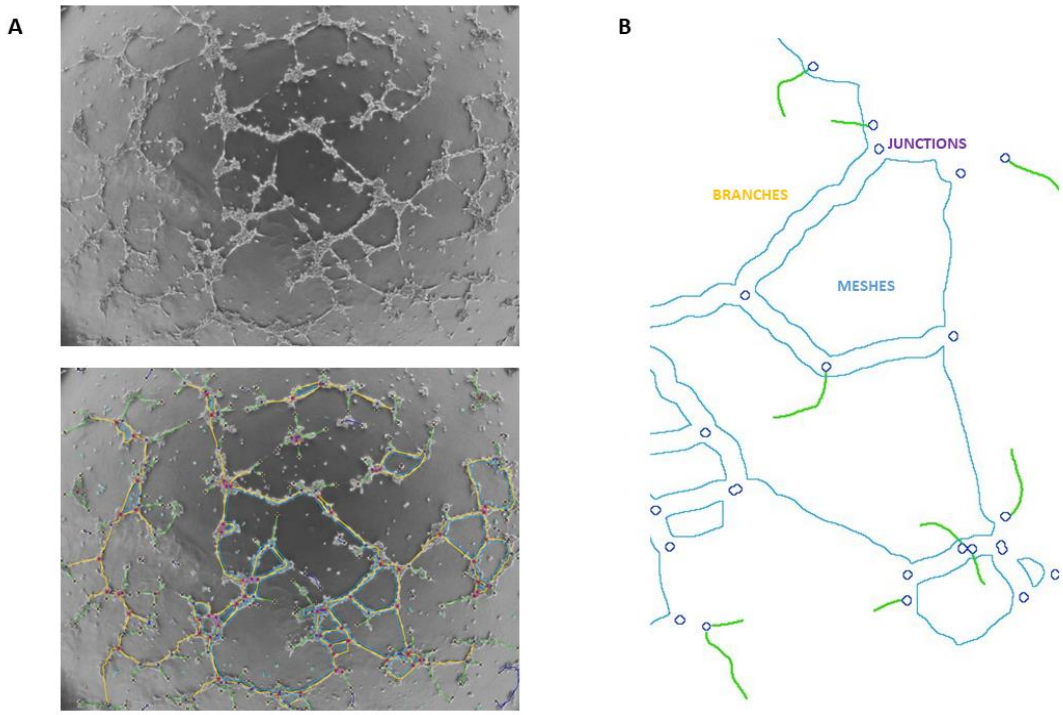


Figure 43. Influence of EVs on the angiogenic response of HUVEC cells. HUVEC cells were treated with EVs from infected HCM, cardiac CD individuals and healthy donors. After 48h, HUVEC cells were cultured in Matrigel for the analysis of angiogenic growth. (A) Representative image of the angiogenesis growth of HUVEC cells after being treated with the positive control. Pink points represent cellular junctions, yellow lines the length of the branches, and blue constructions the meshes. (B) Scheme of the quantified junctions, branches and meshes.

Interestingly, HUVEC cells angiogenic response was dependent on the EV stimulus. HUVEC treated with EVs from *T. cruzi* infected HCM presented less number of junctions, meshes and branches than HUVEC cells treated with EVs from uninfected cells (Figure 44A). Thus, although these results were non-significant, the angiogenesis response was clearly decreased in cells treated with EVs from infected cardiac HCM.

Regarding HUVEC cells treated with circulating EVs isolated from plasma of cardiac CD patients, we observed an increase in the angiogenesis compared to cells treated with EVs from healthy donors (Figure 44B). Again, although the results were non-significant, higher number of junctions, meshes and branches were detected in the HUVEC cells treated with EVs from cardiac CD patients compared to healthy donors. All cardiac CD patients included in the experiment were PCR positive. This increase in the angiogenesis response observed is not related to the EVs secreted by infected cardiac cells. EVs derived from other *T. cruzi* infected cells or from inflammatory cells could be involved. Further studies including a larger number of samples and *T. cruzi* infected patients presenting other forms of the disease are needed.

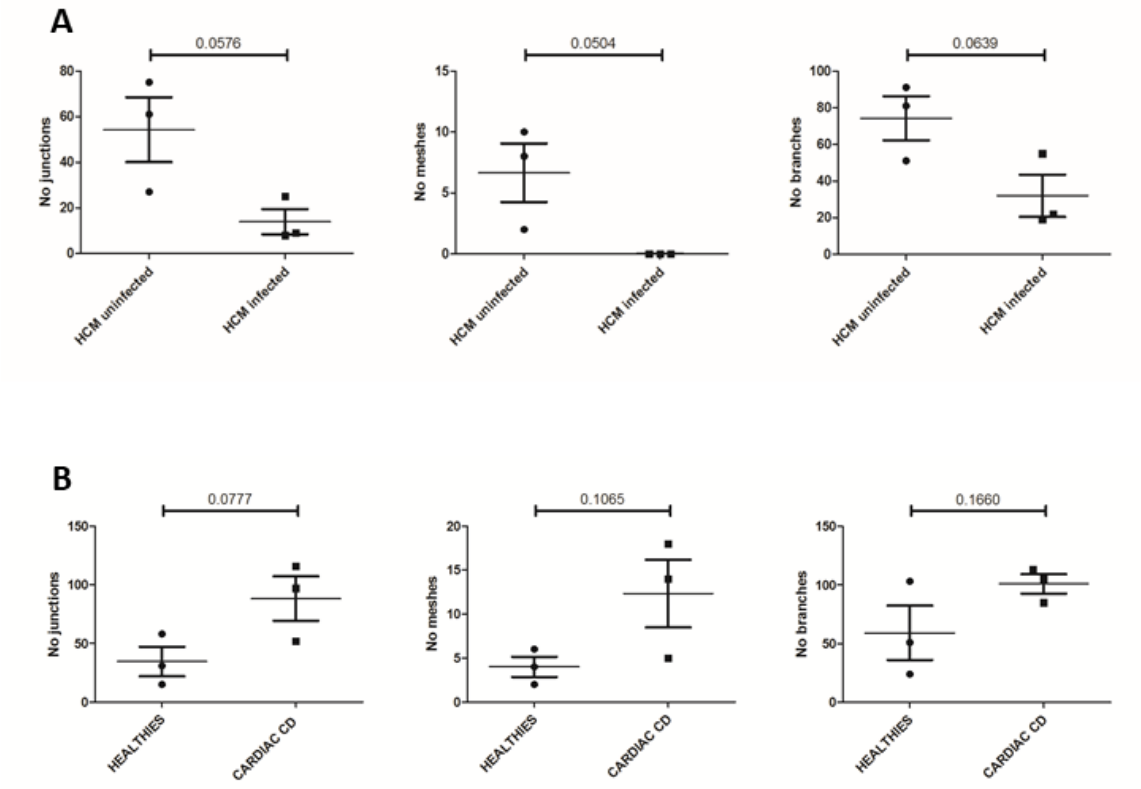


Figure 44. Analysis of the effect of EVs in the HUVEC cells angiogenesis progression. (A) HUVEC cells treated with EVs from infected HCM presented a clear tendency of diminishing the number of junctions, meshed and branches compared to HUVEC cells treated with EVs from uninfected cells. However, these results were non-statistically significant (unpaired *t*-test). (B) HUVEC cells treated with EVs from CD cardiac individuals presented higher number of junctions, meshed and branches than HUVEC cells treated with EVs from healthy donors. Again, although a clear tendency is observed, the results were non-statistically significant (unpaired *t*-test).

5.4.2. Summary

In this chapter we have presented the results of a preliminary functional assay assessing if the HUVEC cells angiogenic response was dependent on the EV stimulus. Curiously, HUVEC cells treated with EVs from infected HCM presented a decreased angiogenesis when compared to EVs from uninfected HCM. However, HUVEC cells treated with EVs derived from plasma of cardiac CD patients showed a clear tendency to increase angiogenesis. Further studies using samples of patients presenting the indeterminate form of the disease and increasing the sample size are needed.

6. DISCUSSION AND FUTURE WORK

The identification of an early biomarker of therapeutic response and disease outcome for Chagas Disease (CD) is a major need and would mean a breakthrough in the management and control of the disease. However, although many research groups are focus on the search of both host-derived and *T. cruzi*-derived biomarkers, we are still far from reaching this goal (12).

Nowadays, there is not a test to determine whether a patient who has been treated for chronic CD has been cured or not, considering cure as "elimination of *T. cruzi* parasites from the patient's body following treatment" (308). Besides, there is not even a consensus on what therapeutic response means in the context on *T. cruzi* infection treatment: there is an open debate on how parasitic response modulates clinical outcomes (309). The gold standard to assess therapeutic response is conventional serology seroconversion (1,34). However, as previously mentioned, this method is not viable, as it can take decades for a patient presenting chronic *T. cruzi* infection to assess seroconversion. Some researchers suggest using clearance of the parasite or decrease in parasitemia (evaluated by qPCR) as therapeutic response criterion. However, in the chronic phase of the disease, *T. cruzi* infected patients present low and intermittent parasitemia. Thus, the detection of circulating parasites after treatment by molecular amplification techniques may be useful for determining treatment failure, but never as a surrogate of cure(308). On the other hand, there is an important need of biomarkers of disease outcome, as clinical progression of CD is silent. The availability of biomarkers of therapeutic response would allow clinicians to adequately triage those chronically infected patients that will likely develop cardiac and/or digestive complications from those that will not, administering the limited resources to follow-up and manage *T. cruzi* infected patients in a most efficient manner (12).

In the last decades, research on the biology, function and potential applications of EVs has grown exponentially. One of the most interesting biomedical utilities of EVs is its potential for biomarkers discovery, as EVs provide specificity and sensitivity, are very stable, and can be easily identified in several biofluids (15,217,219,222,255,256,261,289).

In that context, we hypothesized that the use of EVs isolated from plasma of *T. cruzi* infected individuals could provide novel insights in biomarkers development for CD. Thus, in this thesis, we aimed at isolating and assessing the proteomic composition of circulating EVs from *T. cruzi* infected patients presenting different forms of the disease to identify potential biomarkers

of disease outcome for CD. Moreover, we also studied the change of the EVs proteome in cardiac CD patients before and after BZD treatment to identify biomarkers of treatment response. To our knowledge, this is the first molecular characterization and proteomic profiling of plasma-derived EVs purified directly from plasma of *T. cruzi* infected patients presenting different clinical manifestations.

6.1. EVS FROM *T. cruzi* INFECTED PATIENTS: POTENTIAL SOURCE OF BIOMARKERS

6.1.1. Isolation and characterization of EVs from *T. cruzi* infected patients

Although the potential utility for EVs as disease biomarkers has attracted unprecedented interests over the last decade, this field is particularly challenging and complex, especially when working with biological samples. First, there is a very limited quantity of sample material when compared to EVs derived from in vitro cultures. Also, many details of the biospecimen have to be taken into consideration before processing the sample, such as the sample collection procedure, the storage, transportation and handling prior to arrival at the laboratory. Moreover, the composition of EVs is likely to change depending on the characteristics of the donor, and on intrinsic and environmental factors during the infection. However, even though working with biological samples is challenging, EV isolated from plasma of patients will reflect the heterogeneity and complexity of the pathology much better than EVs derived from in vitro cultures (310,311).

As previously mentioned, the EVs field is a recent area of study (177). Consequently, it is constantly evolving, and unfortunately, nowadays there is not a gold standard method to obtain highly pure and well-characterized EVs. Previous studies have shown that the choice of the isolation method severely impacts the purity of the EVs population and the data obtained (311). The research presented in this thesis is a reflection of the constant progression of the field in terms of exploring new approaches to isolate EVs adequate to reach our final goal (Figure 45).

Several research groups have focus on the study of the proteomic content of EVs secreted by *T. cruzi* metacyclic trypomastigotes and epimastigotes in vitro (252,264,265,312). However, this is the first proteomic characterization of circulating plasma-derived EVs isolated from *T. cruzi* infected patients. Two groups of *T. cruzi* infected individuals have been included: one of patients presenting the cardiac form of the disease, and one of individuals presenting the indeterminate form. Moreover, two control groups have been included: one of cardiac patients non presenting CD, and one of healthy individuals.

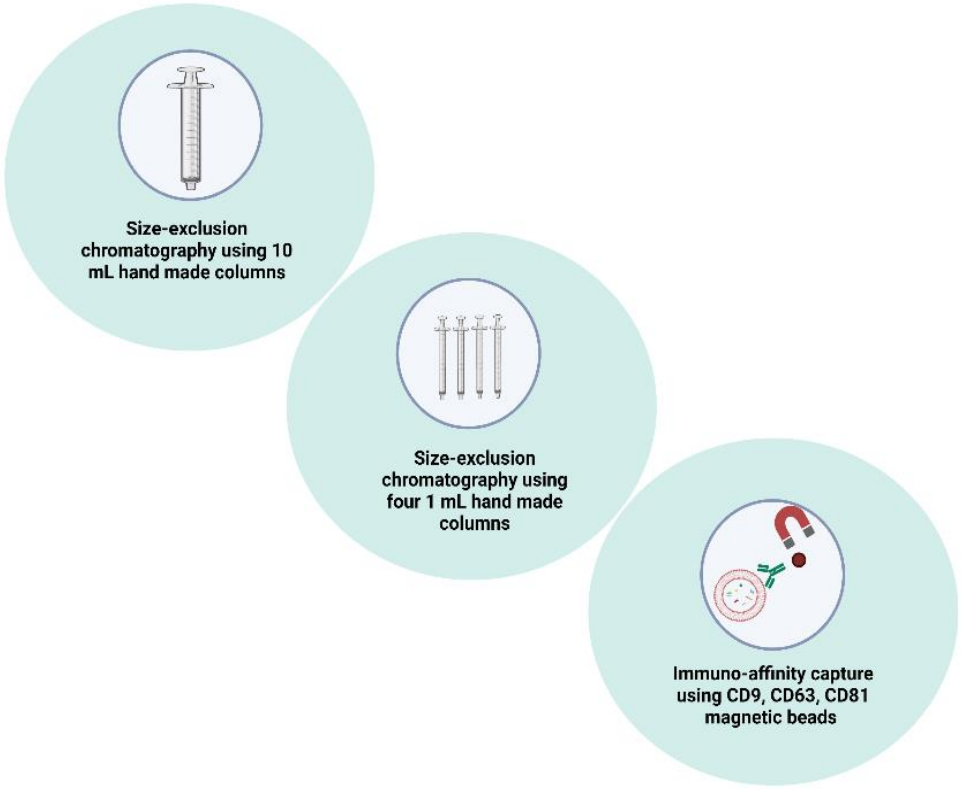


Figure 45. Summary of the EVs isolation techniques assessed during this research. Created with BioRender.com.

The first proteomic analysis, based on one particular cardiac CD patient presenting very specific clinical characteristics, allowed us to identify one parasite (PPDK) and 288 human proteins. The PPDK plays a central role in the metabolism of *T. cruzi* glycosomes and has been shown to be upregulated when trypomastigote forms are incubated with the extracellular matrix, an obligatory step before host-cell invasion and differentiation of trypomastigote into amastigote forms (313). Unfortunately, the PPDK has not been detected in any of the other proteomic analysis performed in this thesis (including the ones performed in infected cardiac cells). However, other proteomic studies have detected other PPDK proteins in EVs secreted by *T. cruzi* epimastigotes and in vesicles released by *T. cruzi* infected THP-1 cells (264,285).

When focusing on human proteins, we were able to detect a cluster of EVs associated proteins clearly upregulated during *T. cruzi* infection that disappeared or were downregulated after treatment (Table 9, Figure 22). This was, for example, the case for mannan-binding lectin serine protease 2, which has been associated with chronic chagasic cardiomyopathy (314,315). In this first proteomic analysis, we also detected human proteins that have already been described as promising biomarkers of therapeutic response for CD, such as the APOA1 and FBN. APOA1 is a common plasma protein that co-isolate with EVs, but is not directly related to vesicles

(316), while FBN can be associated to EVs (317). Both proteins were found upregulated before treatment and returned to normal values three years after treatment (110). Here, its levels were clearly upregulated during *T. cruzi* infection. However, we did not find relevant differences before and after treatment with BZD.

Although the preliminary results were very promising, these were interpreted with caution because they represent a single and very particular clinical case. This cardiac CD patient presented severe cardiopathy, heart transplantation, reactivation of CD after immunosuppression therapy, and positive PCR in the samples collected before anti-parasitic treatment. Moreover, in this case, the EVs were isolated by SEC using 10 mL columns, and a pool of fractions 7-10 was analyzed by LC-MS/MS. The SEC technique separates according to the size of the particles, enabling the isolation of circulating EVs secreted by all cell types and organisms, similarly to the in vivo scenario. Previous work from our group has demonstrated that SEC enables the enrichment of circulating EV populations in early fractions, excluding most of the plasma contaminants (294). However, abundant plasma proteins are always present in EVs preparations obtained by SEC (294). Taking this into consideration, the PPDK and the human proteins detected could be associated to EVs, or could be plasma proteins eluting in the last fractions containing EVs added to the pool. Thus, we decided to apply a new strategy for EVs isolation, adapting the SEC protocol to one mL sepharose handmade columns.

The EVs isolated with one mL columns were characterized by multiples techniques, such as BBA and microscopy. As can be observed by the BBA profile, the use of this new approach allowed us to concentrate EVs in one specific fraction, eliminating abundant plasma contaminants that can elute in distant fractions (Figure 23,

Figure 24 24). We also performed a detailed morphological analysis of the EVs found in plasma of *T. cruzi* infected individuals by cryoEM methodology, observing a high heterogenic profile among isolated vesicles (Figure 25A). These were not unexpected results: previous work from other research groups has shown a great morphological diversity on EVs isolated from biological fluids (318,319), even among EVs purified from a single cell type (299). Thus, high morphological heterogeneity was expected. Consistently with previous publications (299,319), most vesicles were classified as single vesicles. Double and triple vesicles followed in terms of abundance. Less common were oval vesicles, tubule vesicles, incomplete and pleomorphic EVs (Figure 25B). The large majority of the vesicles were between 0 to 200nm (Figure 25C). In our colleagues' work, however, most vesicles presented a diameter around 0 to 100nm (299,319). These variations could be explained by the differential methods used for EVs isolation or the differences among biological fluids.

Once the EVs were isolated and characterized, we performed a second proteomic analysis increasing the sample size and including samples of *T. cruzi* infected patients presenting the indeterminate form of the disease. In this analysis, several human proteins were identified, including a large cluster of EV markers (Table 10, Figure 26B). Among the human proteins, we detected APOA1, previously described as potential biomarker for therapeutic response. However, no we did not find any differences in its levels among pre and post-treatment samples. Unfortunately, parasite proteins were not detected.

At this point, we decided to implement a new approach to isolate the EVs. Previous results from our group have shown that in *P. vivax* infection, the immunoaffinity capture technique using CD71 allows the detection of high number of parasite proteins in plasma-derived EVs (251). The immunoaffinity capture technique is based on the separation of specific EVs according to their surface protein expression. Biomarkers of exosomes are heterogeneous, and are not ubiquitous among different cell types. Thus, using the immunoaffinity capture approach the EV preparations obtained are always a vesicle subpopulation expressing the marker(s) used for isolation. In the case of *P. vivax* infection, the cells preferentially invaded by *P. vivax* parasites are reticulocytes, which express CD71 in their surface. As there are not known specific EV-markers derived from *T. cruzi* infected cells, we performed an in vitro analysis of the EV markers found in vesicles secreted by infected HCF and HCM, one of the main target cells for *T. cruzi* parasites (Figure 27). The finding of a specific EV marker from infected cardiac cells would mean a breakthrough in order to be able to isolate this specific EVs sub-population.

As we identified CD9, CD63 and CD81 in the cell surface and EVs secreted by HCM and HCF (Figure 29A), we decided to perform the immunoaffinity capture using a commercially available kit coupling magnetic beads to anti-CD9, CD63 and CD81 antibodies in a larger number of samples (Figure 34). Other studies using magnetic beads coupled to tetraspanins or specific EV markers of the cell population of interest have shown that immunoaffinity capture kits are interesting options for EV isolation (251,320). Tetraspanins can be found in EVs secreted by most cell types, are not exclusively found in EVs secreted by cardiac cells. Nevertheless, *T. cruzi* can infect all cell types, it is not exclusively found in the cardiac tissue. Moreover, the fact that nowadays there is not a specific EV marker of cardiac cells limited our options (300,301).

In the last proteomic analysis we identified 62 parasite proteins with at least 2 UP normalized (Table 13). Among these, 27 were uncharacterized and have not been previously described in EVs secreted by the parasite, neither in EVs secreted by infected THP-1 (252,264,280,285,312). A trans-sialidase was identified with more than two UP. Interestingly, it presented the same accession number than one of the trans-sialidases previously described in

EVs secreted by trypomastigotes (252). Other parasite proteins identified have been previously described in EVs from in vitro parasite culture (although in most of the cases with a different accession number). This is the case of surface glycoproteins (252,312), serine/threonine protein kinase (264), mitogen-activated protein kinase (264), MASP (252,264,312), mucin TcMUCII (252), ATP-dependent DEAD/H RNA helicase (identified only with one UP) (264), RHS (264,312), helicase (264), receptor-type adenylate cyclase (264), Dynein heavy chain (264), protein kinase domain (identified only with one UP) (264), and ATP-binding cassette transporter ABCA1 (312).

In the later analysis, we have been able to identify 62 parasite proteins with at least two UP normalized, increasing significantly the detection of *T. cruzi* proteins. However, although this is the first time that we are working with these figures in terms of high detection of parasite proteins, many of these are only found in a few samples of the cohort. A high number of factors including the parasitemia, the heterogeneity of *T. cruzi* parasites, and the clinical characteristics of the patients might contribute to the variability of parasite proteins identified per patient.

Interestingly, there are a few parasite proteins that are identified in a large number of samples. This is the case of one mucin-associated surface protein (V5AN53), found in one sample of CD cardiac pre-treatment, one sample of CD cardiac post-treatment, and seven samples of *T. cruzi* indeterminate patients; one *T. cruzi* uncharacterized protein (K2NIL0), detected in one sample of CD cardiac pre-treatment, one sample of CD cardiac post-treatment, and six samples of *T. cruzi* infected indeterminate patients, and finally one *T. cruzi* uncharacterized protein (A0A2V2US84), found in three samples of cardiac CD patients before treatment, two of cardiac CD patients after treatment, and two *T. cruzi* infected patients at indeterminate phase. These parasite proteins could be considered markers of the disease.

Although the results were not statistically significant, higher levels of parasite proteins were detected in samples of cardiac CD patients not treated, compared to the same patients after treatment. These results show the potential of EVs as biomarkers of therapeutic response. Interestingly, all patients were PCR negative after treatment, indicating that the detection of parasite proteins in the EVs is a very sensitive technique.

We identified a cluster of 17 parasite proteins found exclusively in EVs secreted by *T. cruzi* infected indeterminate patients. We also found 24 *T. cruzi* proteins exclusively identified in EVs from cardiac CD patients. On the other hand, we did not find differences in terms of parasite proteins identified in patients presenting different cardiac severity (Kushnir I and II). All together, these results indicate that parasite proteins found in EVs are potential biomarkers of disease

outcome. Surprisingly, we have identified more parasite proteins in EVs from *T. cruzi* indeterminate patients than in patients presenting the cardiac form of the disease

Several of the parasite proteins identified present an immunogenic profile, and might be of interest for a better understanding of the disease pathology. Previous studies have shown that proteins contained in EVs secreted by trypomastigotes are immunoreactive antigens (312). Bautista-Lopez and collaborators incubated extracts of EVs secreted by *T. cruzi* parasites with immunoaffinity resins coupled to IgG antibodies from uninfected donors or cardiac Chagas patients, identifying 111 *T. cruzi* immunoreactive proteins, including trans-sialidases, mitochondrial proteins, RHS proteins, proteases, and multiple uncharacterized proteins (312). Interestingly, several of these proteins, such as the RHS and the surface glycoprotein, have been identified in our study exclusively in EVs from cardiac CD patients.

Regarding the parasitemia, high differences were found among the two groups of *T. cruzi* infected individuals (G1 and G2). Most CD patients presenting the cardiac form of the disease were PCR positive before treatment. On the other hand, almost all *T. cruzi* infected patients presenting the indeterminate form were PCR negative before BDZ treatment. Several evidences have shown that the pathogenesis of the chronic myocarditis inducing the cardiac form of CD is directly related to the presence of the parasite, although additional immunological mechanisms can also be involved (309,321). Recent studies using immunohistochemistry and PCR have demonstrated higher frequencies of *T. cruzi* antigens in cardiac CD patients than in patients presenting other forms of the disease (322–324). *T. cruzi* antigens were detected in 100% of myocardium samples from chronic chagasic patients that died due to heart failure (322–324). Moreover, an association between the presence of *T. cruzi* antigens and severe or moderate inflammation has also been demonstrated (323,324). The differences observed in our cohort in terms of parasitemia between G1 and G2 can also be attributed to demographic differences, as the group of cardiac CD patients was recruited mostly in Bolivia, while the group of *T. cruzi* indeterminate patient mostly in Spain. Interestingly, although all *T. cruzi* infected patients presented undetectable parasite levels at the end of the treatment, EVs isolated from three samples collected after parasite treatment in the group of cardiac CD patients still presented parasite proteins. This feature could indicate that the parasite cannot be found in the bloodstream, but could remain hidden in some of the target tissues, indicating treatment failure in these specific patients. Further studies analyzing the follow-up samples of these patients could be very useful to determine the utility of EVs to assess therapeutic response in *T. cruzi* infection.

Parasite strain heterogeneity is something that must be considered. Most of the *T. cruzi* infected patients included in this study are from Bolivia, where exists a high diversity in terms of *T. cruzi* strains (325).

High differences in terms of human proteins can be observed among the different groups. In our analysis, each clinical group of patients clearly showed a distinct pattern. The group of cardiac *T. cruzi* negative individuals is the one presenting higher variability, together with the group of *T. cruzi* infected patients presenting the cardiac form of the disease. Samples from cardiac CD patients before and after treatment presented the closest pattern, and were not able to be distinguished when performing the 2D PLSDA plot. However, when performing the 3D PLSDA plot, both populations could be separated (Figure 38).

Although the average number of human proteins identified in each sample this last analysis was lower than usual, 25 EV markers were found, showing that the preparation was clearly enriched in EVs. Again, we identified APOA1, FBN and CRP, which have been previously described as interesting proteins for CD pathology (110). Interestingly, we detected a cluster of human proteins upregulated in samples of *T. cruzi* infected patients, independently of the clinical status, that disappeared or were downregulated in the two groups of non *T. cruzi* infected patients. The opposite situation has also been observed: human proteins not found in samples of *T. cruzi* infected patients, and identified in samples from cardiac non CD individuals and healthy donors (Figure 40, Annex 6). Moreover, we have identified a cluster of proteins specifically found in samples of *T. cruzi* infected patients presenting the indeterminate form of the disease (Figure 40, Annex 7) or in samples from cardiac CD patients (Figure 40, Annex 8). Thus, these proteins are potential candidates as biomarkers of the disease, and specific markers of disease outcome.

Notably, among the human proteins, we identified a cluster that was present in samples of cardiac CD before treatment that disappeared after treatment with BZD. These proteins, which were not found in G3 and G4, can be potential candidates of biomarkers of therapeutic response (Figure 41). Among these molecules, we identified one MHC class I antigen protein. Curiously, a different MHC class I antigen protein was previously described as a potential biomarker of therapeutic response in our preliminary study performed with circulating EVs from one heart transplanted cardiac CD patient. Unfortunately, although several of the other human proteins previously identified in EVs as potential biomarkers of therapeutic response were found in our preparation when increasing the sample size (this is the case, for example, of the Coagulation factor XIII A, L-lactate dehydrogenase A chain, or Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein), no differences were found among samples before and after treatment. Unfortunately, we could not include EVs samples from patients presenting the

indeterminate form of the disease after treatment. Further studies are needed to assess if those potential markers of therapeutic response present the same behavior in indeterminate *T. cruzi* infected patients.

In some parasitic diseases, such as malaria or African trypanosomiasis, higher circulating levels of EVs have been found in infected individuals compared to healthy donors, and vesicle levels have been associated with clinical symptoms and severity of the disease (258–260,326). However, it is not clear if this is the case of CD. While some studies did not find any significant statistical differences in the number of vesicles in *T. cruzi* infected patients compared to healthy controls (285), others did find differences in terms of concentration, showing that treated patients presented lower concentration of circulating EVs than healthy donors (256). In our case, the first approach showed higher number of human proteins in EVs from the cardiac CD patient than in EVs isolated from plasma of healthy donors (Figure 22). However, as previously mentioned, this was a very particular clinical case. When increasing the sample size and analyzing a larger group of individuals, almost no differences have been found in terms of number of human proteins identified between the different patients' groups. Although a slight decrease was observed, similar numbers of human proteins have been also identified in samples from cardiac patients pre-treatment and post- treatment. However, this could change when analyzing samples extracted several months after treatment. All together, these results were corroborated by the NTA: when analyzing the plasma samples of the different groups, no differences in terms of particle concentration or particle size were found. However, again, a slight decrease in the concentration of particles could be observed between cardiac CD patients pre and post-treatment (Figure 35A).

Although the proteome of EVs released by *T. cruzi* parasites in vitro has been previously reported, the proteomic content of the vesicles found in circulation in CD patients has never been determined. Overall, our study represents the first proteomic analysis of circulating EVs from *T. cruzi* infected patients presenting several forms of the disease (Figure 46). We have demonstrated the association of parasite proteins to EVs isolated from plasma of *T. cruzi* infected individuals. Interestingly, some of the parasite and human proteins identified are disease specific, presenting potential as biomarkers of disease outcome. We have also found important differences in terms of parasite and human proteins among samples before and after BZD treatment.

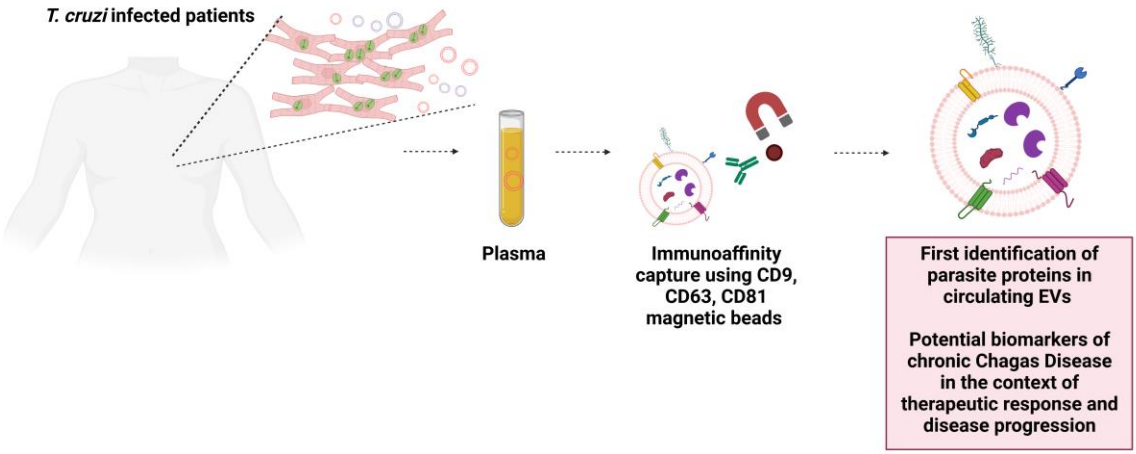


Figure 46. Scheme summarizing our main findings. Plasma-derived EVs from *T. cruzi* infected cells were purified by immunoaffinity capture from chronic CD patients and different potential biomarkers have been identified by mass spectrometry. Created with BioRender.com.

6.1.2. Looking for EV-markers of cardiac *T. cruzi* infected cells

As previously mentioned, we performed an in vitro analysis of the proteomic content of infected HCF and HCM EVs to identify specific markers of *T. cruzi* infected cells. To our knowledge, this is the first proteomic analysis of EVs secreted by *T. cruzi* infected an uninfected cardiac cells. Interestingly, we identified human and parasite proteins exclusively found in EVs secreted by cardiac infected cells which could be used in the future to immunoaffinity capture this specific subpopulation of vesicles.

Previous results from Cronemberger-Andrade and colleagues analyzed the proteomic content of EVs isolated from *T. cruzi* infected and uninfected THP-1 cells, observing a distinct pattern in terms of human and parasite proteins which could contribute to maintain the host inflammatory response (280). *T. cruzi* infected host-cell derived EVs carry proteins from the parasite, as previously described (280,285). Here, we identified 12 parasite proteins in EV secreted by infected HCF, and one parasite protein in EVs secreted by infected HCM (Table 11). Not common parasitic proteins were found between both cardiac cell types. The number of parasite proteins identified is similar to previous findings from other research groups when working with different cell types (280,285).

Here, the isolation of the EVs secreted by infected and uninfected cardiac cells was performed by immunoaffinity capture using CD9, CD63 and CD81 markers, which are only found in eukaryotic cells. Thus, as previously explained, it is very unlikely that the parasite proteins found in the EVs were not directly associated with the EVs from infected HCF and HCM, and were released in the culture SN by *T. cruzi* trypomastigotes. Moreover, as a positive control, the

proteomic content of EVs secreted by *T. cruzi* trypomastigotes was also characterized. Interestingly, we did not find any of the proteins described in EVs secreted by infected cardiac cells in EVs secreted by trypomastigotes (Figure 31).

Most of the parasite family proteins identified have been previously described in EVs from in vitro parasite culture or from in vitro infected cells (again, in most of the cases with a different accession number). This is the case of chaperonin HSP60 (264), tubulin (264,312), glucose-regulated protein 78 (252,312), and mucin like glycoprotein 1 (identified with the same accession number) (252). Again, five trans-sialidases were also identified in EVs from infected HCF. As previously mentioned, several members of this family proteins have been identified in EVs from *T. cruzi* trypomastigotes and in EVs secreted by THP-1 infected cells (252,264,280,285,312). Notably, two of the trans-sialidases previously found in EVs secreted by trypomastigotes by Ribeiro and collaborators were also identified in our study (252). Also, trans-sialidases were identified in EVs isolated from plasma of cardiac *T. cruzi* infected samples pre and post-treatment. In addition to the trans-sialidases, other of the identified proteins has also been found in EVs isolated from plasma of *T. cruzi* infected patients. This is also the case of the leishmanolysin-like peptidase, which has been found in EVs secreted by infected HCF and in two samples of cardiac CD patients collected after treatment (although both were identified with less than two UP).

A total of 1547 human protein groups were identified in HCF, and 1931 in HCM. Higher number of human protein groups were detected in uninfected HCF and HCM compared to infected cells, obtaining similar results than other research groups (280) (Figure 32). Interestingly, we identified a cluster of human proteins present exclusively in infected cardiac cells: fourteen protein groups in EVs from infected HCF, and 32 in EVs from infected HCM (Figure 32). Unfortunately, no common proteins were found among both groups, so we did not identify a universal marker for both infected cardiac cell types. Notably, some of these proteins are specific for the cardiac tissue. The use of several of these markers in combination to further immunoaffinity capture the EVs found in circulation in plasma of *T. cruzi* infected patients could allow to capture specifically those EVs secreted by infected cardiac cells.

6.2. FUNCTIONAL ROLE OF CIRCULATING EVS IN *T. cruzi* INFECTION

Even though the main goal of this thesis was EV biomarkers discovery, we were also interested in trying to have a first glance on the physiological role of EVs found in circulation in CD. It is known that EVs released by *T. cruzi* trypomastigotes or *T. cruzi* infected cells directly affect the host immune-response, potentiating parasite evasion and regulating the inflammatory cells, maintaining a proinflammatory profile (303). However, many questions remain regarding

the role of EVs found in circulation in plasma of *T. cruzi* infected patients. For that reason, we performed classical angiogenic experiments using HUVEC cells, observing that its angiogenic response was dependent on the EV stimulus (Figure 44).

Here, we have shown that EVs found in circulation in plasma of CD patients increase the angiogenic response compared to EVs from healthy donors. To our knowledge, this is the first preliminary study assessing the role of circulating EVs from CD patients in the angiogenic response. Other research groups are currently studying the effect of the EVs secreted by patients presenting different cardiological complications (such as acute myocardial infarction, angina pectoris or stroke) in the angiogenesis, observing a differential response (AP Dantas, unpublished data).

Inflammation and angiogenesis are processes closely interconnected. During inflammation, angiogenesis is initiated by the activation of different cell populations, such as endothelial cells, macrophages, and fibroblasts, which secrete pro-angiogenic factors, promoting neovascularization (327). Moreover, there is growing evidence that the angiogenesis prolongs and intensify the inflammatory response, as new vessels may enhance tissue inflammation by promoting the migration of inflammatory cells (307). The increase of the angiogenesis observed in HUVEC cells treated with EVs from plasma of cardiac CD patients could be related to the high inflammatory profile presented in these individuals. Interestingly, this effect is not caused by EVs secreted by infected HCM, and could be driven by EVs secreted by other infected cell types or EVs secreted by inflammatory cells.

Although these results must be taken as a grain of salt, as it included only three samples for each condition, these are the first essays assessing the effect of circulating EVs from CD patients in the angiogenic response. Further studies including a larger number of samples and patients presenting other forms of the disease are needed.

6.3. FUTURE WORK

6.3.1. Reassessment of the EVs composition

A reassessment of the composition of circulating EVs in *T. cruzi* infections using other EVs isolation methods could be performed to validate the described results. Here, several techniques have been tested, such as SEC, UC and immunoaffinity capture using tetraspanins markers. Among these methodologies, the immunoaffinity is the one that has shown a greater performance for detection of parasite proteins. However, CD9, CD63 and CD81 are highly conserved among eukaryotic cells. Adapting the immunoaffinity capture using specific markers of

cardiomyocytes associated exclusively to *T. cruzi* infected cells could maximize the chances of detection of parasite proteins. Here, we performed a proteomic study of the EVs secreted by *T. cruzi* infected cardiac cells, finding markers of infection associated to the cardiac tissue. The use of these markers to immunoaffinity capture the specific subpopulation of vesicles secreted by infected cardiac cells could result in a very interesting approach.

Apart from parasite proteins, EVs derived from *T. cruzi* infected cells have been shown to contain parasitic nucleic acids (328,329). Moreover, the study of the lipidomic content of the vesicles is also a growing field. Whether these non-proteomic elements are able to regulate the immune response, or interesting for the discovery of new biomarkers in the context of CD, is yet to be determined.

6.3.2. Targeted proteomics

Targeted proteomics is a popular mass spectrometry based protein quantification technique based on the detection of specific proteins of interest. Most targeted proteomics assays are limited to a few dozen proteins per run (330,331). Assessing the protein content of EVs from *T. cruzi* infected patients focusing on these parasite proteins immunogenic, disease specific or potential biomarkers of therapeutic response could be a very interesting approach.

6.3.3. Exploring other biofluids for a better management of the patients

So far, all biomarker candidates described for *T. cruzi* infection have been detected in blood samples. However, blood collection is invasive, and it demands specially trained personnel. In CD endemic areas, health-care access is limited. Developing new tools easy to use and adapted to the reality of affected populations and health systems is still a substantial need (332). Thus, assessing if our findings can be validated in other biofluids easier to collect, store and transport, such as saliva or urine, could be very useful tool particularly for endemic areas.

6.3.4. Larger follow-up of *T. cruzi* infected treated patients

Here, we have analysed plasma EVs from cardiac patients before and after BZD treatment to assess treatment response. Interestingly, although all treated patients were PCR negative at the end of the treatment, parasite proteins could still be detected in EVs in post-treatment samples. The analysis of the proteomic content of those EVs collected six and twelve months after treatment could elucidate the potential role of circulating EVs as biomarkers of treatment response. Moreover, the analysis of post-treatment EVs secreted by *T. cruzi* individuals

presenting the indeterminate form of the disease would be desirable, together with the inclusion of longer longitudinal follow-up of participants.

6.3.5. Biological role of EVs found in plasma of *T. cruzi* infected patients

Classical angiogenic experiments using HUVEC cells showed that the angiogenic response was dependent on the EV stimulus. However, those are very preliminary results, as were performed including only three samples for each clinical group. Further experiments are needed, increasing the sample size and adding samples of *T. cruzi* infected patients presenting the indeterminate form of the disease. Assessing if the increase of the angiogenesis observed when using EVs isolated from cardiac CD patients is maintained when using EVs collected after BDZ treatment may also be of great interest.

Although many questions remain, assessing the biological role of plasma EVs from *T. cruzi* infected patients on the human immune response remains as one of the main ones. In that context, a collaboration with Dr. Torrecilhas was initiated recently. Preliminary studies evaluating the effect of circulating EVs when incubated with THP-1 cells in the secretion of cytokines have been performed (256). Further work is needed in order to understand how the EVs found in plasma of *T. cruzi* infected patients are involved into the immunomodulation occurring in CD.

7. CONCLUSIONS

- To our knowledge, this is the first molecular characterization and proteomic profiling of plasma-derived EVs purified directly from plasma of chronic CD patients with different clinical manifestations.

- Immunoaffinity capture using CD9, CD63, and CD81 is presented as the best method to purify circulating EVs from chronic CD patients.

- Circulating EVs derived from *T. cruzi* infected cells (amastigote forms) and purified by immunoaffinity capture showed a clear enrichment of parasite proteins.

- We have identified human and parasite proteins which are present or upregulated in plasma-derived EVs from cardiac chronic CD patients before treatment that are absent or downregulated following treatment, showing the potential of EVs to identify new biomarkers of therapeutic response for chronic CD.

- We have identified human and parasite proteins specifically associated to the indeterminate or cardiac form of the disease. These results encourage the use of circulating EVs as potential biomarkers of disease progression.

- EVs derived from in vitro *T. cruzi*-infected cardiac cells and purified by immunoaffinity capture using CD9, CD63, and CD81 showed a clear enrichment of parasite proteins. Molecular cargo present in EVs secreted from infected cells is different from the EVs secreted directly by the parasite.

- Specific human proteins have also been identified in EVs secreted by cardiac infected cells, which could be used to immunoaffinity capture this particular subpopulation of EVs in biological samples of *T. cruzi* infected patients.

- A preliminary functional assay using EVs derived from three cardiac CD patients showed a clear tendency to increase angiogenesis. Further studies using samples of patients presenting the indeterminate form of the disease and increasing the sample size are needed.

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9. CONTRIBUTIONS

This chapter presents the main publications and manuscripts that have been produced in the context of this thesis. Note that the contributions are ordered in the same way the findings related to them were presented in the thesis:

1. Cortes-Serra N, Mendes MT, Mazagatos C, Segui-Barber J, Ellis CC, Ballart C, Garcia-Alvarez A, Gállego M, Gascon J, Almeida IC, Pinazo MJ, Fernandez-Becerra C. **Plasma-Derived Extracellular Vesicles as Potential Biomarkers in Heart Transplant Patient with Chronic Chagas Disease.** Emerg Infect Dis. 2020 Aug;26(8):1846-1851. doi: 10.3201/eid2608.191042. PMID: 32687028; PMCID: PMC7392439.
2. Cortes-Serra N, Gualdron-Lopez M, Pinazo MJ, Torrecilhas AC, Fernandez-Becerra C. **Extracellular Vesicles in *Trypanosoma cruzi* Infection: Immunomodulatory Effects and Future Perspectives as Potential Control Tools against Chagas Disease.** Journal of Immunology Research, vol. 2022, Article ID 5230603, 11 pages, 2022. https://doi.org/10.1155/2022/5230603 (accepted article).
3. Cortes-Serra N, Losada-Galvan I, Pinazo MJ, Fernandez-Becerra C, Gascon J, Alonso-Padilla J. **State-of-the-art in host-derived biomarkers of Chagas disease prognosis and early evaluation of anti-*Trypanosoma cruzi* treatment response.** Biochim Biophys Acta Mol Basis Dis. 2020 Jul 1;1866(7):165758. doi: 10.1016/j.bbadis.2020.165758.

Other relevant contributions outside the main objectives of this thesis:

1. Cortes-Serra N, Pinazo MJ, de la Torre L, Galizzi M, Gascon J, Bustamante JM. **Diagnosis of *Trypanosoma cruzi* Infection Status using Saliva of Infected Subjects**. Am J Trop Med Hyg. 2018 Feb;98(2):464-467. doi: 10.4269/ajtmh.17-0141. Epub 2018 Jan 4. PMID: 29313472; PMCID: PMC5929177.
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State-of-the-art in host-derived biomarkers of Chagas disease prognosis and early evaluation of anti-*Trypanosoma cruzi* treatment response

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ABSTRACT

Chagas disease is caused by infection with the parasite *Trypanosoma cruzi*, which might lead to a chronic disease state and drive to irreversible damage to the heart and/or digestive tract tissues. Endemic in 21 countries in the Americas, it is the neglected disease with a highest burden in the region. Current estimates point at ~6 million people infected, of which ~30% will progress onto the symptomatic tissue disruptive stage. There is no vaccine but there are two anti-parasitic drugs available: benznidazole and nifurtimox. However, their efficacy is variable at the chronic symptomatic stage and both have frequent adverse effects. Since there are no prognosis markers, drugs should be administered to all *T. cruzi*-infected individuals in the indeterminate and early symptomatic stages. Nowadays, there are no tests-of-cure either, which greatly undermines patients follow-up and the search of safer and more efficacious drugs. Therefore, the identification and validation of biomarkers of disease progression and/or treatment response on which to develop tests of prognosis and/or cure is a major research priority. Both parasite- and host-derived markers have been investigated. In the present manuscript we present an updated outlook of the latter.

1. Introduction

Chagas disease is caused by infection with the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). It affects ~6 million people around the world, mostly in Latin America [1], and it is estimated that approximately ~30% of them will eventually progress to the symptomatic life-threatening stages of the disease that entail cardiac and/or digestive tissues disruptions [1]. This organ involvement, which can lead to the Chagas disease characteristic mega-syndromes, does not occur immediately but it is rather observed upon several years of infection (even 10 to 30 years) [2,3]. Unfortunately, at present there is not a way to predict this possible progression. Being able to anticipate it would be crucial to tailor the needs of patients based on their risk of progression. This would mean a breakthrough in the management of the disease considering the limited resources available. Thus, finding prognosis markers to assess the risks of progression and predict the early stages of organ involvement is one of the main objectives of biomarker research projects in the field.

On the other hand, despite there are two drugs to treat *T. cruzi* infection, benznidazole (BNZ) and nifurtimox (NFX) [2,3], both have a

Abbreviations: ADA, adenosine deaminase; ACE2, angiotensin-converting enzyme 2; ApoA1, apolipoprotein A1; BNP, brain natriuretic peptide; cTnT, cardiac troponin T; CRP, C-reactive protein; CKMB, creatine kinase-MB; DNA, deoxyribonucleic acid; ETP, endogenous thrombin potential; EVs, extracellular vesicles; FBN, fibronectin; F1 + 2, fragment 1 + 2; Gal-1, galectin-1; GWAS, genome-wide association study; GPx, glutathione peroxidase; HLA, human leukocyte antigen; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; LAMP, loop-mediated isothermal amplification; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; miRNAs, micro RNAs; NO, nitric oxide; NT-proBNP, N-terminal portion brain natriuretic peptide; PAP, plasmin-antiplasmin complexes; PCR, polymerase chain reaction; ROS, reactive oxygen species; RPA, recombinase polymerase amplification; RNA, ribonucleic acid; Se, selenium; SNPs, single nucleotide polymorphisms; SNO, S-nitrosylation; TIMP-1, tissue inhibitor of metalloproteinase 1; TIMP-2, tissue inhibitor of metalloproteinase 2; TNF-α, tumor necrosis factor alpha; TGFB1, transforming growth factor beta 1; TGFB2, transforming growth factor beta 2; VCAM-1, vascular cell adhesion molecule-1

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poor safety profile that forces clinicians to discontinue a high percentage of treatments [4–6]. Besides, although BNZ and NFX have a very good efficacy against the acute stage of the disease, it is at the chronic stage that the infection is generally diagnosed and treated. By then drugs efficacy is reduced. Limitations of currently available drugs unveil the requirement of biomarkers for the early assessment of treatment response so that a closer and accurate follow-up of treated patients can be made. Availability of such biomarkers would allow the development of tests of cure based on them. These tests would also be very useful in the search of safer and more efficient new therapeutic options. A major obstacle for the clinical evaluation of new treatments is that the current "gold standard" of cure is the negative seroconversion of treated patients, which occurs years or decades after treatment [7,8]. This time scale is impractical from the viewpoint of the daily clinical management of patients, as well as for the interpretation of the outcome of clinical trials with new drugs or with new regimens of existing ones. Thus, it is urgent to identify and validate biomarkers for the early assessment of therapeutic efficacy too.

Finding reliable biomarkers of disease progression and/or treatment response would mean the greatest leap forward in the history of Chagas disease since its discovery in 1909 by Dr. Carlos Chagas. For this reason there are numerous research groups devoted to the search of both host-derived and *T. cruzi*-derived biomarkers [9]. With the aim to host discussions on the subject and nurture joint investigations appeared the NHEPACHA network [10]. Since its inception in 2012 it has produced some important advances in the field as a result of multidisciplinary and transnational collaboration, like a paradigm shift proposal for the treatment of chronically infected people [11], or the elaboration of the first Target Product Profile document for a test to early address treatment response [9]. Standing on these and other works, it is the purpose of this article to review the research on host-derived biomarkers for Chagas disease providing an updated overview of the advances made, and outlining state-of-the-art investigations in the field.

2. Host-derived biochemical markers for the management of Chagas disease

Research on biochemical markers gathered attention in the last years due to their potentially simple analysis and low cost, which makes them easy to implement in middle- and low-income countries [9]. In the case of Chagas disease, several biomarkers have been proposed for the assessment of cardiac and/or digestive involvement [12] (Fig. 1). In addition, there are also some examples of biochemical markers proposed for the early evaluation of treatment response [12]. We summarize all biochemical markers identified so far in the context of Chagas disease and their proposed application in Table 1. Unfortunately, despite recent advancements, we are yet far from having one single molecule that meets all the required expectations. Due to the complexity of the chronic stage of the disease, the option of using a battery of biomarkers rather than relying on a single one to assess treatment response and/or anticipate pathological progression of the infection appears more likely [9].

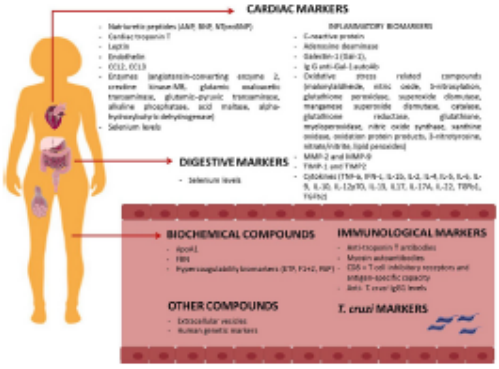

Fig. 1. Summary of Chagas disease biomarkers under research.

2.1. Biochemical markers for the evaluation of treatment response

Setting the focus on biomarkers of treatment response, there are two main groups of molecules identified up to now: those related to the metabolism of lipids [13,14], and those associated to the hypercoagulability state [15,16].

2.1.1. Markers based on lipid metabolism intermediates

A study by Santamaria and co-workers with sera from 37 adult Chagas disease patients and 37 healthy subjects showed that apolipoprotein A1 (ApoA1) and certain fragments of ApoA1 and fibronectin (FBN) proteins were interesting for the evaluation of treatment response. Higher levels of fragments of ApoA1 and FBN were found in serum samples of *T. cruzi* seropositive patients in comparison to the control group. Contrarily, full-length Apoa1 levels were lower in *T. cruzi* infected individuals compared to healthy donors [13]. Such altered levels of ApoA1, its specific fragments thereof, and a fragment of FBN returned to normal in 43% of the studied *T. cruzi*-infected subjects three years after NFX treatment [13]. These results have been recently validated in a cohort of 30 *T. cruzi* seropositive children treated with BNZ [14]. In the latter study, ApoA1 and FBN fragments were absent at the end of BNZ treatment in a significant part of the cohort (66.6% and 53.3% of the children respectively for ApoA1 and FBN) [14]. Also, correlation between seroconversion of the children upon treatment and absence of detection of ApoA1 and FBN fragments in serum samples was observed in 100% and 96.6% of the cases, respectively [14].

2.1.2. Hypercoagulability state biomarkers

The presence of a hypercoagulability state in *T. cruzi*-infected patients was described a few years ago [15,16], which contrasted with other report by Melo and co-workers [17]. Notwithstanding, at the clinical level, the presence of a coagulation state condition could be hinted upon the description of the occurrence of thromboembolic events in *T. cruzi*-infected patients who did not show any signs of cardiomyopathy [18]. In 2016, some of us published the results of a clinical study with plasma samples from 56 chronically *T. cruzi*-infected adult patients and reported that a high percentage of them had statistically significant altered levels of the hypercoagulability markers prothrombin fragment 1 + 2 (F1 + 2) and endogenous thrombin potential (ETP) [19]. These two markers, which had not been looked upon by Melo et al., were abnormally expressed in respectively 77% and 50% of the patients [19]. Moreover, after BNZ treatment, both markers returned to and remained at their normal levels in respectively 76% and 96% of the patients by 36 months upon end of treatment [19]. Among the rest of hypercoagulation markers that were evaluated in that study, plasmin-antiplasmin complexes (PAP) also showed good results in terms of percentage of patients that returned to normal levels upon treatment (94% of them) [19]. However, PAP was found altered in only 32% of the studied *T. cruzi*-infected participants before treatment and it was thus discarded from further consideration [19].

2.2. Biochemical markers of disease progression

Besides the identification and validation of biomarkers for the early evaluation of treatment response, the other main area of research encompasses a whole series of studies to find biomarkers of pathogenesis progression. Their availability would promote the development of diagnostic tools based on them that could allow clinicians to adequately triage those chronically infected patients that will likely develop cardiac and/or digestive tissue disruptions from those that will not. Having such tests would permit to administer the limited resources to follow-up and manage Chagas disease patients in a most efficient manner.

Table 1
List of biochemical markers described up to date. Markers are ordered as they appear in the text. (Table 1 continues in next page).

Biomarker name (acronym)	Application evaluated (higher levels correlate to)	References
ApoA1, FBN	Therapeutic response[a]	[13,14]
F1 + 2 and ETP	Therapeutic response[a]	[19]
BNP	Cardiac damage progression	[20,22,23,25–27,30,43]
NTproBNP	Cardiac damage progression	[20,24,28]
cTnT[b]	Cardiac damage progression	[28,47]
Leptin	Cardiac damage progression	[29]
ACE2	Cardiac damage progression	[30]
GOT, GPT, ALP, AM, alpha-HBDH	Cardiac damage progression	[31]
Selenium (Se)	Cardiac and digestive disease progression	[32]
Endothelin	Cardiac damage progression (controversial)	[34,35]
CRP[b]	Cardiac damage progression	[37–39]
ADA[b]	Cardiac damage progression	[37]
Gal-1, anti-Gal-1 auto-antibodies	Cardiac damage progression	[40]
NO[b] and SNO[b]	Cardiac damage progression	[41,48]
MMP-2[b], MMP-9[b]	Cardiac damage progression (controversial)	[20,42–46]
TIMP-1[b], TIMP-2[b]	Cardiac damage progression	[20,46]
CKMB, troponin I, TGFβ1, and TGFβ2	Cardiac damage progression	[20,36,43,47]
Biomarker name (acronym)	Application evaluated (higher levels correlate to)	References
GPx, superoxide dismutase[b]	Cardiac damage progression	[48]
Malonylaldehyde, glutathione, glutathione peroxidase, superoxide dismultase, manganese superoxide dismutase	Cardiac damage progression	[49,51]
Myeloperoxidase, nitric oxide synthase, xanthine oxidase, oxidation protein products, 3-nitrotyrosine, nitrate/nitrite	Cardiac damage progression	[50,51]
Lipid peroxides	Cardiac damage progression	[51]

[a] Therapeutic response is associated with a decrease in the markers levels.
[b] These markers with inflammatory mediators function are described separately in their own section in the text.

Cardiac damage is the most common clinical outcome observed in the symptomatic state of the disease [2,3]. Thereby, markers of cardiac damage progression have been the most studied for Chagas disease (Fig. 1). It is estimated that between 20% - 30% of *T. cruzi* chronically infected people will develop cardiomyopathy, and an early indicator of disease prognosis would help to prioritize treatment to those patients with a high risk of developing complications. Multiple biomarkers have been associated with Chagas disease cardiomyopathy (Table 1). However, although it has been proved that their levels increase accordingly to the severity of the damage, most of them are not able to distinguish between Chagas disease cardiomyopathy and other cardiomyopathies [20].

Natriuretic peptides (brain and atrial) were among the first markers of cardiac disease progression ever considered. These are released under conditions of myocardial stress and have been shown to be increased in *T. cruzi* experimentally infected animals [21], and in Chagas disease patients with cardiomyopathy [22]. Furthermore, levels of natriuretic peptides correlated to clinical prognosis [23]. The N-terminal portion of brain natriuretic peptide (NT-proBNP) could be a better predictor than BNP itself, due to its high stability [24]. Both peptides are strong predictors of mortality, and are some of the most well characterized markers for assessing early cardiac damage and predicting heart failure outcome. Measurement of BNP levels has also been suggested for the prognosis of patients presenting left ventricular systolic dysfunction, one of the typical signs of cardiac Chagas disease [22,25–27].

The use of natriuretic peptides in the context of Chagas disease has also been studied in combination with other host-derived molecules. For example with cardiac troponin T (cTnT), which is a marker of ischemia and inflammation (see next section for further details). A study assessing it in combination with NT-proBNP that included samples from 137 *T. cruzi*-infected patients with several forms of the disease found that levels of both markers were increased in those individuals with cardiomyopathy in comparison to the asymptomatic ones [28]. Moreover, their values were increased accordingly to the severity of the cardiomyopathy [28]. Plasma leptin levels and their relation to different forms of the disease were also studied in combination with NT-proBNP in 52 *T. cruzi*-infected patients, and those patients with heart failure had higher levels of NT-ProBNP and lower levels of leptin than controls [29].

Angiotensin-converting enzyme 2 (ACE2) is another potential prognostic biomarker that has been evaluated. Wang and colleagues showed that ACE2 activity was significantly increased in those patients with signs of heart failure, but it was not so in patients without systolic dysfunction [30]. Moreover, plasma ACE2 levels significantly correlated with clinical severity and echocardiographic (ECHO) parameters indicative of this [30]. Similarly to cTnT and leptin, ACE2 activity was also compared to BNP levels in order to predict cardiac death and need of heart transplant, finding an additive predictive value when both markers were used in combination [30]. Other enzymes that have been suggested as possible markers for early cardiac damage are glutamic oxaloacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT), alkaline phosphatase (ALP), acid maltase (AM) and alpha-hydroxybutyric dehydrogenase (alphaHBDH), and their levels were found to be significantly altered in chagasic patients [31]. Very interestingly, authors of that reference suggested that the finding of those released enzymes in patients without clinical evidence could represent good biomarkers of early myocardial damage [31].

On the other side, the measurement of selenium (Se) levels has also been valued as a disease progression marker for cardiac as well as also for digestive manifestations (Fig. 1; Table 1). In a cohort of 170 *T. cruzi*-infected people, Rivera and co-workers found that Se levels were lower in patients presenting the cardiac form of the disease compared with healthy donors or asymptomatic individuals [32]. Moreover, such decrease of normal levels of Se was significantly correlated with malfunction of the ventricular ejection fraction. Low Se levels were also found in 6 out of the 10 *T. cruzi*-infected patients with digestive megasyndromes [32]. In fact the use of Se as dietary supplementary was suggested as a possible therapeutic strategy to preserve heart function in patients presenting the indeterminate form of the disease [33].

Finally, another biochemical biomarker that has been researched for Chagas disease is endothelin 1, but the available data about it is yet controversial. A study performed in 2001 showed that plasmatic levels of endothelin 1 were elevated in patients presenting *T. cruzi* infection

related cardiomyopathy [34]. However, Garcia-Alvarez and colleagues were not able to replicate those results, and reported in another study that similar plasmatic levels of endothelin 1 were observed in *T. cruzi* seropositive patients and control individuals [35]. Moreover, the group of infected subjects presenting the undetermined form of the disease had even lower levels of endothelin 1 than controls [35].

2.2.1. Biochemical markers of inflammation

Although the molecular mechanisms of Chagas disease pathogenesis are yet largely unknown, the presence of an inflammatory environment is a common feature to both cardiac and/or digestive tissue disruptions. Such continuous inflammation would lead to the occurrence of organ mega-syndromes, severe indicators of symptomatic chronic *T. cruzi*-infection. Thereby, the prognostic value of host biochemical markers with inflammatory mediator function has also been evaluated. For instance C-reactive protein (CRP), which is liberated during the acute phase of inflammation and its serum levels associate to vascular inflammation and development of cardiovascular events [36]. Several studies have evaluated this protein as a marker for the progression of Chagas disease, showing an association between chronic inflammation, cardiac manifestations and CRP levels [37,38].

Other interesting marker under research is the enzyme adenosine deaminase (ADA). ADA regulates adenosine levels and its activity increases as a consequence of hypoxia and inflammation associated with immunologic events [39]. In the same study, which included serum samples from 28 healthy individuals and from 82 *T. cruzi*-infected people presenting asymptomatic and symptomatic (cardiac) forms of the disease, it was shown that CRP and ADA levels linearly increased in connection to disease severity, and further correlated with the observed ECHO and electrocardiographic (ECG) parameters indicative of this state [39].

Galectin-1 (Gal-1), found in human heart tissue, is also involved in immunological and inflammatory processes. A study with serum samples from healthy donors and patients in the acute and chronic phases of Chagas disease showed that anti-Gal-1 IgG auto-antibodies specifically correlated with cardiac damage severity caused by *T. cruzi* infection as they were shown to be absent in non-related cardiomyopathies [40]. Moreover, levels of Gal-1 were upregulated in cardiac tissue from patients presenting chronic *T. cruzi* infection, compared to cardiac tissue from healthy individuals [40].

In the presence of excessive oxidative and nitric oxide (NO) stress, a post-translational modification of cysteine residues of host proteins can occur. This modification, called S-nitrosylation (SNO), can affect cellular homeostasis and contribute to disease development. Recently, Zago and co-workers have shown that SNO modifications found in peripheral blood mononuclear cells from 53 Chagas disease patients were differentially abundant according to disease state, having the potential to identify disease severity [41].

Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) have also been investigated in the context of *T. cruzi* infection, but their participation in the disease progression is yet subject to controversy. A cross-sectional study with plasma samples from 144 patients at different stages of Chagas disease and 44 samples from healthy donors showed that patients had increased levels of MMP-2 and MMP-9 [42]. This work also reported that patients presenting ECG abnormalities had a significant increase of both enzymes compared to patients presenting the indeterminate form [42]. In contrast, another study with serum samples from 193 *T. cruzi*-infected individuals observed an increase of the MMP-2/MMP-9 ratio that was associated with severity of the cardiac form of the disease [20]. The study authors claimed that this ratio could be useful for assessing the progression from early inflammation to late fibrosis [20]. *T cruzi*-infected subjects presented progressively higher levels of MMP-2, tissue inhibitor of metalloproteinase 1 (TIMP-1, inhibitor of MMP-9) and tissue inhibitor of metalloproteinase 2 (TIMP-2, inhibitor of MMP-2) that paralleled cardiac severity [20]. In line with that, Clark and colleagues have shown that serum levels of MMP-2 and TIMP-1 increased progressively in 85 individuals presenting cardiac structural changes, either in early or late stages of the disease [43]. In comparison, other studies performed using serum and whole blood samples suggest that MMPs are differentially involved in Chagas disease cardiomyopathy: while MMP-2 would be associated to regulatory cytokines, MMP-9 would be correlated with inflammatory ones [44,45]. Thus, MMP-2 would present a cardiac-protective and regulatory function, favoring the indeterminate form of the infection, and MMP-9 would promote an inflammatory atmosphere, favoring the development of the cardiac form [44,45].

Similarly to what occurs with BNP and NT-proBNP, high levels of creatine kinase-MB (CKMB) indicate extensive damage and worse prognosis in patients suffering heart failure. Thus, this compound is commonly used as a measure of heart failure severity, and has been suggested as a possible biomarker for Chagas disease [20,46]. A first study by Okamoto and colleagues assessing serum levels of BNP, NTproBNP, CKMB, troponin I, MMP-2, MMP-9, TIMP-1, TIMP-2, transforming growth factor beta 1 (TGFB1), and transforming growth factor beta 2 (TGFB2), showed no differences in biomarkers levels when stratifying patients by cardiac stage and *T. cruzi* infection status [20]. This was the first time that CKMB was examined as a prognostic marker in Chagas cardiomyopathy. In that study, Okamoto and co-workers saw that troponin I levels (among others) rose in relation with an increasing severity of the disease stage, but unfortunately it did not distinguish between Chagas cardiomyopathy and other cardiomyopathies [20]. However, Sherbuk and colleagues showed that CKMB, together with BNP, NTproBNP and MMP2, were significantly associated with mortality among patients presenting severe Chagas cardiomyopathy [46]. Similarly, the conclusions from the cross-sectional case control retrospective study by Keating and co-workers, which measured CKMB, troponin, myoglobin, VCAM, NTproBNP as well as the cytokines IFN-γ, IL-6, IL-10 and TNF-α (see next section for more information about studies involving cytokines) pointed out that a clear pattern of inflammatory biomarkers was solely observed in those subjects presenting with the more severe cardiac symptomatic stages [36].

As it was mentioned above, measuring serum levels of cTnT was analyzed as prognosis test of cardiac damage progression. Determining cTnT levels with a highly sensitive assay in serum samples from 26 healthy subjects and 179 chronically infected subjects concluded that those were significantly higher in patients suffering cardiomyopathy compared to the rest of groups [47]. Moreover, authors of that work indicated that cTnT value correlated with the severity of that cardiomyopathy [47]. Notably, CRP and IL-6 levels followed the same trend as cTnT changes [47].

Finally, NO levels have been also reported to be significantly higher in patients with cardiomyopathy compared with asymptomatic patients and healthy donors [48–51]. This increase of NO levels in serum correlated with an increase of TNF-α, and a reduction in glutathione peroxidase (GPx) and superoxide dismutase [48]. Alterations in the oxidant/antioxidant balance had been previously reported in a cohort of 80 *T. cruzi* infected patients, 50 healthy individuals, and 20 non-chagasic cardiomyopathy subjects [49]. That study showed that *T. cruzi*-infected patients presented higher levels of malonylaldehyde and lower levels of glutathione, glutathione peroxidase, superoxide dismutase and manganese superoxide dismutase than healthy individuals. Patients presenting cardiomyopathy but negative for *T. cruzi* infection presented insignificant higher plasma malonylaldehyde levels compared to healthy patients, and their plasma antioxidant defense capacity was not compromised [49]. Years later, the same group of researchers studied the role of inflammatory mediators such as myeloperoxidase, inducible nitric oxide synthase (iNOS) and xanthine oxidase, in the stimulation and sustenance of oxidative and nitrosative stress response in plasma samples of *T. cruzi* seropositive and seronegative patients [50]. Those infected presented a significant increase in myeloperoxidase activity and protein level, advanced oxidation protein products, and 3-nitrotyrosine levels compared to healthy donors. However, plasma levels

of xanthine oxidase and nitrate/nitrite contents were not altered. A correlation between increased myeloperoxidase activity and protein 3-nitrotyrosine formation was found, suggesting that myeloperoxidase could contribute to protein nitration and thus to oxidative and nitrosative damage in Chagas disease patients [50]. More recently, the role of other markers of inflammation and oxidant/antioxidant status was studied in a cohort of 116 *T. cruzi* seropositive patients characterized as clinically-symptomatic or asymptomatic, 45 seronegative healthy individuals, and 102 *T. cruzi* seronegative patients presenting cardiac problems. Consistent with previous findings, seropositive subjects showed an increase in sera or plasma levels of myeloperoxidase, advanced oxidation protein products, nitrite, lipid peroxides and malonylaldehyde, and a decrease in superoxide dismutase and glutathione compared to healthy controls. Interestingly, myeloperoxidase and lipid peroxides levels correlated with clinical disease state, being potential biomarkers candidates for evaluating Chagas disease clinical severity [51].

2.3. Markers associated with the host immune state

The study of cytokines has also been suggested as a relevant tool for assessing cardiac disease progression [36]. These and other biomarkers associated with the host immune state identified so far for Chagas disease are summarized in Table 2. In 2014, a study by Sousa et al. involving plasma samples of 176 *T. cruzi* infected people and 24 healthy individuals showed that the expression of plasma inflammatory cytokines, such as IFN-γ, TNF-α, IL-6, and IL-1β, was higher in those with cardiac form of the disease [52]. These results were consistent with previous findings where high levels of TNF-α were found in patients suffering Chagas disease cardiomyopathy [48,53]. Poveda and colleagues also found a higher expression of IFN-γ in serum samples from Chagas disease patients with cardiomyopathy compared to those with the indeterminate form [54]. By contrast, indeterminate *T. cruzi* infected patients had a higher expression of IL-10 when compared with that of individuals with cardiac damage [54]. Interestingly, IL-10 expression was associated with better cardiac function as determined by left ventricular ejection fraction and left ventricular diastolic diameter values [54]. These results confirmed previous findings from Costa and co-workers, which suggested a cardiac-protective role for IL-10 in *T. cruzi* infection [55].

In line with the former, other work has shown that high levels of IL-17 could correlate with better cardiac function, although this is considered a pro-inflammatory cytokine [56]. Nonetheless, the role of IL-17 is still open to discussion because a recent study assessing plasma samples from 57 children showed that IL-17A levels were significantly higher in *T. cruzi* infected in comparison to seronegative individuals. Interestingly, those higher IL17A levels decreased to normal one year after treatment [57].

At present, despite the identification of cytokine signatures indicative of a pattern of pathogenesis progression has been pursued, the only tests that would independently have value for clinical decision would be the aforementioned NTproBNP (see Section 2.2) and the detection of *T. cruzi* DNA by PCR, as far as they are accompanied with electrocardiographic (ECG) and ECHO clinical assessments [36].

Other studies have focused on the potential role of adaptive immune response mediators as biomarkers. In relation to humoral immunity, the possible impact of anti-troponin T and myosin autoantibodies has been suggested [58]. Serum samples from 131 patients presenting different clinical forms of Chagas disease, healthy donors, and patients with ischemic cardiomyopathy were included in a study whose results showed that specific anti-*T. cruzi* antibodies and autoantibodies against myosin and troponin T are frequently co-detected in high levels in patients with chronic Chagas disease [58]. Even though anti-troponin T autoantibodies levels were very similar in patients presenting the indeterminate form of the disease and in patients presenting cardiac symptoms, the study found a correlation between cardiac Chagas disease, the production of anti-troponin T and anti-myosin autoantibodies, and a diminished left ventricular ejection fraction, which is an important indicative of systolic dysfunction [58]. Anti-*T. cruzi* specific antibodies and their correlation with disease progression and cardiac damage have also been evaluated. In a cohort of 55 *T. cruzi* infected patients (20 presenting the indeterminate form of the disease and 35 suffering cardiac damage) an inverse correlation between anti-*T. cruzi* IgG1 titers and left ventricular ejection fraction was found in patients presenting the cardiac form of the disease, indicating a worse prognosis for those *T. cruzi*-infected patients presenting high titers of anti-*T. cruzi* IgG1 [59]. Thus, anti-*T. cruzi* IgG1 levels could be an interesting biomarker to predict the severity of chronic Chagas disease cardiomyopathy.

In terms of T cell immune responses, it has been recently described that Chagas patients with cardiac tissue damage present higher expression levels of T-cell inhibitory receptors and lower antigen-specific capacity compared with that of asymptomatic patients [60]. These features were partially reversed by BNZ treatment in both asymptomatic and symptomatic patients: the co-expression of inhibitory molecules was reduced, the multifunctional antigen-specific response of $CD8^+$ T cells was enhanced, and an increase in the subset of cells with cytotoxic proprieties and production of IFN-γ was observed. These results point at a potential application of the analysis of those immunological signatures as biomarkers of disease progression and treatment response [60].

3. Other types of host-derived Chagas disease biomarkers

3.1. Extracellular vesicles

Extracellular vesicles (EVs) are cell-derived membranous nanoparticles released by most cells, present in all body fluids, and implicated, as intercellular communicators, in diverse pathological processes [61]. Mainly for these reasons, EVs hold an enormous potential as predictive biomarkers for human diseases, including several parasitic diseases [62,63].

Table 2
List of immune-related molecules studied as markers for the evaluation of cardiac damage progression.

Name of the marker or combinations of markers	Observed effect of higher marker expression	Reference
TNF-α	Progression	[48,52,53]
Chemokine C–C ligands 2 and 3 (CCL2, CCL3)	Progression	[53]
IL-6	Progression	[37,47]
IL-10	Protection	[55]
Anti-troponin T antibodies, myosin autoantibodies	Progression	[58]
IFN-γ, IL-1β	Progression	[52]
IL-2, IL-4, IL-5, IL-9, IL-12p70, IL-13, IL-22	Progression/protection	[54]
IL17, IL-17A	Controversial	[55–57]
$CD8^+$ T cell inhibitory receptors and antigen-specific capacity	Cardiac damage progression and therapeutic response	[60]
Anti-*T. cruzi* IgG1 levels	Cardiac damage progression	[59]

In Chagas disease, the possible role of EVs as active communicators between *T. cruzi* and its host, as well as the implications of this interaction in parasite tropism and changes in immune status during the chronic phase of the disease, has been recently reviewed [64]. Moreover, the use of EVs as potential diagnostic biomarkers during the course of the disease has been recently reported in several studies. Diaz Lozano and collaborators showed that EVs secreted by the infective forms of *T. cruzi* are targeted by the immune system forming immune complexes that could be used as biomarkers of prognosis for digestive Chagas disease [65]. In addition, EVs released by human THP-1 cells after interacting with different stages of *T. cruzi* parasites are differently recognized by antibodies present in sera from infected individuals with the indeterminate or the cardiac form of the disease [66]. Moreover, it has also been shown that circulating micro-particles secreted by Chagas disease patients induce pro-inflammatory activation and nitrosative response in THP-1 macrophage cells, which is dependent of the clinical stage of the patients [67].

Proteomic analysis to determine the molecular composition of EVs secreted by different *T. cruzi* parasite stages has been reported in recent years [68–70]. However, to the best of our knowledge the protein-cargo associated to EVs circulating in peripheral blood of *T. cruzi*-infected subjects has not been evaluated in the context of therapeutic response. In this regards, unpublished results by Cortes-Serra et al. describe for the first time, human and *T. cruzi* proteins present or upregulated in plasma-derived EVs from a chronic Chagas disease patient before chemotherapy and that are absent or downregulated following treatment. Although these results derive from the analysis of a single heart-transplanted patient presenting severe cardiac complications, they represent a proof-of-principle of the potential of this approach to discover new biomarkers of therapeutic response.

3.2. Markers derived from human genetic studies

Chagas disease has a multifactorial etiology that involves complex host-parasite interactions governed by parasite and host genetics, which can be as well influenced by environmental factors. As it was mentioned before, the mechanisms of pathogenesis of chronic *T. cruzi* infection are yet largely unknown. But immune system mediators have been described to participate in driving heart and/or gut tissues inflammation, either through response to the parasite presence and, to a certain level, by autoimmune reactions [71]. Thus, most of the genetic studies performed so far have searched for sequence variations that could be associated to chronic Chagas cardiomyopathy susceptibility in immune system related genes. These searches followed a hypothesis-driven approach to find single nucleotide polymorphisms (SNPs) in genes known or suspected to play a role in those inflammatory phenomena [72]. Among the genes studied there are: human leukocyte antigen (HLA) class I and class II alleles, cytokines (e.g.: IL-1β, IL-10, TNF-α, IL-17, IL-18) and chemokines and their receptors (MCP1/CCL2, CCR5, MIG/CXCL9, IP10/CXCL10) (reviewed in [72]), as well as inflammasome genes [73]. Some variations were associated to chronic Chagas cardiomyopathy, but in other traits like TNF-α controversy is open mainly due to the limited sample size analyzed and the ample genetic heterogeneity of the studied cohorts. These features have also limited the only genome-wide association study (GWAS) performed so far in Chagas disease [74]. With the power of its "hypothesis-free" and "hypothesis-generating" nature, unfortunately this study did not report any significant associations with chronic cardiomyopathy at the genomic level. However it interestingly suggested that SNPs in the solute carrier family gene *SLCO1B1* associated to a cardiomyopathy phenotype [74].

Other kind of genetic studies, such as transcriptomics and epigenetics works, will be also required in order to functionally expand and integrate the aforementioned genomics data, as well as to comprehend the impact of environmental factors in the susceptibility to the disease. In this regard, a whole-blood transcriptome of *T. cruzi*-infected subjects and uninfected controls identified a signature of 27 genes, mainly related to natural killer (NK) and $CD8^+$ T cells, which would mark a disease progression pattern [75]. Whereas Frade et al. analyzed the whole-transcriptome of heart biopsies from chronic Chagas cardiomyopathy patients and identified a long non-coding ribonucleic acid (RNA) molecule that had been associated to heart failure [76]. Long non-coding RNAs are > 200 nt long RNA transcripts that have been described to have a broad functionality in the regulation of gene expression at transcriptional, post-transcriptional and epigenetics levels [76].

Other series of studies analyzed the differential expression of micro RNAs (miRNAs) in chronic cardiomyopathy Chagas patients, either versus those suffering from idiopathic dilated cardiomyopathy [77], or comparing them to *T. cruzi*-infected subjects at the indeterminate stage and a group of non-infected subjects [78,79]. These miRNAs are small non-coding RNA molecules with a cell and tissue specific expression pattern that are involved in post-transcriptional regulation and might target up to 60% of the human genes [80]. A work integrating miRNA and gene expression profiles of *T. cruzi* acutely infected mice heart tissues suggested a correlation between those miRNAs and the observed pathobiology [81]. Such miRNAs can be epigenetic regulators and be regulated epigenetically at the same time [82]. One of the most common epigenetic modifications is deoxyribonucleic acid (DNA) methylation, and the results from a whole-genome cardiac fingerprinting study revealed that up to 399 genes were differentially methylated and expressed in chronic Chagas cardiomyopathy patients in comparison to healthy controls [83].

The above mentioned studies are very necessary to achieve a deeper understanding of Chagas disease complex pathogenesis events. However, so-called genomic medicine yet lies far from being applicable for Chagas disease. The usefulness of those genetic markers in the field as potential markers of disease prognosis will largely depend on a much awaited generalization of molecular-based diagnostics, or the development of easier-to-use molecular based detection methods such as loop-mediated isothermal amplification (LAMP) or recombinase polymerase amplification (RPA) assays based on them.

4. Conclusions

We are still far from having a licensed test for the early assessment of treatment efficacy or to accurately anticipate Chagas disease progression. Many candidate molecules, parasite- and host-derived, have been evaluated so far. Most of the studies have generally been limited to the evaluation of a few dozen of samples. Larger study cohorts, which should ideally involve individuals from varied geographic origins and accurate clinical stratification according to the distinct disease stages, should be pursued in order to obtain robust results as well as to shed light onto those markers that still have controversial results. In addition, the length of the follow up periods should be extended to up to 5 years post-treatment particularly for studies on therapeutic response biomarkers. In relation to studies on biomarkers to evaluate pathogenesis progression, an even longer longitudinal follow-up of participants would be desirable. This is because in the majority of studies performed so far cardiac alterations (assessed by ECG and ECHO) are already present at the time of triaging the study groups. With current designs it would not be possible to assign a clinical decision on the patients' management upon observed changes in the markers' levels, as these were found in patients already symptomatic. Thus, those markers that appear altered at indeterminate stages with no evidences of clinical signs should be therefore the most promising.

Regardless of their application, assessment of the markers with the same cohort of samples could also contribute to draw a clearer picture. In this sense, multinational scientific networks like NHEPACHA [10] are very interesting initiatives so as to bring together academic, clinical and industry groups, providing them with the environment to collaboratively work towards the identification of the most useful biomarkers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Review Article

Extracellular Vesicles in *Trypanosoma cruzi* Infection: Immunomodulatory Effects and Future Perspectives as Potential Control Tools against Chagas Disease

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Chagas disease, caused by the protozoa parasite *Trypanosoma cruzi*, is a neglected tropical disease and a major public health problem affecting more than 6 million people worldwide. Many challenges remain in the quest to control Chagas disease: the diagnosis presents several limitations and the two available treatments cause several side effects, presenting limited efficacy during the chronic phase of the disease. In addition, there are no preventive vaccines or biomarkers of therapeutic response or disease outcome. Trypomastigote form and *T. cruzi*-infected cells release extracellular vesicles (EVs), which are involved in cell-to-cell communication and can modulate the host immune response. Importantly, EVs have been described as promising tools for the development of new therapeutic strategies, such as vaccines, and for the discovery of new biomarkers. Here, we review and discuss the role of EVs secreted during *T. cruzi* infection and their immunomodulatory properties. Finally, we briefly describe their potential for biomarker discovery and future perspectives as vaccine development tools for Chagas Disease.

1. Chagas Disease

Chagas disease (CD) or American trypanosomiasis is a neglected tropical disease (NTD) caused by the protozoan intracellular parasite *Trypanosoma cruzi*. The disease is widely distributed across Latin America, with an estimated 6 to 7 million individuals infected, affects vulnerable populations, and has an important impact on the health, social and economic well-being of infected individuals (WHO, 2022, accessed March 14, 2022, https://www.who.int/news-room/fact-sheets/detail/chagas-disease-american-trypanosomiasis). In the last decades, CD has become an emerging infectious disease in nonendemic regions such as Europe, North America, Japan, and Australia, with the immigration of infected people from endemic countries contributing to the spread of the infection [1–3]. Importantly, many challenges remain to control CD effectively in nonendemic areas, such as access to diagnosis (with up to 90% of cases being undiagnosed), access to treatment, and screening of pregnant women and blood banks [1, 4–6].

The transmission of the parasite occurs through contact with the infected feces/urine of blood-sucking triatomine bugs (bug vector), congenital transmission, blood transfusion, and oral ingestion of contaminated food [7]. The

disease consists of two stages: the acute phase, occurring up to one week after infection and being mostly asymptomatic, and the chronic phase, with 30-40% of chronic patients manifesting cardiac and digestive symptoms [7, 8].

CD control presents multiple challenges: the parasite-host interactions are not yet completely understood; the diagnosis has several limitations; the two available treatments present several side effects and limited efficacy during the chronic phase of the disease; and there are no preventive vaccines for human or veterinary use. Additionally, there are no prognosis markers or biomarkers of therapeutic response [4, 9, 10]. In this scenario, the development of new therapeutic tools is urgently needed.

2. Extracellular Vesicles

Extracellular vesicles (EVs) are small particles formed by a lipid bilayer secreted by all cell types into the extracellular microenvironment and present in all body fluids [11]. Particles are divided into several subtypes, such as exosomes, microvesicles, and apoptotic bodies, according to their origin, size, and molecular composition [11–13]. Their content, which reflects the cell of origin, includes cytosolic and cell-surface proteins, nucleic acids, lipids, and metabolites. EVs mediate intercellular communication in a great variety of biological processes during homeostasis and in pathological conditions, where they act as messenger entities that deliver specific cargo to recipient cells, thereby altering their physiological status. The molecular signatures and functional properties of EVs, together with their remarkable stability in biofluids and systemic distribution, endow them with great potential to be used as biomarkers for the diagnosis and prognosis of diseases, as therapeutic vehicles for drug and gene therapy, and for developing new vaccine platforms against infectious diseases [14–18].

EVs have been described and are currently being studied in several protozoan parasites and helminths, such as *T. cruzi*, *Leishmania* spp., *T. brucei*, *Toxoplasma gondii*, *Plasmodium* spp., *Giardia intestinalis*, *Schistosoma mansoni*, and *Fasciola hepatica* [19–29]. In the context of the parasitic diseases that these organisms cause, EVs are known to play a major role in intercellular communication between the parasite and the host. Importantly, EVs can modulate the host immune response, increase parasite invasion, and alter the integrity and function of cells and tissues, resulting in different disease outcomes [20, 28, 30–34]. Moreover, the EV's capacity to mediate immune evasion through a broad type of mechanisms contributes to the exacerbation of infection [15]. On the other hand, EVs can also trigger protective responses by activating an immune cell effector mechanism that benefits the host, controlling parasite replication, and promoting host survival [17, 18, 23, 35, 36]. EVs released by parasites induce an immune modulatory effect and, together with their proven capacity for direct and indirect antigen presentation in the adaptive immune response [16], make them promising tools for vaccine or immunotherapy development [17, 18, 37].

Although EVs have not yet been tested as vaccines in clinical trials for parasitic diseases, several studies have demonstrated their potential. In Toxoplasmosis, EVs derived from dendritic cells incubated with *T. gondii* antigens induce a systemic immune response in mouse models. The vaccinated animals demonstrated increased survival and lower cerebral parasite burden after parasite challenge [38–40]. Another group has shown that EVs released by cells infected with *T. gondii* alter cell proliferation, causing changes in neighboring cells, which is the most likely mechanism for modulating the host's immune system [41]. In addition, using *P. yoelii* as a murine model of malaria, it has been shown that EVs generated from infected reticulocytes when administered in the presence of CpG as adjuvant elicited a potent host humoral immune response, decreased parasitemia, and protected mice against a challenge with a lethal strain of *P. yoelii* [23, 42].

EVs released in parasitic diseases contain parasite and host proteins, nucleic acids, and glycoconjugates or lipids from the parasite membrane [33, 43–46]. This characteristic, together with the fact that EVs are found in all biological fluids and present a specific molecular signature, dependent on the cell of origin, makes them interesting tools for biomarker discovery [47–49]. In *P. falciparum* and *P. vivax*, high circulating levels of EVs have been associated with the clinical symptoms and severity of the disease, showing that EV concentrations may have applications as biomarkers of malaria severity [50, 51]. In schistosomiasis, schistosomal miRNAs were detected in EVs isolated from patients before treatment. These levels decreased after treatment, indicating that EVs could be used as new diagnostic tools for patients presenting low parasitic burden, and as new biomarkers for therapeutic response [52].

3. Molecular Composition and Virulent Factors Associated with the Shedding of EVs by *T. cruzi* Parasite

The shedding of EVs by *T. cruzi* was first described in epimastigote form in 1979 [53]. Later, Gonçalves's group demonstrated that infective trypomastigote form from four different *T. cruzi* strains (Y, YuYu, CA1, and RA) released surface antigens bound to the particles by a spontaneous process [54]. However, it was not until 2013 that the first proteomic analysis of the *T. cruzi* secretome was performed [55]. In this study, EVs released by noninfective epimastigotes and infective metacyclic trypomastigote forms were isolated by ultracentrifugation, and two EV subtypes (larger and smaller), as well as vesicle-free fractions, were analyzed by mass spectrometry. The results showed a rich collection of proteins involved in metabolism, host-parasite interaction, signaling, nucleic acid binding, parasite survival, and virulence [55]. From then on, other proteomic studies emerged to characterize the exoproteome of trypomastigote forms [56] and to detect antigens associated with vesicles secreted by *T. cruzi* trypomastigotes [57]. Later on, another proteomic analysis of trypomastigote EVs was performed using two different strains (Y and YuYu), known to modulate the host immune responses differentially [45]. The analysis confirmed previous protein identification and showed

quantitative and qualitative differences in the EV cargo of the two strains, which correlated with differences in their infection profile [45]. Recently, another study characterized the proteome and the nanomechanical properties of EVs released from trypomastigotes and epimastigotes, finding marked differences in the EV cargo between both stages [58]. The first proteomic characterization of plasma-derived EVs purified directly from a heart transplant patient with chronic Chagas disease (CCD) was performed recently, identifying both human and parasite proteins in circulating EVs [46, 59].

The molecular cargo released in EVs by the different stages of *T. cruzi* parasites cultured *in vitro* is summarized in Figure 1. Among the proteins identified in EVs released to the conditioned medium by different *T. cruzi* stages, the presence of virulent factors is worth highlighting, as their expression in the parasite is fundamental for disease establishment and progression of infection. This is the case for trans-sialidases (TS), mucin, mucin-associated surface protein (MASP), cruzipain, and phosphatases, among others [19] (Table 1). These molecules, whose functions have been studied for years in the context of infection and the parasite, are involved in attachment and invasion of the host cells, protecting the parasite from complement-mediated lysis system, and may act as proinflammatory agents [60–65]. However, the function of these molecules in EVs is not yet well known and requires further investigation.

4. Immunomodulatory Role of EVs Derived from Trypomastigote Forms and *T. cruzi*-Infected Cells

The early events of *T. cruzi* infection are crucial for the establishment of the disease. The parasites contain molecules that induce the host innate immune response [62]. As macrophages and other mononuclear cells are among the host's first line of defense, several research groups have focused on the study of these cells and their interaction with EVs secreted by the parasite. EVs are among the mechanisms used by the parasite to escape the immune system. It has been shown that microvesicles released by THP-1 cells, after interacting with trypomastigotes in the early stages of infection, are able to inhibit the C3 convertase, protecting the parasite against the complement system and increasing its chances of survival [66]. These authors also demonstrated that a subpopulation of microvesicles carrying transforming growth factor beta (TGF-β), after incubation with Vero cells, increased *T. cruzi* invasion [66] (Figure 2(a)). Previous studies have also shown that *T. cruzi* infection requires the activation of the TGF-β signaling pathway to increase parasite invasion in epithelial and cardiac cells and that TGF-β is also involved in the development of CCD cardiomyopathy, being crucial for the formation of cardiac fibrosis [67, 68].

It has been observed that EVs released by several *T. cruzi* strains (Y, Colombiana, CL-14, and YuYu) modulate the inflammatory response of macrophages via the TLR2-dependent pathway, involving the signaling pathways of mitogen-activated protein kinases (MAPK), and trigger an inflammatory response mediated by proinflammatory TNF-α and IL-12, IL-6, and NO [69] (Figure 2(b)). In the same line, other studies exploring the EV's contribution to the proinflammatory response of THP1 macrophages showed that vesicles isolated from the plasma of CCD patients and experimentally infected mice also triggered the synthesis of proinflammatory cytokines and oxidative and nitrosative products [70]. Interestingly, the expression levels of proinflammatory genes observed in this study depended on the patient's disease stage, being higher in CCD patients presenting symptoms than in individuals suffering the indeterminate form of the disease [70] (Figure 2(b)). Notably, an unbalanced immune response favoring a proinflammatory environment is one of the main features responsible for disease progression. In this scenario, therapies capable of preventing tissue damage or reprogramming macrophages to increase microbicide and effector functions could be a useful tool for CD treatment. More recently, Vasconcelos and collaborators showed that the viability and/or integrity of the parasite are necessary factors for the release of EVs, which trigger a proinflammatory response in the host cell *in vitro*, and may be a strategy developed by the parasite that is aimed at creating a more favorable environment for establishing infection in the host [71]. However, other studies have shown that bone marrow-derived macrophages treated with *T. cruzi*-derived EVs (strain Y) induced lipid body and prostaglandin E2 (PGE2) formation prior to infection. Twenty-four hours after *T. cruzi* infection, these EV-treated macrophages decreased the production of PGE2, TNF-α, and IL-6, decreasing the production of proinflammatory cytokines and oxidative and nitrosative products, which favored parasite infection and persistence (Figure 2(c)) [72].

The therapeutic potential of EVs is variable in different models and needs to be addressed carefully. Infected macrophages can also modulate the activation of other human THP-1 cells, promoting an inflammatory response [69]. Using a NF-κB activation reporter CHO cell line, the authors showed that EVs secreted from infected cells induced the translocation of NF-κB after interacting with TLR2 in this model. Moreover, both EVs from trypomastigotes forms and from infected macrophages altered the gene expression of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, and signal transducer molecules, such us STAT-1 and STAT-3 in THP1 macrophages (Figure 2(b)) [69]. In another report, the mechanism of NF-κB-mediated proinflammatory cytokine response was studied further and identified two proteins involved in sensing DNA damage, cGAS, and PARP1 (a DNA repair enzyme), as factors responsible for the proinflammatory phenotype induced by the parasite and infected cell-derived EVs. Oxidized DNA was detected in EVs secreted by infected macrophages and in EVs from the plasma of chronically infected mice. Interestingly, the inhibition of PARP1 decreased the overall proinflammatory response and heart inflammation of chronically infected mice, suggesting that chemical inhibitors of this enzyme could become potential therapeutic targets for CD [73]. Notably, in some of the described *in vitro* models, *T. cruzi*-infected cells released higher levels of EVs compared to

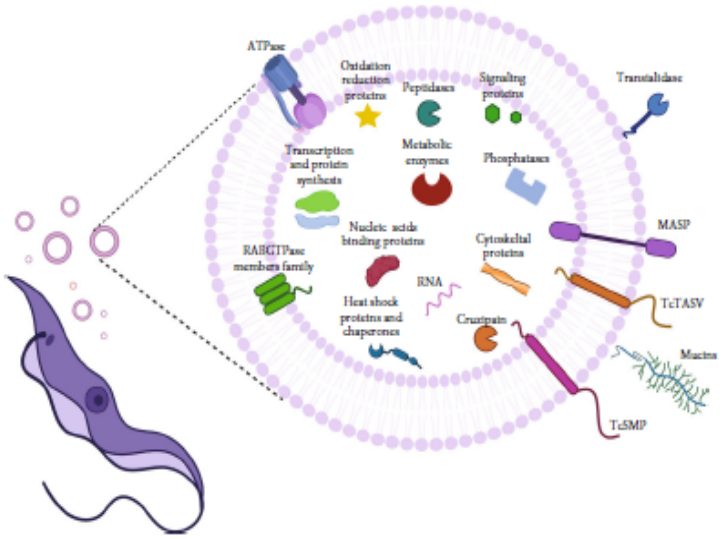

FIGURE 1: Schematic illustration summarizing the molecules identified in extracellular vesicles secreted by *T. cruzi* trypomastigotes. Several proteomic studies have identified *T. cruzi* virulence factors in EVs isolated from *T. cruzi* trypomastigotes, such as *trans*-sialidases, mucins, MASP proteins, the protease cruzipain, phosphatases, TcSMP, and TcTASV. Other molecules that can be found in trypomastigote EVs are signaling proteins, peptidases, oxidation/reduction proteins, ATPases, transcription and protein synthesis proteins, metabolic enzymes, cytoskeletal proteins, nucleic acid-binding proteins, heat shock proteins and chaperones, members of the RAB GTPase family and RNA, among others (created with http://BioRender.com).

TABLE 1: Main virulence factors associated to EVs shedding by *T. cruzi* parasites.

T. cruzi virulence factors	Description	EV source	References
trans-sialidase (TS)	*T. cruzi* is unable to synthesize sialic acid (SA) *de novo*, TS transfers α2-3-linked SA from host glycoproteins and glycolipids to acceptors containing terminal β-galactosyl residues present on the parasite surface. Avoiding lysis by serum factors and increasing invasion in the mammalian host (parasite sialylation)	T	55–58
Mucins (mucin-like glycoproteins: tGPI-mucins, eGPI or mtGPI-mucin)	Trypomastigotes (T) (i) Mucins are the main acceptors of SA in the parasite's surface (ii) Activation of the host innate immune system (iii) Induce the production of TNF-α, IL-12, and NO Epimastigotes (E) (i) Similar cell surface glycoprotein complex, called GP24, GP31, and GP37 (ii) Molecules maybe affect parasite migration in the vector Metacyclic trypomastigotes (MT) (i) Reported originally as the 35/50-kDa antigens (ii) Mucins from MT increased infectivity and the ability of the parasite to shed the mucins upon invasion of the host cell	T E MT	28, 55,57,76
Mucin-associated surface proteins (MASP)	MASPs proteins, considered one of the most antigenic *T. cruzi* proteins, are a very diverse protein family, with members involved in host-cell invasion and survival and multiplication of intracellular amastigotes (A)	E T A	55-58
Phosphatases	In *T. cruzi*, phosphatases present multiple roles, such as providing a source of inorganic phosphate, facilitating epimastigotes differentiation, and promoting infection	E MT T	55-58
TcSMP family	TcSMP induce calcium signaling and lysosome mobilization, facilitating the formation of the parasitophorous vacuole and parasite invasion	E MT	55
TcTASV family	Still unknown function, this family has been suggested as a potential target for intervention against *T. cruzi*, mainly due to the observation that some host-molecules trigger TcTASV-C expression *in vivo* during the infection.	T A	60
Cruzipain	The major cysteine peptidase involved in host immune evasion, cell invasion, and intracellular development	E T	28, 55, 57, 76

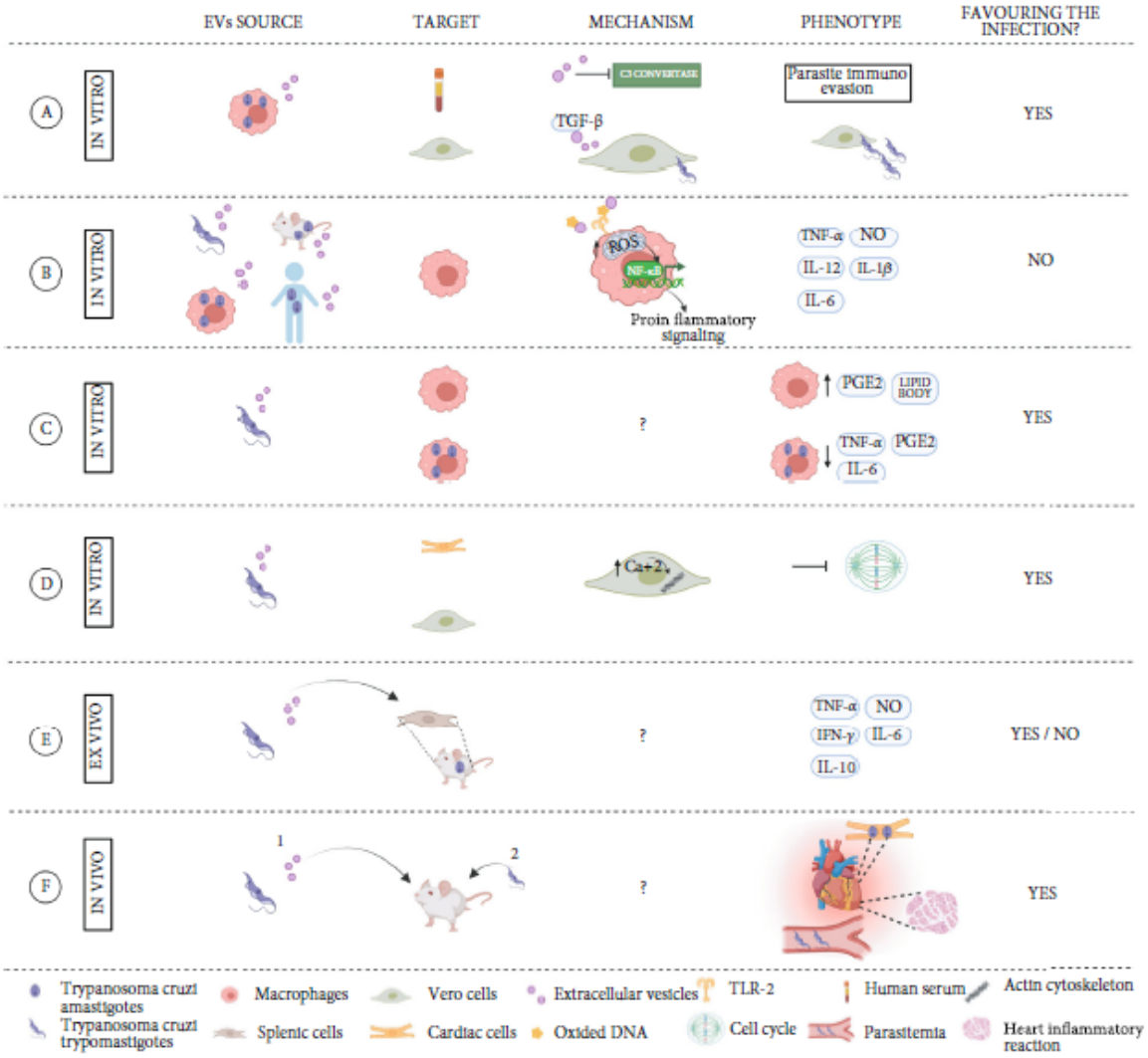

FIGURE 2: Immunomodulatory role of extracellular vesicles derived from *T. cruzi* and *T. cruzi*-infected cells. Summary of the main studies targeting the immunomodulatory effect of EVs in *T. cruzi* infection: EV source, target cell or body fluid, mechanism of action (if known), phenotype, and final effect on the infection process. (a) EVs secreted by infected macrophages can inhibit the C3 convertase, protecting the parasite against the complement system and increasing its chances of survival, and promote rapid cell invasion. (b) EVs from *T. cruzi* trypomastigotes, infected cells, infected individuals, and infected mice are recognized by uninfected macrophages via TLR2, inducing the translocation of NF-κB and modulating the synthesis of proinflammatory cytokines. (c) *T. cruzi* EVs induce the formation of lipid body and PGE2 in noninfected macrophages and downregulate the synthesis of TNF-α, PGE2, and IL-6 in infected macrophages. (d) EVs secreted by the parasite alter cell permeability and intracellular levels of calcium in nonimmune host cells, modifying the dynamics of the cytoskeleton and arresting the cell cycle. (e) EVs secreted by trypomastigotes induce an ex vivo production of pro and anti-inflammatory cytokines in splenic cells from chronically-infected mice. (f) *In vivo* studies in mice, injected with trypomastigotes-derived EVs prior to *T. cruzi* infection, have shown an increase of circulating EVs in plasma and parasitemia, cardiac tropism, and inflammation (created with http://BioRender.com).

noninfected cells, and these differ largely in their protein cargo [69, 74].

Little is known about the mechanisms by which *T. cruzi* EVs alter nonimmune host cells. A recent *in vitro* study showed that epithelial cells (Vero) and cardiac muscle cells (HL-1), incubated with parasite EVs, altered cell permeability and intracellular levels of calcium, which modified the dynamics of the actin cytoskeleton and arrested the cell cycle (Figure 2(d)). All together, these changes could explain the increased host-cell invasion observed in this study [75].

The role of EVs in the immune response of the chronic stage of *T. cruzi* infection has been studied less. Nogueira and collaborators studied the *ex vivo* effect of EVs secreted by different *T. cruzi* strains (Y, Colombiana, CL-14, and

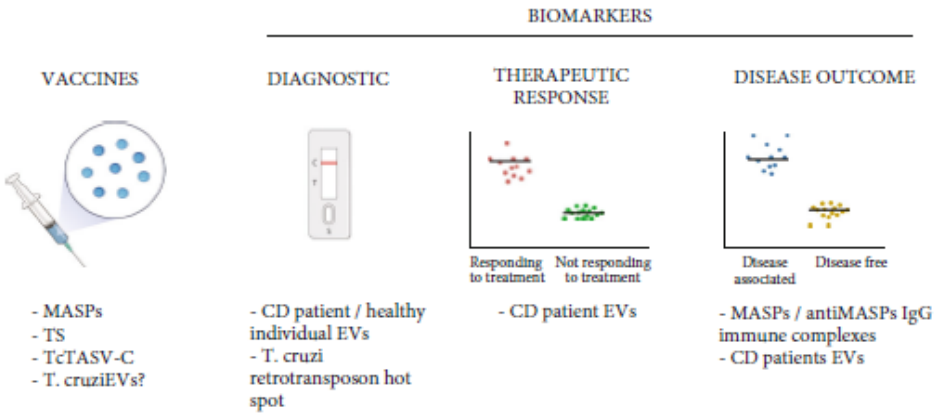

FIGURE 3: Potential role of EVs as new tools for CD prevention and control. EVs from *T. cruzi* or parasite proteins found in the EVs have been tested as vaccine antigens for Chagas disease. EVs isolated from CD patients, the retrotransposon hot spot *T. cruzi* protein, and the immunocomplexes found in CD patients are also being studied for their potential as biomarkers for diagnosis, therapeutic response, and disease outcome (created with http://BioRender.com).

YuYu) when used to stimulate splenocytes from chronically infected mice. Interestingly, the immunomodulatory responses caused by the EV stimulus depended on the parasite strain. As previously reported, in other cell types, splenic cells also produced NO, TNF-α, IL-6, and IFN-γ upon stimulation with parasite EVs. However, an increase in the production of anti-inflammatory cytokine IL-10 by T and B cells was also observed, which is in contrast with the proinflammatory profile found in other studies, and reinforces the importance of IL-10 in modulating the balance between inflammatory and anti-inflammatory responses, avoiding tissue damage (Figure 2(e)) [76]. Several *in vivo* studies addressing the EV effect on the pathological features of CD have also been performed, all of which used well-established mouse models. Some of these studies have shown that animals treated with parasite-derived EVs prior to *T. cruzi* infection are distinguished by increased circulating EVs in plasma, parasitemia, cardiac tropism, and inflammation (Figure 2(f)) [28, 66, 72] . Moreover, some studies found a reduction of NO and TNF-α levels in plasma and a decreased production of TNF-α and IL-6 in spleen cells from infected animals [72]. However, some discrepancies have been observed in the mortality rates linked to EV immunization, with some studies reporting increased mortality [28] while no differences were found in others [72].

There is growing evidence that the immunomodulatory properties of EVs depend on the *T. cruzi* strain and the parasite stage [74, 76, 77]. *T. cruzi*-derived EVs from different strains present different protein cargos, which correlates with differences in the sensitivity to complement-mediated lysis, parasite invasion, infectivity, virulence, and immunomodulatory responses [45, 76]. In relation to the effect of the EVs released by host cells after interacting with different parasite developmental stages, it has been found that all *T. cruzi* stages are able to induce the release of EVs by host cells, with mammalian infective forms causing the highest release [74].

5. EVs as a Potential Source of New Biomarkers in Chagas Disease

The use of EVs as a new platform to identify biomarkers has been described in the last few years for different pathologies, including parasitic diseases [78]. Taking into consideration that one of the biggest challenges for CD is the lack of validated biomarkers to indicate therapeutic response and disease outcomes [59], EVs could become a promising source for developing new biomarkers in infectious diseases.

As previously mentioned, the MASP multigene family is one of the major virulence factors of *T. cruzi*. It plays a fundamental role in cell invasion and has an associated humoral immune response in CD patients. Interestingly, this response is different depending on the clinical stage of the individuals, being lower in the sera of patients presenting cardiac affection compared to sera from those suffering from the gastrointestinal form of the disease [65]. Further research showed that the EVs released by the parasite containing MASP proteins are targeted by the immune system, triggering the formation of circulating immune complexes containing anti-MASP Immunoglobulin Gs (IgGs). The EVs forming immune complexes inhibit the complement system. Interestingly, the highest percentage of inhibition appeared in the digestive group, compared to the asymptomatic and cardiac patients. Taking advantage of this particularity, these immune complexes could be used as biomarkers for the differential diagnosis or prognosis of CD, in particular in patients with digestive manifestations [61]. In the same line, microvesicles also have potential as differential diagnosis or prognosis biomarkers during CD infection. The antibodies contained in the sera from CD patients detected antigens from EVs released by host cells after interacting with the infective forms of the parasite. Interestingly, these molecules were recognized differently by patients presenting the cardiac or indeterminate phase of the disease, indicating the existence of specific markers

associated with a differential diagnosis depending on the organ involved [74].

EV concentration in the body fluids of healthy individuals and patients presenting several forms of the disease has also been studied as a potential biomarker for differential diagnosis, with no clear results. While some studies did not find any statistical differences in the number of vesicles in CD patients compared to healthy controls [74], others did find differences in terms of concentration, showing that treated patients presented lower concentrations of circulating EVs than healthy donors [48]. In this study, human THP-1 cells were incubated with circulating EVs, followed by ELISA to measure cytokines and determine whether the concentration of circulating EVs was associated with differential activation of the immune system. IFN-γ and IL-17 showed a differential profile when compared to chronic Chagas patients and healthy controls, finding that patient samples induced a higher production of IFN-γ, and lower production of IL-17, a profile that could contribute to parasite persistence and tissue damage due to continuous inflammatory signaling [48] .

Two of the features of CD are chronic inflammation and oxidative stress, which are specially exacerbated in individuals suffering the cardiac form of the disease. It has been shown that microparticles generated during *T. cruzi* infection carry the host's signature for oxidative, nitrosative, and inflammatory states. Thus, EVs provide information about the disease's progression and could be useful for evaluating disease severity [70].

In a different study, a group of human and parasite proteins were identified in plasma-derived EVs from a heart transplant patient with chronic CD, while being absent in EVs from the plasma of healthy individuals. Interestingly, several human proteins and one parasite protein (pyruvate phosphate dikinase) were found to be present or upregulated before treatment and were absent or downregulated after treatment. Although these results should be interpreted with caution, as they represent a single clinical case and need to be validated in a larger cohort, they represent a proof-of-principle of the potential of this approach to discover new biomarkers of therapeutic response [46].

Finally, EVs from *T. cruzi* are also attractive candidates for use in the serological diagnosis of CD. In an attempt to identify antigens, present in trypomastigote excreted-secreted EVs, Bautista-López and collaborators incubated trypomastigote-excreted antigens associated with EVs with affinity columns containing IgG antibodies from healthy donors, or Chagas patients with clinical symptoms. Chagasic IgG affinity resin was highly enriched in *trans*-sialidases and showed a significant enrichment in mitochondrial proteins, retrotransposon hot spot (RHS) proteins, paraflagellar rod proteins, proteases, and multiple uncharacterized proteins [57]. RHS and *T. cruzi* paraflagellar rod-3 protein were further explored for their potential as serological antigens for the diagnosis of *T. cruzi* infection, showing robust cross-reactivity with sera from patients presenting all clinical forms of CD. Interestingly, no cross-reactivity with RHS was detected when using sera from patients with other parasitic diseases, which could be relevant for the development of a new diagnostic test with high specificity [57]. The potential control strategies that could be associated with EVs secreted by *T. cruzi* or *T. cruzi*-infected cells, such as biomarker discovery and/or vaccine development, are summarized in Figure 3.

6. Chagas Disease Prevention: Future Perspectives of EVs as New Vaccine Antigens against *T. cruzi* Infection

Even though vaccines could be a very useful cost-effective tool for the prevention and control of *T. cruzi* infection and transmission, we are still a long way from having a beneficial vaccine for CD [79]. The lack of financial support and interest from governments and the pharmaceutical industry, together with the genetic complexity of the parasite, have contributed to the slow progress in its development [80]. Multiple attempts have been made to develop safe and effective vaccines for CD. Currently, there are two main target product profiles for developing vaccines for CD. The first one, which could be used alone or in combination with drug therapy, aims to prevent, or at least delay, the progression of cardiac and digestive manifestations in patients presenting the indeterminate form of the disease [80]. The second one is aimed at developing a preventive vaccine [81]. Unfortunately, although some of the candidates were able to induce a partial protective response, none of them showed complete protective immunity [81]. In this scenario, new approaches and ideas are needed to develop a protective vaccine for *T. cruzi* infection. Immunization with molecules delivered into EVs is an interesting possibility for exploring *T. cruzi* infection.

Interestingly, one of the protein families present in *T. cruzi*-derived EVs, which has been tested as a potential vaccine antigen, is the MASP family. Taking into consideration that MASPs play a major role in host-cell invasion, that they are one of the most important *T. cruzi* virulence factors, and that several MASP family members have predicted MHC-I and MHC-II epitopes, a synthetic MASP-derived peptide was tested as a vaccine candidate in a murine model of CD [65, 82, 83]. Mice immunized with the synthetic MASP peptide conjugated to keyhole limpet hemocyanin showed an 86% survival rate after being infected with trypomastigotes and had a much lower parasite load in the heart, liver, and spleen compared to untreated animals. Moreover, vaccinated animals produced neutralizing antibodies and developed a protective cytokine response against parasite infection. Interestingly, the vaccine engaged both humoral and cellular responses, indicating that MASP proteins are promising targets for the development of a CD vaccine [83].

Another well-known *T. cruzi* virulence factor, which is essential for the invasion process and present in EVs, is the TS family. Several investigators have tested immunization with multiple gene-encoding members of the TS family, in different vaccine platforms (bacterial and viral vectors, or as a recombinant protein) and formulations (alone, together with other *T. cruzi* glycoconjugates, and associated with adjuvants) [81, 84]. Although the results obtained showed

some limitations, some vaccine formulations induced immunity in mouse models challenged with *T. cruzi*, producing antibodies, preventing the development of tissue damage, and having an impact on the mortality of infected animals [84]. In that context, TS antigens conjugated to EVs could be a different approach to developing vaccines for CD.

Another *T. cruzi* protein family secreted in EVs that has been considered for immunization is the *T. cruzi* trypomastigote alanine, valine, and serine (TcTASV-C). To evaluate the performance of TcTASV-C as a vaccine antigen, mice were vaccinated following a DNA-prime protein-boost schedule of immunization. However, when animals were challenged with a highly virulent *T. cruzi* strain two weeks after the final dose, the results obtained were not very promising. Although TcTASV-C-vaccinated mice showed a strong humoral response, there was a delay in the appearance of circulating trypomastigotes, and they presented lower parasitemia, exhibiting only a 30% higher survival rate than controls [60].

Finally, preliminary results have shown that mice immunization with 3 doses of EVs derived from trypomastigote forms of *T. cruzi* (Y strain), administered in the presence of $Al(OH)_3$ as adjuvant, could induce some level of protection against experimental CD. Preliminary results showed that vaccinated mice presented lower parasitemia than non-vaccinated animals. However, no significant changes were observed in the survival of all animal groups. Further investigation needs to be carried out to understand which molecules are responsible for this potential protection. Moreover, experimental assays using EVs isolated from trypomastigote forms from different DTUs are needed to verify the influence of virulence factors in vaccination against experimental CD (Torrecilhas. A. C., unpublished data).

The use of different experimental models, cell types, adjuvants, doses, and vaccination regimens may also determine the development of the protective response. The key questions remaining for the development of new vaccine tools for CD are as follows: further characterization of the immune responses, development of highly efficient antigen delivery systems, animal models mimicking the chronic phase of the disease, assessment of parasite diversity and antigenic variation, study of coinfections, and use of adjuvants and new vaccination regimens together with more studies focusing on parasite tissue distribution [79].

7. Conclusions

In the last decade, research on the biology, function, and potential applications of EVs has grown exponentially. Even though the number of studies regarding *T. cruzi* infection and EVs is increasing every year, there is still a long way to go. Many questions remain in relation to the role of EVs in the pathogenesis of the disease and its mechanisms in pathogen-host interaction. Do the virulent factors maintain their virulent function when associated with EVs? Even though the function of these molecules has been perfectly described on the parasite surface, their specific function in vesicles is still not well known. Do the EVs secreted by the parasite or infected cells protect the host, or otherwise favor the infection? The EVs secreted by trypomastigotes favor host cell invasion and promote parasite immune evasion, increasing its survival in *in vitro* and *in vivo* studies. However, EVs secreted by the parasite, infected cells, infected individuals, and infected mice are also able to modulate macrophages, triggering a proinflammatory response against the parasite. Importantly, this inflammatory response, if unbalanced, is one of the main features responsible for disease progression in Chagas disease. Finally, it is urgent that we continue to explore the potential of EVs for antigen discovery, vaccine development, therapeutic strategies, and biomarkers, as these are among the most important challenges that we face in our efforts to control CD.

Conflicts of Interest

No potential conflicts of interest were reported by the authors.

Authors' Contributions

NC-S, MG-L, ACT, and CF-B wrote the manuscript. NC-S, MG-L, MJP, ACT, and CF-B contributed to the final manuscript editing. Figures have been idealized and were done by NC-S and CF-B. All authors reviewed the manuscript and approved the submitted version.

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DISPATCHES

Plasma-Derived Extracellular Vesicles as Potential Biomarkers in Heart Transplant Patient with Chronic Chagas Disease

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Chagas disease is emerging in countries to which it is not endemic. Biomarkers for earlier therapeutic response assessment in patients with chronic Chagas disease are needed. We profiled plasma-derived extracellular vesicles from a heart transplant patient with chronic Chagas disease and showed the potential of this approach for discovering such biomarkers.

Chagas disease, caused by *Trypanosoma cruzi* parasite, is one of the most prevalent parasitic infections in Latin America and is responsible for millions of clinical cases. However, mainly because of migratory movements, the epidemiology of Chagas disease has changed in recent decades; cases have increased substantially in North America, Europe, and Asia, where it is not endemic (*1*). Thus, raising awareness of this debilitating or deadly neglected tropical disease and promoting the creation of global strategies for its accurate diagnosis, treatment, and control are of paramount importance.

Detection of *T. cruzi*–specific antibodies in serologic assays is the current standard technique for diagnosing chronic Chagas disease. However, this so-called conventional serology is not a valid indicator of chemotherapeutic outcomes because most patients remain seropositive for 10–20 years after treatment (*2*). Therefore, validated biomarkers are lacking for early assessment of therapeutic responses for testing current and new drugs or treatment regimens.

Extracellular vesicles (EVs) are cell-derived membranous nanoparticles present in most biologic fluids. Biofluid-derived EVs are minimally invasive molecular tools for diagnosing and screening diseases (*3*). They can be released by various mammalian cells and pathogens, and their use as predictive biomarkers for disease progression and treatment outcomes has been reported for different pathologic conditions, including parasitic diseases (*3*,*4*).

The Study

The Ethical Committee of Clinical Research of Hospital Clinic (Barcelona, Spain; reference no. Reg. HCB/2015/0616) approved this project. The patient provided written informed consent before sample collection.

In 2009, a 51-year-old patient from Bolivia with a history of chronic Chagas disease, exhibiting severe organ involvement (chronic cardiomyopathy Kuschnir III and megacolon and megaesophagus degree IV) (*5*), was admitted to the International Health Department (Hospital Clinic, Barcelona). Serologic diagnosis for chronic Chagas disease was performed using 2 ELISA kits (Ortho-Clinical Diagnostics, https://www.orthoclinicaldiagnostics.com) and BioELISA Chagas

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Table 1. Timeline of heart transplant patient with chronic Chagas disease from initial diagnosis to last follow-up and death*

Date	Infection	Observation, treatment, outcome
2015 Aug	Cytomegalovirus, detected by serology	Diagnosed only by positive IgG serology, no active infection (no positive IgM serology). No treatment.
2015 Aug	Toxoplasmosis, detected by serology	Diagnosed only by positive IgG serology, no active infection (no positive IgM serology). No treatment.
2015 Nov		Heart transplantation on Nov. 28. Patient started with immunosuppressive therapy (tacrolimus, azathioprine, prednisone) until the end of follow-up.
2016 Jan	Chagas disease reactivation, detection by qPCR	Pretreatment sample collected on Jan 28. Patient started BZN treatment (2.5 mg/kg, twice a day, 60 d) on Feb 3.
2016 Mar	Bronchopulmonary aspergillosis, detected by serology and CT	BZN course interrupted on Mar 21. Completed 80% of the prescribed treatment.
2016 Mar	Bronchopulmonary aspergillosis	Aspergillosis treatment started on Mar 22. Initially with voriconazole and amphotericin B liposomal. Treatment was changed to posaconazole until the end of the follow-up.†
2016 Apr	Chagas disease reactivation, detected by qPCR	On Apr 14, patient started second round of BZN treatment until May 5, completing 100% of the prescribed treatment.
2016 May		Posttreatment sample collected on May 11.
2016 Aug		Late organ rejection. Patient died.

*BZN, benznidazole; CT, computed tomographic scan; qPCR, quantitative PCR.
†Parasite clearance could be related to the prolonged used of posaconazole, as previously reported (6), and/or the combined use of posaconazole and benznidazole because a second round of the latter was started in April 2016.

(Biokit, https://www.biokit.com). Together with clinical management of dysphagia and constipation, a pacemaker in the context of third-degree atrioventricular block was implanted. In July 2015, an echocardiogram revealed iterative cardiac failure and severe ventricular dysfunction (ejection fraction 15%–20%). On November 28, 2015, the patient underwent heart transplantation without incident, and results of follow-up endomyocardial biopsies showed no early signs of transplant rejection.

After transplantation and in the context of immunosuppression therapy (Table 1), quantitative PCR (qPCR) was performed weekly to detect *T. cruzi* in the blood (*Tc*-qPCR) (*7*). First benznidazole treatment was started when several consecutive and positive *Tc*-qPCRs confirmed Chagas disease reactivation. Three weeks after benznidazole treatment, the *Tc*-qPCR became negative. After completion of 80% of the treatment, bronchopulmonary aspergillosis developed, and the benznidazole course was interrupted. The *Tc*-qPCR became positive and a second benznidazole course was initiated; this time the patient completed the initial prescribed dose without evidence of therapeutic failure based on *Tc*-qPCR results. Plasma samples for purification and characterization of EVs were collected before the first benznidazole treatment and just after the second course (Table 1). Unexpectedly, the patient died in August 2016 because of a late organ rejection. Therefore, samples at 6 and 12 months posttreatment, already included in the approved protocol, were not collected.

To determine whether circulating EVs from this patient could have been used as predictive biomarkers to evaluate therapeutic response and disease outcome in the Chagas disease context, we collected pretreatment and posttreatment plasma samples, and EVs were enriched by size-exclusion chromatography (SEC) and characterized as described (*8*) (Figure 1, panel A). As negative controls, plasma samples from 2 healthy donors were also subjected to SEC. We characterized eluting EVs by bead-based assay and Nanoparticle Tracking Analysis (Figure 1, panels B, C). We pooled aliquots (100 μL) from SEC fractions 7–10 and determined protein composition using 2D-liquid chromatography–tandem mass spectrometry (2D-LC-MS/MS). In brief, samples were digested with trypsin and resulting peptides were resolved by high-pH reversed-phase peptide fractionation (*9*), followed by C18 reversed-phase nanoflow ultrahigh-performance liquid chromatography coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (QE Plus MS; Thermo Fisher Scientific, https://www.thermofisher.com), as described (*10*). Raw 2D-LC-MS/MS data were analyzed using Proteome Discoverer version 2.1.1.21 software (Thermo Fisher Scientific), followed by Scaffold perSPECtives version 4.8.7 (Proteome Software; http://www.proteomesoftware.com). A protein database with combined human, *T. cruzi*, and potential contaminants was generated from UniProt (https://www.uniprot.org). Using a false-discovery rate <1% and 1 unique peptide per protein, we identified 12 *T. cruzi* proteins and 338 human proteins (Appendix, https://wwwnc.cdc.gov/EID/article/26/8/19-1042-App1.xlsx). However, when we applied the more stringent criterion of ≥2 unique peptides per protein, we detected only 1 *T. cruzi* protein (i.e., pyruvate phosphate dikinase [PPDK]), and 288 human proteins, of which we identified 19 only in pretreatment samples (Table 2). PPDK has been identified by proteomic

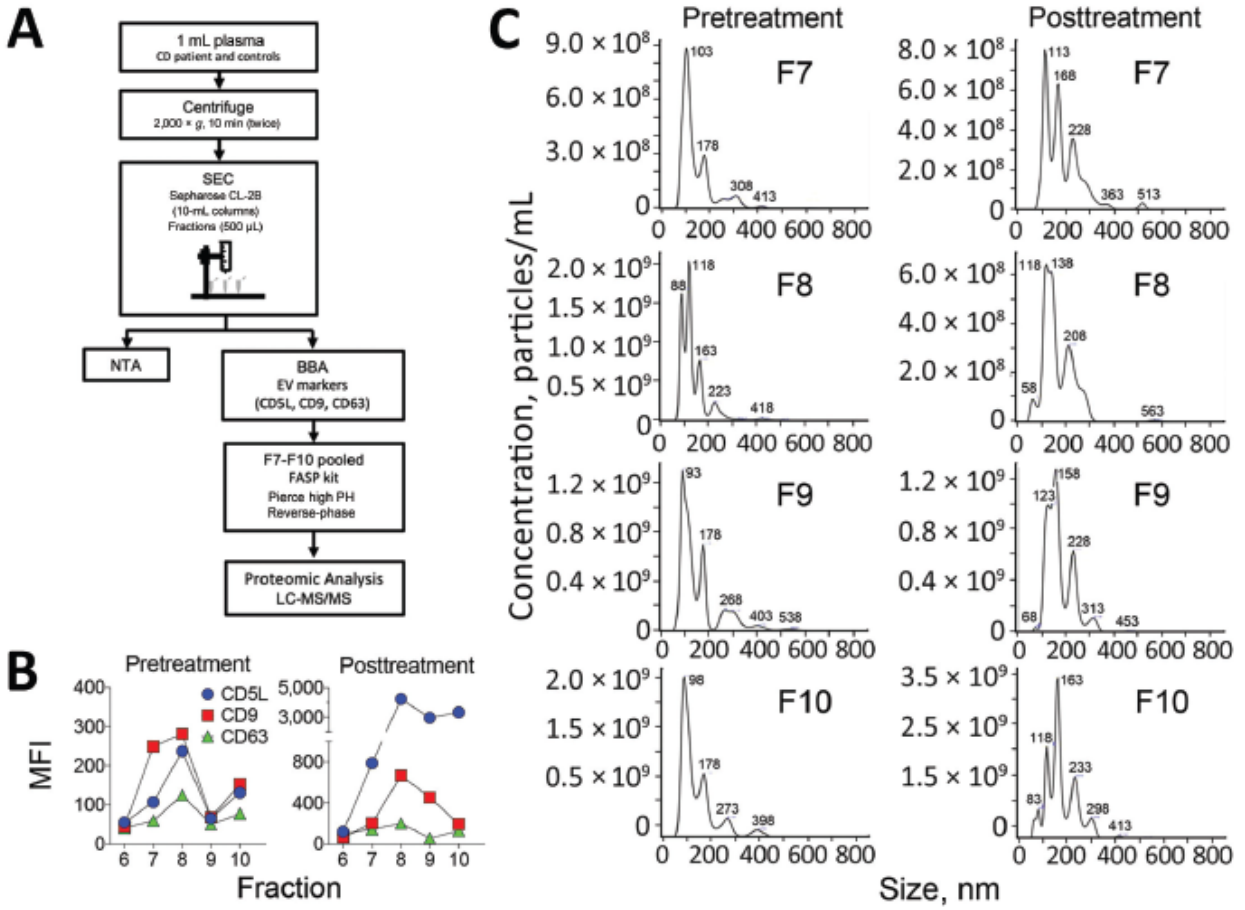

Figure 1. Isolation and characterization of plasma-derived EVs. A) Schematic diagram of the isolation and characterization of EVs derived from plasma samples. The details of each step are explained in The Study section. B) EVs were characterized by BBA using the classical EV markers CD5L, CD9, and CD63. C) NTA of SEC fractions F7–10. BBA, bead-based assay; EV, extracellular vesicle; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MFI, median fluorescence intensity; NTA, nanoparticle tracking analysis; SEC, size-exclusion chromatography.

analysis of *T. cruzi* total secretome and EVs (*10*–*12*). This protein plays a central role in the metabolism of *T. cruzi* glycosomes and has been shown to be upregulated when trypomastigote forms are incubated with the extracellular matrix, an obligatory step before host-cell invasion and differentiation of trypomastigote into amastigote forms (*13*). The specific role of PPDK in EVs secreted by this patient remains to be determined.

Among the 19 human proteins uniquely identified in EVs from the patient with chronic Chagas disease before treatment, the mannan binding lectin serine protease 2 (MASP2) is worth highlighting. A recent study with human samples showed that *MASP2* gene polymorphisms and MASP2 levels are associated with high risk for chronic Chagas disease cardiomyopathy (*14*). Furthermore, mannose-binding lectin, which activates complement on *T. cruzi* through MASP2, has been related to a decrease in blood and tissue parasite load and in myocarditis and cardiac fibrosis in experimental *T. cruzi* infection (*15*). In this study, mRNA levels of collagen-1 and -6 increased in the infected animals' hearts (*15*). These results could support our findings because collagen α-1 is one of the proteins identified exclusively in EVs before patient treatment (Table 2).

Another important observation is the identification of a higher number of human proteins in patient-derived EVs than in the 2 healthy donor–derived EV samples (Figure 2; Appendix). Of the total proteins identified, in which statistical analysis was feasible, 4 were significantly upregulated in patient-derived EVs before treatment, particularly for the proteins complement C1s subcomponent, isoform CRA_b, FLJ00385 protein, and cDNA FLJ75416 (Appendix). Complement C1s subcomponent recently

was identified among the 6 upregulated EV biomarkers with potential for clinical applications in myocardial infarction (*3*).

Conclusions

Proteins associated with EVs secreted by *T. cruzi* have been identified in the conditioned medium of different parasite stages (*11*–*13*) but not in biofluids from Chagas disease patients. We described the proteomic profiling of plasma-derived EVs purified directly from a heart transplant patient with chronic Chagas disease who exhibited reactivation after immunosuppression. We identified human and parasite proteins present or upregulated in plasma-derived EVs from a chronic Chagas disease patient before chemotherapy and that are absent or downregulated after treatment. We thus hypothesize that EV proteins released by the host or parasite during infection might be potential biomarker candidates for evaluating therapeutic response and disease outcome in chronic Chagas disease, independently of the immunologic status of patients.

Table 2. *Trypanosoma cruzi* and human proteins identified in plasma-derived EVs from a heart transplant patient with chronic Chagas disease before benznidazole chemotherapy but absent after treatment and in healthy donors

Protein name†	UniProt accession no.	Unique peptides			
		ChD Pre-BZN‡	ChD Post-BZN	Healthy 1	Healthy 2
T. cruzi					
Pyruvate. phosphate dikinase OS = Trypanosoma cruzi marinkellei GN = MOQ_000480 PE = 3 SV = 1	K2MVM1_TRYCR	2 (0.96)	0	0	0
Homo sapiens					
Collagen α-1(VI) chain OS = Homo sapiens GN = COL6A1 PE = 1 SV = 3	CO6A1_HUMAN	3 (1.44)	0	0	0
Group of Angiopoietin-related protein 6 OS = Homo sapiens GN = ANGPTL6 PE = 1 SV = 1+1	ANGL6_HUMAN (+1)	3 (1.44)	0	0	0
sp|PPIA_HUMAN|	sp|PPIA_HUMAN|	3 (1.44)	0	0	0
Mannan binding lectin serine protease 2 OS = Homo sapiens GN = MASP2 PE = 1 SV = 4	MASP2_HUMAN	2 (1.92)	0	0	0
Myosin regulatory light chain 12B OS = Homo sapiens GN = MYL12B PE = 1 SV = 2	ML12B_HUMAN	2 (1.92)	0	0	0
Collagen α-2(VI) chain OS = Homo sapiens GN = COL6A2 PE = 1 SV = 4	CO6A2_HUMAN	2 (1.44)	0	0	0
Collectin subfamily member 10 (C-type lectin). isoform CRA_a OS = Homo sapiens GN = COLEC10 PE = 4 SV = 1	tr|A0A024R9J3|A0A024R9J3_HUMAN	2 (1.44)	0	0	0
Group of Coagulation factor XIII A chain OS = Homo sapiens GN = F13A1 PE = 1 SV = 4+2	F13A_HUMAN (+2)	2 (1.44)	0	0	0
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. eta polypeptide. isoform CRA_b OS = Homo sapiens GN = YWHAH PE = 3 SV = 1	tr|A0A024R1K7|A0A024R1K7_HUMAN	2 (1.44)	0	0	0
Fibrinogen-like protein 1 OS = Homo sapiens GN = FGL1 PE = 1 SV = 3	FGL1_HUMAN	2 (0.96)	0	0	0
Group of L-lactate dehydrogenase A chain OS = Homo sapiens GN = LDHA PE = 1 SV = 2+1	LDHA_HUMAN (+1)	2 (0.96)	0	0	0
Group of Laminin subunit α-2 OS = Homo sapiens GN = LAMA2 PE = 1 SV = 1+1	A0A087WX80_HUMAN (+1)	2 (0.96)	0	0	0
Group of MHC class I antigen (Fragment) OS = Homo sapiens GN = HLA-A PE = 3 SV = 1+3	tr|D2KZ27|D2KZ27_HUMAN (+3)	2 (0.96)	0	0	0
Group of Serum amyloid A protein OS = Homo sapiens GN = SAA1 PE = 1 SV = 1+2	E9PQD6_HUMAN (+2)	2 (0.96)	0	0	0
Group of Transforming growth factor β-induced 68kDa isoform 2 (Fragment) OS = Homo sapiens GN = TGFBI PE = 2 SV = 1+1	tr|A0A0S2Z4K6|A0A0S2Z4K6_HUMAN (+1)	2 (0.96)	0	0	0
Heparan sulfate proteoglycan 2 (Perlecan). isoform CRA_b OS = Homo sapiens GN = HSPG2 PE = 4 SV = 1	tr|A0A024RAB6|A0A024RAB6_HUMAN	2 (0.96)	0	0	0
Neurogenic locus notch homologue protein 3 OS = Homo sapiens GN = NOTCH3 PE = 1 SV = 2	NOTC3_HUMAN	2 (0.96)	0	0	0
V1–16 protein (Fragment) OS = Homo sapiens GN = V1–16 PE = 4 SV = 1	tr|Q5NV81|Q5NV81_HUMAN	2 (2.88)	0	0	0
Rheumatoid factor RF-ET6 (Fragment) OS = Homo sapiens PE = 2 SV = 1	tr|A2J1N5|A2J1N5_HUMAN	2 (5.29)	0	0	0

*BZN, benznidazole; ChD, Chagas disease.
†All proteins were identified by at least 2 unique peptides. A unique peptide is defined as a peptide found only in ≥1 protein members of the same protein family of a certain proteome.
‡Normalized total spectrum count values are indicated in parenthesis. Complete mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRoteomics IDEntifications Database (PRIDE, https://www.ebi.ac.uk/pride/) partner repository, with the dataset identifier PXD014668 and 10.6019/PXD014668.

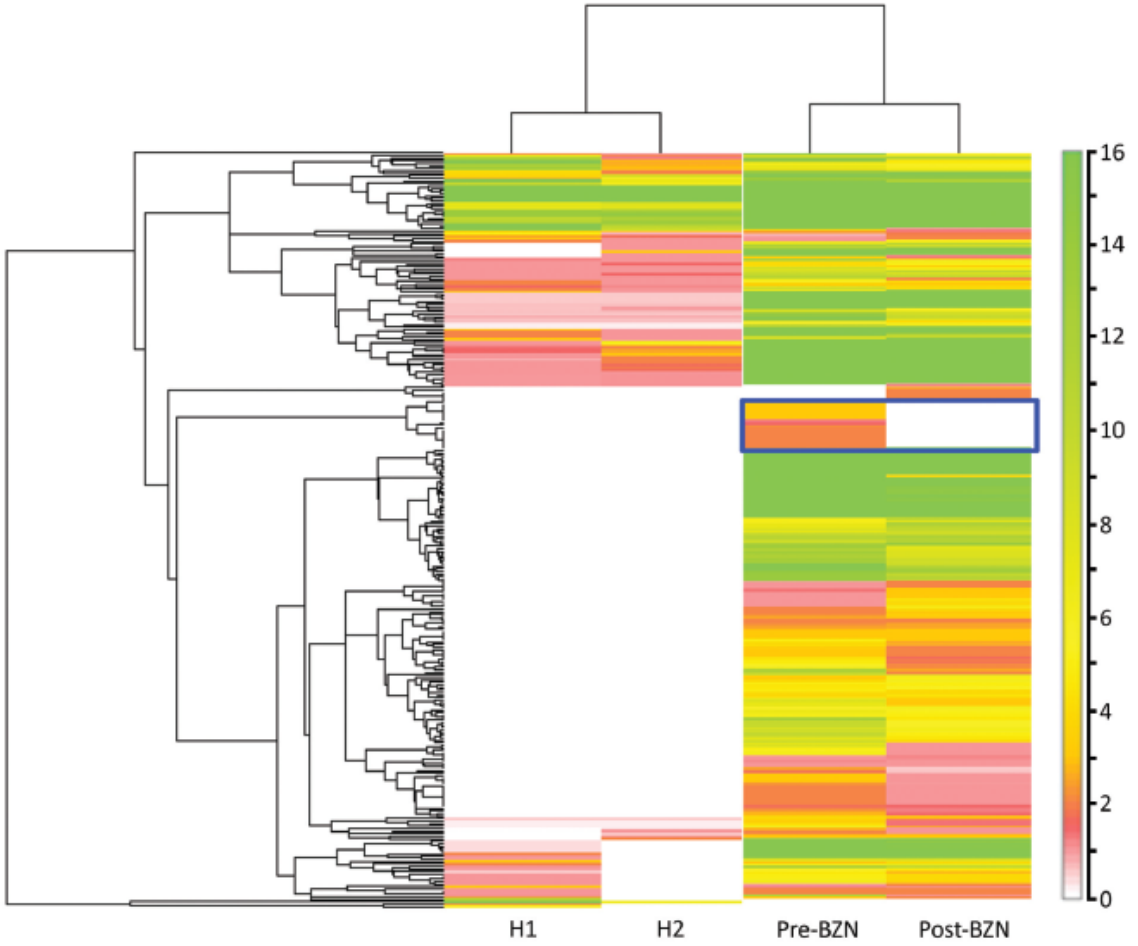

Figure 2. Human proteomic profile of plasma-derived EVs from a heart transplant patient with chronic Chagas disease before and after BZN treatment and from 2 healthy donors. Heatmap of the identified human EV-derived proteins. Heatmap was generated from proteomic data by the Scaffold perSPECtives software (Proteome Software; http://www.proteomesoftware.com). Hierarchical clustering (on the left), using single-linkage clustering with a Euclidean distance metric, was performed based on the normalized weighted spectrum counts of the identified proteins. Color gradient scale bar (Y-axis) indicates normalized spectrum counts (numerical matrix) translated into a color image, as described in the Scaffold perSPECtives version 3.0 User's Manual (http://www.proteomesoftware.com/pdf/scaffold_perspectives_users_guide.pdf?v=232de555). More human proteins were found in EVs isolated from the patient, before and after BZN treatment than from EVs derived from the 2 healthy donors (H1, H2). BZN, benznidazole; EV, extracellular vesicle.

However, our results should be interpreted with caution because they represent a single clinical case. Further research is needed to validate and provide stronger evidence that circulating EVs in patients with chronic Chagas disease can serve as biomarkers in disease progression and early assessment of therapeutic outcomes. Moreover, the future incorporation of such validated biomarkers in a point-of-care device could help in the detection of very low parasites in circulation, particularly when concentrations are below the PCR detection level (2).

Acknowledgments

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Ms. Cortes-Serra is a PhD student in ISGlobal, Hospital Clinic–Universitat de Barcelona, Barcelona, Spain. Her research interests include extracellular vesicles and neglected infectious diseases, especially Chagas disease.

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10. ANNEX

ANNEX 1. LIST OF CLASSICAL EV MARKERS.

Protein name	Gene name	References
Actin, cytoplasmic 1	ACTB	(294,300,301)
Actinin-1	ACTN1	(300)
Actinin-4	ACTN4	(300,301)
ADAM10	ADAM10	(300)
Ago1	AGO1	(301)
Ago3	AGO3	(301)
Ago4	AGO4	(301)
Fructose-bisphosphate aldolase A	ALDOA	(294)
Annexin A1	ANXA1	(301)
Annexin XI	ANXA11	(300,301)
Annexin A2	ANXA2	(294,300,301)
Annexin A5	ANXA5	(294,301)
Annexin A6	ANXA6	(294)
Annexin VII	ANXA7	(301)
ARRDC1	ARRDC1	(301)
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	(301,333)
Basigin	BSG	(333)
Leukocyte surface antigen CD47	CD47	(333)
CD5L	CD5L	(294)
CD63	CD63	(294,300,301,334)
CD81	CD81	(294,301,334)
CD82	CD82	(294)
CD9	CD9	(294,300,301,334)
Cofilin-1	CFL1	(294)
Clathrin heavy chain 1	CLTC	(294,333)
EEF1A1	EEF1A1	(294,301)
eEF2	eEF2	(300,301,333)
EGFR	EGFR	(301)
EHD-4	EHD4	(300)
Enolase 1	ENO1	(301)
Ezrin	EZR	(294,300)
Prothrombin	F2	(300)
Fatty acid synthase	FASN	(294)
Flotillin-1	FLOT1	(300,301,334)
Flottilin-2	FLOT2	(301,334)
GAPDH	GAPDH	(294,300,301,333)
Rab GDP dissociation inhibitor beta	GDI2	(294)
Guanine nucleotide-binding protein G(i) subunit alpha-2	GNAI2	(333)
Guanine nucleotide-binding protein G(i) subunit alpha-3	GNAI3	(333)
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	(333)
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2	(333)
HSP70	HSP70	(300,334)
HSP90	HSP90AA1	(300,301,334)
GP96 (Endoplasmin)	HSP90B1	(300)
HSC70	HSPA8	(300,301)
Mitofilin	IMMT	(300)
Integrin α2	ITGA2	(301)
Integrin ß1	ITGB1	(301,333)
LC3B	LC3B	(301)
Galectin-3-binding protein	LGALS3BP	(294,333)
Lactadherin	MFGE8	(294)
MHCI	MHCI	(300,334)
MHCII	MHCII	(300,334)
Moesin	MSN	(294,300)

PARK7	PARK7	(301)
ALIX	PDCD6IP	(294,301,333,334)
Phosphoglycerate kinase 1	PGK1	(294)
Pyruvate kinase	PK1	(294,301)
Peroxiredoxin-1	PRDX1	(294)
Na/K ATPase	PXK	(301)
Ras-related protein Rab-10	RAB10	(333)
Rab11	Rab11	(301)
Rab27A	Rab27A	(301)
Rab7	Rab7	(301)
GTP-binding nuclear protein Ran	RAN	(333)
Ras-related protein Rap-1b	RAP1B	(294,333)
Radixin	RDX	(294)
Transforming protein RhoA	RHOA	(294)
Rho-related GTP-binding protein RhoC	RHOC	(294)
Ubiquitin-40S ribosomal protein S27a	RPS27A	(333)
RPS8	RPS8	(301)
Ras-related protein R-Ras	RRAS	(333)
Syntenin-1	SDCBP	(294,300,301,333)
Neutral amino acid transporter B(0) (ATB(0))	SLC1A5	(333)
4F2 cell-surface antigen heavy chain (4F2hc) (4F2 heavy chain antigen)	SLC3A2	(333)
p62	SQSTM1	(301)
TSG101	TSG101	(294,300,301,333,334)
Tubulin	TUBA1A	(300,301)
14-3-3 protein beta/alpha	YWHAB	(294,301)
14-3-3 protein epsilon	YWHAE	(294,301)
14-3-3 protein gamma	YWHAG	(294,301)
14-3-3 protein theta	YWHAQ	(294,301)
14-3-3 protein zeta/delta	YWHAZ	(294,301)

ANNEX 2. APPROVAL BY THE ETHICS COMMITTEE OF THE HOSPITAL CLINIC OF BARCELONA.

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DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

NEUS RIBA GARCIA, Secretaria del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona**

Certifica:

Que este Comité ha evaluado la propuesta del promotor, para que se realice el estudio:

DOCUMENTOS CON VERSIONES:

Tipo	Subtipo	Versión
Hoja Información de Paciente	Versión 2. 22.09.2015	v2. 22.09.2015
Protocolo	Versión 1.10.06.2015	v1.10.06.2015

CIF – G-08431173

TÍTULO: Uso de Vesículas extracelulares (EVs) como herramienta para la identificación de nuevos biomarcadores en dos enfermedades parasitarias desatendidas: malaria y chagas

INVESTIGADOR PRINCIPAL: CARMEN FERNÁNDEZ-BECERRA
JOAQUIM GASCÓN BRUSTENGA

y considera que, teniendo en cuenta la respuesta a las aclaraciones solicitadas (si las hubiera), y que:

- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles.
- La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.
- Que se han evaluado la compensaciones económicas previstas (cuando las haya) y su posible interferencia con el respeto a los postulados éticos y se consideran adecuadas.
- Que dicho estudio se ajusta a las normas éticas esenciales y criterios deontológicos que rigen en este centro.
- Que dicho estudio se incluye en una de las líneas de investigación biomédica acreditadas en este centro, cumpliendo los requisitos necesarios, y que es viable en todos sus términos.

Este CEIC acepta que dicho estudio sea realizado, debiendo ser debiendo ser comunicado a dicho Comité Ético todo cambio en el protocolo o acontecimiento adverso grave.

y hace constar que:
1º En la reunión celebrada el día 23/09/2015, acta 16/2015, se decidió emitir el informe correspondiente al estudio de referencia.
2º El CEIC del Hospital Clínic i Provincial, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95)
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- JOSE MIGUEL SOTOCA (Farmacéutico Atención Primaria, CAP Les Corts)
- ANTONI TRILLA GARCIA (Médico Epidemiólogo, HCB - Director UAPS)
- OCTAVI SANCHEZ LOPEZ (Representante de los pacientes)
- MARIA JESÚS BERTRAN LUENGO (Médico Epidemiólogo, HCB)
- MARTA AYMERICH GREGORIO (Médico Hematólogo, HCB)

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En el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, este se ausentará de la reunión durante la discusión del proyecto.

Para que conste donde proceda, y a petición del promotor,

Barcelona, a 22 de octubre de 2015

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NEUS RIBA GARCIA, Secretario del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona.**

Certifica:
Que este Comité ha evaluado la propuesta del promotor, para que se realice:

Nueva HIP/CI

HIP/CI Versión 4.0 de 14.06.2017

del estudio:

CÓDIGO: NÚMERO EUDRACT:

TÍTULO: Uso de Vesículas extracelulares (EVs) como herramienta para la identificación de nuevos biomarcadores en dos enfermedades parasitarias desatendidas: malaria y chagas

PROMOTOR:

y emite

DICTAMEN FAVORABLE

Y hace constar que:

1º En la reunión celebrada el día 13/07/2017, acta 13/2017 se decidió emitir el informe correspondiente a la enmienda de referencia.

2º El CEIC del Hospital Clínic de Barcelona, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95).

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- NURIA SOLER BLANCO (Farmacéutica Hospitalaria, HCB)
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Que en el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, éste se ausentará de la reunión durante la discusión del proyecto.

Barcelona, a 18 de julio de 2017

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DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

NEUS RIBA GARCIA, Secretario del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona.**

Certifica:
Que este Comité ha evaluado la propuesta del promotor, para que se realice:

Noves versions en protocol i FIP

Protocol Versión 4.0 de 30.04.2018 FIP Versión 5.0 de 30.04.2018

del estudio:

CÓDIGO: NÚMERO EUDRACT:

TÍTULO: Uso de Vesículas extracelulares (EVs) como herramienta para la identificación de nuevos biomarcadores en dos enfermedades parasitarias desatendidas: malaria y chagas

PROMOTOR:

y emite

DICTAMEN FAVORABLE

Y hace constar que:

1º En la reunión celebrada el día 28/06/2018, acta se decidió emitir el informe correspondiente a la enmienda de referencia.

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- JOSE RIOS GUILLERMO (Estadístico. Plataforma de Estadística Médica. IDIBAPS)
- OCTAVI SANCHEZ LOPEZ (Representante de los pacientes)
- MARIA JESÚS BERTRAN LUENGO (Médico Epidemiólogo, HCB)
- JOAQUÍN SÁEZ PEÑATARO (Médico Farmacólogo Clínico, HCB)
- SERGIO AMARO DELGADO (Médico Neurólogo, HCB)
- JULIO DELGADO GONZÁLEZ (Médico Hematólogo, HCB)
- EDUARD GUASCH I CASANY (Médico Cardiólogo, HCB)
- VIRGINIA HERNANDEZ GEA (Médico Hepatólogo, HCB)
- NURIA SOLER BLANCO (Farmacéutica Hospitalaria, HCB)
- MARINA ROVIRA ILLAMOLA (Farmacéutico Atención Primaria, CAP Eixample)

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Hospital Universitari

- JOSE LUIS BLANCO ARÉVALO (Médico Medicina Interna, HCB)
- MIRIAM MÉNDEZ GARCÍA (Abogada, HCB)
- MERCÈ VIDAL FLOR (Enfermera, HCB)

Que en el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, éste se ausentará de la reunión durante la discusión del proyecto.

RIBA GARCIA NEUS - 46540984R

Signat digitalment per RIBA GARCIA NEUS - 46540984R
DN: c=ES, serialNumber=IDCES-46540984R, givenName=NEUS, sn=RIBA GARCIA, cn=RIBA GARCIA NEUS - 46540984R
Data: 2018.07.03 12:57:36 +02'00'

Barcelona, a 03 de julio de 2018

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Generalitat de Catalunya
Departament de Salut

Institut UNIVERSITAT DE BARCELONA

ANNEX 3. INFORMED CONSENT.

HOJA INFORMATIVA Y CONSENTIMIENTO INFORMADO

TÍTULO DEL ESTUDIO: USO DE VESÍCULAS EXTRACELULARES (EVS) COMO HERRAMIENTA PARA LA IDENTIFICACIÓN DE NUEVOS BIOMARCADORES EN DOS ENFERMEDADES PARASITARIAS DESATENDIDAS: MALARIA Y CHAGAS

CÓDIGO DEL ESTUDIO: HCB / 2015 / 0616

PROMOTOR: INSTITUTO DE SALUD GLOBAL (ISGlobal)

INVESTIGADORES PRINCIPALES: MARIA DEL CARMEN FERNANDEZ BECERRA (Profesora Asistente de Investigación en el ISGlobal, telf.: 932275400 ext: 4276) Y JOAQUIM GASCÓN BRUSTENGA (Jefe de Servicio de Salut Internacional-Hospital Clinic, telf.: 932275400 ext: 3288)

CENTRO: HOSPITAL CLINIC – ISGLOBAL – Institut d'Investigació Germans Trias i Pujol (IGTP)

INTRODUCCIÓN

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por un Comité de Ética de la Investigación con medicamentos y por la Agencia Española de Medicamentos y Productos Sanitarios, de acuerdo a la legislación vigente, el Real Decreto 1090/2015 de 4 de diciembre y el Reglamento Europeo 536/2014 de 16 de abril, por los que se regulan los ensayos clínicos con medicamentos.

Nuestra intención es que usted reciba la información correcta y suficiente para que pueda decidir si acepta o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir. Además, puede consultar con las personas que considere oportuno.

PARTICIPACIÓN VOLUNTARIA

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar o cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su tratamiento.

DESCRIPCIÓN GENERAL DEL ESTUDIO

El paludismo o malaria es causado por un parásito denominado *Plasmodium* que se transmite a través de la picadura de mosquitos infectados. En el organismo humano, los parásitos se multiplican en el hígado y después infectan los glóbulos rojos. Los síntomas de la malaria generalmente aparecen de 10 a 15 días después de la picadura del mosquito. Si no se trata, el paludismo puede poner en peligro la vida del paciente en poco tiempo, pues altera el aporte de sangre a órganos vitales. En España, personas que viajan a regiones donde existe este parásito pueden infectarse durante el viaje y presentar malaria.

La enfermedad de Chagas o tripanosomiasis americana es una infección provocada por un parásito, denominado *Trypanosoma cruzi*, ampliamente distribuido por América Central y Sudamérica, que se transmite al hombre por la picada de un insecto llamado "vinchuca". En

España, existen personas infectadas por *T. cruzi* debido a los flujos migratorios provenientes de América Latina. En nuestro país, el parásito puede transmitirse a los hijos durante el embarazo o a otras personas mediante la donación de sangre u órganos. La persona que padece la enfermedad puede no tener ningún tipo de síntoma durante años y sólo ser diagnosticada una vez que el parásito ya ha producido alteraciones graves a nivel del aparato digestivo o del corazón.

En este estudio queremos saber si existen moléculas específicas provocadas por las infecciones por *T. cruzi* y plamodios, que se eliminan a través de la sangre, saliva y/u orina, y si éstas desaparecen después del tratamiento contra el parásito. Ello podría ser útil para facilitar el diagnóstico futuro de estas infecciones. También se quiere saber si estos factores podrían relacionarse con la severidad de la enfermedad.

Participarán personas con enfermedad de Chagas, con malaria y también personas sanas, como grupo de control, para poder comparar los resultados obtenidos.

A las personas con estas infecciones que entren en el estudio se les ofrecerá la posibilidad de tratamiento con benznidazol (a las personas afectadas por infección por *T. cruzi)* y con artesunato o malarone (a las personas con malaria*)*. Estos son los fármacos habituales para tratar estas enfermedades, y no representan una novedad.

Para la realización de este estudio es necesario realizar las siguientes pruebas:

- Extracción de sangre: es necesario realizar unos análisis de sangre antes y después del tratamiento. Esta extracción post-tratamiento se realizará en los pacientes con malaria 7 días después del tratamiento, mientras que en los pacientes con infección por *T.cruzi*, al terminar el tratamiento y a los 6 y 12 meses después del tratamiento. Estas extracciones sirven para realizar las pruebas necesarias para detectar las moléculas específicas del parásito.

- Obtención de muestra de saliva y orina: en el caso de las personas con infección por *T. cruzi,* es necesario obtener una muestra de saliva y orina antes y después del tratamiento, para la detección de las moléculas específicas del parásito.

- *Electrocardiograma y ecocardiograma*: siguiendo el protocolo habitual del SSI, a los pacientes con enfermedad de Chagas se les realizará un electrocardiograma y un ecocardiograma, para valorar el grado de afectación cardíaca.

Siguiendo el protocolo habitual de tratamiento para la enfermedad de Chagas, deberá acudir al hospital cada 15 días durante el tratamiento para realizar una visita médica y extracción de sangre, tal y como se hace de forma regular. Después del tratamiento tendrá 2 visitas más, a los 6 y 12 meses. En total, su participación en el estudio duraría 12 meses desde el inicio del tratamiento.

A las personas con paludismo, se les hará un control a los 7 días después del inicio del tratamiento.

A las personas sin infección por *T. cruzi* y *Plasmodium* (grupo control) se les realizará la extracción sanguínea una única vez.

Las muestras de sangre, saliva y orina sólo se utilizarán para las determinaciones indicadas en el protocolo, y, si fuera necesario, para usos futuros en estudios similares, aunque en ningún caso serán usados para estudios genéticos. El material biológico se guardará en el Laboratorio de Malaria del ISGlobal/ SSI hasta su utilización y harán parte de la colección del Instituto de Salud Global.

Este proyecto ha sido aprobado por el Comité Ético de Investigación clínica (CEIC) de la Corporación Sanitaria Clínic, garantizándose el cumplimiento de toda la normativa bioética aplicada a la investigación biomédica. Su único fin es el de contribuir al conocimiento actual de la enfermedad de Chagas y malaria.

BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO

* *Riesgos del estudio*: La obtención de muestras de sangre, saliva y orina no comporta ningún riesgo adicional valorable, aparte de las habituales (mínimo sangrado y molestias locales). La ecocardiografía no comporta ningún riesgo adicional.

* *Posibles beneficios derivados de estudio:* No hay beneficios directos por participar en el estudio, no se prevé ningún pago. No obstante, del estudio pueden obtenerse beneficios futuros en el diagnóstico y seguimiento de los pacientes afectados por la enfermedad de Chagas y por la malaria.

CONFIDENCIALIDAD

El Hospital Clínic de Barcelona, con CIF 0802070C, como responsable del tratamiento de sus datos, le informa que el tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los participantes se ajustará al cumplimiento del Reglamento UE 2016/679 del Parlamento Europeo y del Consejo de 27 de abril de 2016 relativo a la protección de las personas físicas en cuanto al tratamiento de datos personales y la libre circulación de datos, siendo de obligado cumplimiento a partir del 25 de mayo del 2018. La base legal que justifica el tratamiento de sus datos es el consentimiento que da en este acto, conforme a lo establecido en el artículo 9 del Reglamento UE 2016/679.

Los datos recogidos para estos estudios se recogerán identificados únicamente mediante un código, por lo que no se incluirá ningún tipo de información que permita identificar a los participantes. Sólo el médico del estudio y sus colaboradores con un permiso específico podrán relacionar sus datos recogidos en el estudio con su historia clínica.

Su identidad no estará al alcance de ninguna otra persona a excepción de una urgencia médica o requerimiento legal. Podrán tener acceso a su información personal identificada, las autoridades sanitarias, el Comité de Ética de Investigación y personal autorizado por el promotor del estudio, cuando sea necesario para comprobar datos y procedimientos del estudio, pero siempre manteniendo la confidencialidad de acuerdo a la legislación vigente.

Sólo se cederán a terceros y a otros países los datos codificados, que en ningún caso contendrán información que pueda identificar al participante directamente (como nombre y apellidos, iniciales, dirección, número de la seguridad social, etc.). En el supuesto de que se produjera esta cesión, sería para la misma finalidad del estudio descrito y garantizando la confidencialidad.

Si se realizara una transferencia de datos codificados fuera de la UE, ya sea a entidades relacionadas con el centro hospitalario donde usted participa, a prestadores de servicios o a investigadores que colaboren con su médico, sus datos quedarán protegidos por salvaguardas como contratos u otros mecanismos establecidos por las autoridades de protección de datos.

Además de los derechos que ya contemplaba la legislación anterior (acceso, modificación, oposición y cancelación de datos, supresión en el nuevo Reglamento) ahora también puede limitar el tratamiento de datos que sean incorrectos, solicitar una copia o que se trasladen a un tercero (portabilidad) los datos que usted ha facilitado para el estudio. Para ejercitar estos derechos, o si desea saber más sobre confidencialidad, deberán dirigirse al

investigador principal del estudio o o al Delegado de Protección de Datos del Hospital Clínic de Barcelona a través de protecciodades@clinic.cat. Así mismo tienen derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho/a.

Los datos ya recogidos no se pueden eliminar aunque usted abandone el estudio, para garantizar la validez de la investigación y cumplir con los deberes legales y los requisitos de autorización de medicamentos. Pero no se recogerán nuevos datos si usted decide dejar de participar.

El Investigador y el Promotor están obligados a conservar los datos recogidos para el estudio al menos hasta 8 años tras su finalización. Posteriormente, la información personal solo se conservará por el centro para el cuidado de su salud y por el promotor para otros fines de investigación científica si el paciente hubiera otorgado su consentimiento para ello, y si así lo permite la ley y requisitos éticos aplicables.

GASTOS Y COMPENSACIÓN ECONÓMICA

Usted no tendrá que pagar por los medicamentos ni por pruebas específicas del estudio. Su participación en el estudio no le supondrá ningún gasto adicional a la práctica clínica habitual. No obstante, no se prevé ningún pago por la participación en este estudio.

OBTENCIÓN Y UTILIZACIÓN DE MUESTRAS BIOLÓGICAS

La participación en este estudio conlleva la obtención de muestras de sangre, saliva y orina. De conformidad con lo que establece la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011 por el que se regula la utilización de muestras biológicas en investigación, al firmar este documento usted acepta que se utilicen las muestras que se obtendrán para las finalidades del presente estudio. Las muestras se mantendrán almacenadas en la planta 1 del Institut d'Investigació Germans Trias i Pujol (IGTP) hasta su utilización para los objetivos de este estudio. Una vez finalizado, las muestras sobrantes serán destruidas.

Se utilizará un código para identificar su muestra y no se utilizará ningún dato suyo que pueda desvelar su identidad. Únicamente el médico del estudio y sus colaboradores podrán relacionar la muestra con usted.

Los datos que se deriven de la utilización de estas muestras se trataran del mismo modo que el resto de datos que se obtengan durante este estudio.

La cesión de muestras biológicas para este estudio es gratuita y voluntaria. Esto supone que usted no tendrá derechos sobre posibles beneficios comerciales de los descubrimientos que pudieran derivarse del resultado de la investigación biomédica.

Si se obtuviera información relevante que pudiera afectar a su salud o a la de sus familiares, se le notificará. En caso que fuera necesario contactar con usted, se utilizarían los datos que constan en su historia clínica. No obstante, se respetará su derecho a decidir que no se le comuniquen éstos, para lo que puede marcar la casilla que se encuentra en el formulario de consentimiento.

Asimismo, en caso que se realicen análisis genéticos no se le comunicarán ni a usted ni a su médico los resultados que se obtuvieran, aunque usted tiene derecho a solicitarlos dirigiéndose al médico del estudio. Tenga en cuenta que al tratarse de estudios de investigación exploratorios, no proporcionarán información útil ni se podrán utilizar para guiar su tratamiento ni para diagnóstico.

OTRA INFORMACIÓN RELEVANTE

Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos y puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de nuevos análisis.

También debe saber que puede ser excluido del estudio si el promotor o los investigadores del estudio lo consideran oportuno, ya sea por motivos de seguridad, por cualquier acontecimiento adverso que se produzca por la medicación en estudio o porque consideren que no está cumpliendo con los procedimientos establecidos. En cualquiera de los casos, usted recibirá una explicación adecuada del motivo que ha ocasionado su retirada del estudio.

Al firmar la hoja de consentimiento adjunta, se compromete a cumplir con los procedimientos del estudio que se le han expuesto.

Cuando acabe su participación recibirá el mejor tratamiento disponible y que su médico considere el más adecuado para su enfermedad, pero es posible que no se le pueda seguir administrando la medicación del estudio. Por lo tanto, ni el investigador ni el promotor adquieren compromiso alguno de mantener dicho tratamiento fuera de este estudio. Al otorgar su consentimiento a participar en el estudio, asegura que no tendría objeciones a dichas consideraciones.

Consentimiento Informado

Datos del estudio para el que se otorga el consentimiento

Titulo del proyecto: USO DE VESÍCULAS EXTRACELULARES (EVS) COMO HERRAMIENTA PARA LA IDENTIFICACIÓN DE NUEVOS BIOMARCADORES EN DOS ENFERMEDADES PARASITARIAS DESATENDIDAS: MALARIA Y CHAGAS

Yo, *(nombre y apellidos del participante)*

- He leído la hoja de información que se me ha entregado sobre el estudio.

- He podido hacer preguntas sobre el estudio.

- He recibido suficiente información sobre el estudio.

- He hablado con: *(nombre del investigador)*

- Comprendo que mi participación es voluntaria.

- Comprendo que puedo retirarme del estudio:

- Cuando quiera.

- Sin tener que dar explicaciones.

- Sin que esto repercuta en mis cuidados médicos.

- De conformidad con lo que establece la Ley Orgánica 15/1999, de 13 de Diciembre de Protección de Datos de Carácter Personal, declaro haber sido informado de la existencia de un fichero o tratamiento de datos de carácter personal, de la finalidad de la recogida de éstos y de los destinatarios de la información.

- Presto libremente mi conformidad para participar en el estudio.

Firma del participante Firma del investigador

Fecha: ____/____/____ Fecha: ____/____/____

Deseo que me comuniquen la información derivada de la investigación que pueda ser relevante para mí salud:

o SI o NO

Firma del participante Firma del investigador

Fecha: ____/____/____ Fecha: ____/____/____

ANNEX 4. SUMMARY OF THE SAMPLES USED FOR EACH ASSAY.

STUDY PATIENT IDENTIFIER	STUDY GROUP	POST-TTM SAMPLE?	PCR	SEC 10 mL PROTEOMICS	SEC 1 mL PROTEOMICS	DIC PROTEOMICS	FUP DIC PROTEOMICS	BBA	NTA	MICROSCOPY	WB	FUNCTIONAL ASSAY
EVINDET 1	G1		•			•			•			
EVINDET 2	G1	•	•			•			•		•	
EVINDET 3	G1		•			•			•			
EVINDET 4	G1		•			•			•		•	
EVINDET 5	G1		•			•			•			
EVINDET 6	G1		•			•			•			
EVINDET 7	G1		•			•			•			
EVINDET 8	G1		•			•			•			
EVINDET 9	G1	•	•			•			•			
EVINDET 10	G1		•			•			•			
EVINDET 11	G1	•	•		•	•		•	•	•		
EVINDET 12	G1	•	•			•			•			
EVINDET 13	G1	•	•		•	•		•	•			
EVINDET 14	G1	•	•			•			•			
EVINDET 15	G1	•	•			•			•			
EVINDET 16	G1	•	•		•			•	•	•		
EVINDET 17	G1		•									
EVINDET 18	G1	•	•									
EVCARD01	G2	•	•			•	•		•			
EVCARD02	G2	•	•			•	•		•			
EVCARD03	G2	•	•			•	•		•			
EVCARD04	G2	•	•			•	•		•			
EVCARD05	G2		•									
EVCARD06	G2	•	•			•	•		•			
EVCARD07	G2	•	•			•	•		•			
EVCARD08	G2	•	•			•	•		•			
EVCARD09	G2	•	•			•	•		•			
EVCARD10	G2	•	•			•	•		•		•	•
EVCARD11	G2											
EVCARD12	G2											
EVCARD13	G2											
EVCARD14	G2	•	•			•	•		•		•	
EVCARD15	G2	•	•			•	•		•			
EVCARD16	G2	•	•			•	•		•			•
EVCARD17	G2	•	•			•	•		•			•
EVCARD18	G2	•	•			•	•		•			
EVCARD19	G2	•	•			•	•		•			
EVCARD20	G2	•	•	•				•	•			
EVCARD21	G2	•										
EVCARD22	G2											
EVCARD23	G2		•									
EVCARD24	G2	•	•									
EVCARD25	G2	•										
EVCARD26	G2	•										
EVCARD27	G2	•										
EVCARD NO CHAGAS 01	G3					•					•	
EVCARD NO CHAGAS 02	G3					•						
EVCARD NO CHAGAS 03	G3					•					•	
EVCARD NO CHAGAS 04	G3					•						
EVCARD NO CHAGAS 05	G3					•						
EVCARD NO CHAGAS 06	G3					•						
EVCARD NO CHAGAS 07	G3					•						
EVCARD NO CHAGAS 08	G3					•						
EVCARD NO CHAGAS 09	G3					•						
EVCARD NO CHAGAS 10	G3					•						
EVCARD NO CHAGAS 11	G3											
HEALTHY 1	G4					•			•			
HEALTHY 2	G4					•			•			•
HEALTHY 3	G4					•			•			
HEALTHY 4	G4					•			•			
HEALTHY 5	G4					•			•			
HEALTHY 6	G4					•			•			•
HEALTHY 7	G4				•	•		•	•		•	
HEALTHY 8	G4					•			•			
HEALTHY 9	G4					•			•		•	•
HEALTHY 10	G4					•			•			
HEALTHY 11	G4				•			•				
HEALTHY 12	G4											
HEALTHY 13	G4											
HEALTHY 14	G4											
HEALTHY 15	G4											
HEALTHY 16	G4											
HEALTHY 17	G4											
HEALTHY 18	G4											
HEALTHY 19	G4											
HEALTHY 20	G4											
HEALTHY 21	G4											

ANNEX 5. CLINICAL AND MORPHOLOGICAL DATA OF THE INDIVIDUALS INCLUDED IN THE STUDY

PATIENT	GENDER	DATE OF BIRTH	ORIGIN (COUNTRY AND DEPARTMENT)	COMORBIDITIES	SEROLOGY	PCR	CLINICAL GROUP	IF CARDIAC PATHOLOGY, KUSHNIR	ELECTROCARDIOGRAM	ECOCARDIOGRAM	TOTAL PRESCRIVED DOSE BZD (mg)	TOTAL RECEIVED DOSE BZD	TREATMENT DURATION (DAYS)
EVCARD01	F	10/11/1990	BOLIVIA (CERCADO)	-	POSITIVE	POSITIVE (28,74)	CARDIAC	I	LEFT ANTERIOR HEMIBLOCK	-	18.000	18.000	60
EVCARD02	M	25/07/1982	BOLIVIA (CARRASCO)	-	POSITIVE	POSITIVE (28,52)	CARDIAC	II	RIGHT BUNDLE BRANCH BLOCK+ LEFT ANTERIOR HEMIBLOCK + VENTRICULAR EXTRASYSTOLE	-	18.000	18.000	61
EVCARD03	F	09/10/1979	BOLIVIA (CERCADO)	-	POSITIVE	POSITIVE (28,77)	CARDIAC	I	LEFT ANTERIOR HEMIBLOCK	-	18.000	18.000	60
EVCARD04	M	25/11/1977	BOLIVIA (CERCADO)	-	POSITIVE	POSITIVE (28,13)	CARDIAC	II	BRADYCARDIA (44 BPM) + INCOMPLETE RIGHT BUNDLE BRANCH BLOCK	-	18.000	18.000	60
EVCARD05	F	11/05/1985	BOLIVIA (COCHABAMBA-SACABA-CHAPARE)	-	POSITIVE	POSITIVE (27,05)	CARDIAC	I	BRADYCARDIA (45 BPM)	-	18.000	18.000	60
EVCARD06	F	28/12/1985	BOLIVIA (QUILLACOLLO)	-	POSITIVE	POSITIVE (28,96)	CARDIAC	I	LEFT ANTERIOR HEMIBLOCK	-	18.000	18.000	60
EVCARD07	F	18/06/1968	BOLIVIA (QUILLACOLLO / TIQUIPAYA)	DIGESTIVE DISEASE (GASTRITIS BY CLINICS)	POSITIVE	POSITIVE (29,42)	CARDIAC	I	LEFT ANTERIOR HEMIBLOCK	-	18.000	18.000	60
EVCARD08	F	12/07/1987	BOLIVIA (CERCADO)	DIGESTIVE DISEASE (GASTRITIS BY CLINICS)	POSITIVE	NEGATIVE	CARDIAC	II	RIGHT BUNDLE BRANCH BLOCK	-	18.000	18.000	60
EVCARD09	F	18/09/1994	BOLIVIA (CERCADO)	-	POSITIVE	POSITIVE (31,27)	CARDIAC	I	BRADYCARDIA (40 BPM)	-	18.000	18.000	60
EVCARD10	F	18/10/1992	BOLIVIA (CERCADO)	-	POSITIVE	NEGATIVE	CARDIAC	I	BRADYCARDIA (50 BPM)	-	18.000	18.000	60

EVCARD11	F	19/04/1995	BOLIVIA (CERCADO)	-	POSITIVE	-	CARDIAC	I	BRADYCARDIA (48 BPM)	-	-	-	-
EVCARD12	F	30/08/1996	BOLIVIA (CERCADO)	-	POSITIVE	-	CARDIAC	I	FIRST-DEGREE ATRIOVENTRICULAR BLOCK	-	-	-	-
EVCARD13	-	-	-	-	-	-	-	-	-	-	-	-	-
EVCARD14	F	25/03/1976	BOLIVIA (CAPINOTA)	-	POSITIVE	POSITIVE (27,09)	CARDIAC	II	INCOMPLETE RIGHT BUNDLE BRANCH BLOCK + FIRST-DEGREE ATRIOVENTRICULAR BLOCK	-	18.000	18.000	60
EVCARD15	M	05/08/1993	BOLIVIA (MIZQUE)	-	POSITIVE	POSITIVE (31)	CARDIAC	II	BRADYCARDIA (38 BPM) + RIGHT BUNDLE BRANCH BLOCK	-	18.000	16.500	60
EVCARD16	F	02/08/1967	BOLIVIA (CERCADO)	HYPERTHYROIDISM	POSITIVE	POSITIVE (27,15)	CARDIAC	I	LEFT ANTERIOR HEMIBLOCK	-	18.000	18.000	60
EVCARD17	M	28/08/1979	BOLIVIA (QUILLACOLLO)	DIGESTIVE DISEASE (GASTRITIS BY ENDOSCOPY CAUSED BY *Helicobacter Pilory*)	POSITIVE	POSITIVE (28,45)	CARDIAC	I	BRADYCARDIA (42 BPM)	-	18.000	18.000	60
EVCARD18	F	24/04/1973	BOLIVIA (CERCADO)	-	POSITIVE	POSITIVE (30,32)	CARDIAC	II	INCOMPLETE RIGHT BUNDLE BRANCH BLOCK+ VENTRICULAR EXTRASYSTOLE	-	18.000	18.000	60
EVCARD19	M	13/11/1962	BOLIVIA (CERCADO)	DIGESTIVE DISEASE (GASTRITIS BY ENDOSCOPY CAUSED BY *Helicobacter Pilory*)	POSITIVE	POSITIVE (31,21)	CARDIAC	II	RIGHT BUNDLE BRANCH BLOCK	-	18.000	18.000	60
EVCARD20	M	26/05/1964	BOLIVIA (SANTA CRUZ)	MULTIFACTORIAL HEPATITIS, HYPERTENSION, MEGACOLON, MEGASIGMA, MEGAESOPHAGUS	POSITIVE	POSITIVE (25,25)	CARDIAC	III	SEVERE VENTRICULAR DYSFUNCTION, FREQUENT VENTRICULAR EXTRASYSTOLES	15-20%	17.400	17.400	58
EVCARD21	F	06/11/1976	BOLIVIA (SANTA CRUZ)	ANXIETY, OBESITY, ESOPHAGITIS PHASE III, LATENT TUBERCULOSIS	POSITIVE	NEGATIVE	CARDIAC	I	LEFT VENTRICULAR HYPERTROPHY	60%	28.200	28.200	70

EVCARD22	F	26/04/1968	ECUADOR	HEART TRANSPLANT, HYPERKALAEMIA	POSITIVE	-	CARDIAC	III	RIGHT BUNDLE BRANCH BLOCK + SEVERE VENTRICULAR DYSFUNCTION	65%	-	-	-
EVCARD23	M	19/07/1972	BOLIVIA (SUCRE)	-	POSITIVE	NEGATIVE	CARDIAC	I	RIGHT BUNDLE BRANCH BLOCK, LEFT ATRIAL ENLARGEMENT, LEFT ANTERIOR FASCICULAR BLOCK	65%	18.000	18.000	60
EVCARD24	M	16/01/1960	BOLIVIA (SANTA CRUZI)	-	POSITIVE	NEGATIVE	CARDIAC	I	CHRONIC CHAGAS MYOCARDIOPHATY	60%	21.300	21.300	61
EVCARD25	F	04/10/1985	BOLIVIA (COCHABAMBA)	CONGENITAL DEAFNESS, PACEMARKER IMPLANTATION	POSITIVE	-	CARDIAC	II	CHRONIC CHAGAS MYOCARDIOPHATY	58%	27.000	4.400	67
EVCARD26	F	18/04/1956	BOLIVIA (SANTA CRUZ)	HYPOTHYROIDISM, OSTEOPOROSIS, HYPERTENSION, DYSLIPIDEMIA, HYPERPARATHYROIDISM	POSITIVE	-	CARDIAC	II	CHRONIC CHAGAS MYOCARDIOPHATY	50-55%	17.700	17.700	59
EVCARD27	F	29/09/1977	BOLIVIA (CAMARGO)	-	POSITIVE	-	CARDIAC	I	DIASTOLIC FUNCTION ALTERATION + FIRST-DEGREE ATRIOVENTRICULAR BLOCK	60%	26.400	26.400	66

PATIENT	**GENDER**	**DATE OF BIRTH**	**ORIGIN (COUNTRY AND DEPARTMENT)**	**COMORBIDITIES**	**SEROLOGY**	**PCR**	**CLINICAL GROUP**	**TOTAL PRESCRIVED DOSE BZD (MG)**	**TOTAL RECEIVED DOSE BZD (MG)**	**TREATMENT DURATION (DAYS)**
EVINDET01	M	27/07/69	BOLIVIA (COCHABAMBA)	-	POSITIVE	NEGATIVE	INDETERMINATE	22.800	22.800	65
EVINDET02	F	21/05/70	BOLIVIA (SANTA CRUZ)	PSORIASIS, FIBROMYALGIA	POSITIVE	NEGATIVE	INDETERMINATE	21.600	LOST OF FOLLOW-UP	LOST OF FOLLOW-UP
EVINDET03	F	12/10/58	BOLIVIA	HYPERCHOLESTEROLEMIA, MIGRAINE	POSITIVE	NEGATIVE	INDETERMINATE	-	-	-

EVINDET04	F	2/6/58	BOLIVIA (SANTA CRUZ / MINERO)	-	POSITIVE	NEGATIVE	INDETERMINATE	-	-	-
EVINDET05	F	29/06/69	BOLIVIA (SANTA CRUZ / SAN JAVIER)	ARTERIAL HYPERTENSION, IRON DEFICIENCY ANEMIA, HYPERPROLACTINEMIA, DEPRESSION, PARANOID SCHIZOPHRENIA	POSITIVE	NEGATIVE	INDETERMINATE	-	-	-
EVINDET06	F	24/12/80	BOLIVIA (SANTA CRUZ)	-	POSITIVE	POSITIVE (CT 26,44)	INDETERMINATE	-	-	-
EVINDET07	M	09/06/78	BOLIVIA (PUNATA)	-	POSITIVE	NEGATIVE	INDETERMINATE	-	-	-
EVINDET08	F	23/03/1967	BOLIVIA (SAN MARTÍN)	HYPOTHYROIDISM, IRON DEFICIENCY ANEMIA, FIBROMYALGIA, DEPRESSION	POSITIVE	NEGATIVE	INDETERMINATE	-	-	-
EVINDET09	M	13/05/1973	BOLIVIA (SANTA CRUZ)	-	POSITIVE	NEGATIVE	INDETERMINATE	22.200	22.200	63
EVINDET10	M	02/10/1962	BOLIVIA (TARIJA)	-	POSITIVE	POSITIVE (CT 36,06)	INDETERMINATE	-	-	-
EVINDET11	F	24/08/1984	BOLIVIA	-	POSITIVE	NEGATIVE	INDETERMINATE	17.100	16.200	54
EVINDET12	F	25/07/1969	PARAGUAY	-	POSITIVE	NEGATIVE	INDETERMINATE	21.300	21.300	61
EVINDET13	F	04/08/1973	BOLIVIA (COCHABAMBA)	NEUROCYSTICERCOSIS	POSITIVE	NEGATIVE	INDETERMINATE	16.200	16.200	54
EVINDET14	M	28/05/1967	BOLIVIA (SANTA CRUZ)	-	POSITIVE	NEGATIVE	INDETERMINATE	21.000	21.000	60
EVINDET15	M	17/07/1979	BOLIVIA (COCHABAMBA)	-	POSITIVE	POSITIVE (34,68)	INDETERMINATE	25.500	17.500	43
EVNDET16	M	27/12/1973	BOLIVIA	HYPERCHOLESTEROLEMIA	POSITIVE	NEGATIVE	INDETERMINATE	19.500	19.500	65
EVINDET17	F	18/03/1977	BOLIVIA (SANTA CRUZ)	-	POSITIVE	NEGATIVE	INDETERMINATE	23.100	23.100	66
EVINDET18	F	04/09/1966	BOLIVIA (SANTA CRUZ)	-	POSITIVE	POSITIVE (27,21)	INDETERMINATE	24.000	24.000	60

PATIENT	GENDER	DATE OF BIRTH	ORIGIN (COUNTRY AND DEPARTMENT)	COMORBIDITIES	SEROLOGY	CLINICAL GROUP	ELECTROCARDIOGRAM	ECOCARDIOGRAM
EVCARD NO CHAGAS 01	M	22/06/1969	SPAIN	HYPERTENSION, SCHIZOPHRENIA, ENOLIC HEPATOPATHY	NEGATIVE	CARDIAC	SEVERE VENTRICULAR DYSFUNCTION, SEVERE MITRAL INSUFFICIENCY	10%
EVCARD NO CHAGAS 02	F	19/09/1972	SPAIN	ANTIPHOSPHOLIPID SYNDROME, HEPATIC AFFECTATION	NEGATIVE	CARDIAC	SEVERE VENTRICULAR DYSFUNCTION	20%
EVCARD NO CHAGAS 03	F	12/05/69	SPAIN	-	NEGATIVE	CARDIAC	ARRHYTHMOGENIC CARDIOMYOPATHY -	25%
EVCARD NO CHAGAS 04	M	23/03/1971	COLOMBIA	DIABETES	NEGATIVE	CARDIAC	LEFT BUNDLE BRANCH BLOCK, SEVERE VENTRICULAR DILATATION	20%
EVCARD NO CHAGAS 05	F	19/04/1960	SPAIN	MERALGIA PARAESTHETICA	NEGATIVE	CARDIAC	FAMILIAL DILATED CARDIOMYOPATHY (MUTATION MYH7)	35%
EVCARD NO CHAGAS 06	F	15/06/1981	GEORGIA	HYPERTENSION, HEART TRANSPLANT	NEGATIVE	CARDIAC	IDIOPATHIC DILATATED CARDIOMYOPATHY	55%
EVCARD NO CHAGAS 07	M	12/07/1961	SPAIN	-	NEGATIVE	CARDIAC	SEVERE VENTRICULAR DYSFUNCTION, LEFT BUNDLE BRANCH BLOCK III	30%
EVCARD NO CHAGAS 08	M	21/01/1965	VENEZUELA	HYPERTENSION, DIABETES, CHRONIC KIDNEY DISEASE, DIABETIC RETINOPATHY, NON-HODGKIN'S LYMPHOMA IN 2011, TUBERCULOSIS AFTER LYMPHOMA TREATMENT	NEGATIVE	CARDIAC	CHEMOTHERAPY-INDUCED CARDIOMYOPATHY	25%
EVCARD NO CHAGAS 09	M	20/01/1969	SPAIN	HYPOTHYROIDISM, , CHRONIC VENOUS INSUFFICIENCY, STROKE (2019).	NEGATIVE	CARDIAC	NON-ISCHEMIC CARDIOMYOPATHY	10%
EVCARD NO CHAGAS 10	M	22/07/1966	SPAIN	RENAL FAILURE, HYPERURICEMIA, OBSTRUCTIVE SLEEP APNOEA/HYPOPNOEA SYNDROME, AUTOIMMUNE THYROIDITIS, GASTROESOPHAGEAL REFLUX, BARRETT'S ESOPHAGUS, COLONIC POLYPOSIS, VITILIGO	NEGATIVE	CARDIAC	LEFT BUNDLE BRANCH BLOCK	18%
EVCARD NO CHAGAS 11	F	09/03/1964	BOLIVIA	DIABETES, DYSLIPIDEMIA, FATTY LIVER DISEASE	NEGATIVE	CARDIAC	IDIOPATHIC DILATED CARDIOMYOPATHY	30%

PATIENT	GENDER	DATE OF BIRTH	ORIGIN (COUNTRY AND DEPARTMENT)	COMORBIDITIES	SEROLOGY	CLINICAL GROUP
H1	F	09/03/1984	BOLIVIA (COCHABAMBA)	-	NEGATIVE	HEALTHY
H2	F	10/06/1977	BOLIVIA (COCHABAMBA)	-	NEGATIVE	HEALTHY
H3	F	15/09/1982	BOLIVIA (SANTA CRUZ)	-	NEGATIVE	HEALTHY
H4	F	18/03/1989	VENEZUELA (MARACAIBO)	-	NEGATIVE	HEALTHY
H5	F	20/09/1982	BOLIVIA (COCHABAMBA)	-	NEGATIVE	HEALTHY
H6	F	23/12/1970	BOLIVIA (TARIJA)	-	NEGATIVE	HEALTHY
H7	M	09/02/1972	BOLIVIA (SANTA CRUZ)	-	NEGATIVE	HEALTHY
H8	F	16/02/1972	BOLIVIA (COCHABAMBA)	-	NEGATIVE	HEALTHY
H9	F	14/05/1997	BOLIVIA	-	NEGATIVE	HEALTHY
H10	F	11/09/1990	BOLIVIA (SUCRE)	-	NEGATIVE	HEALTHY
H11	M	21/10/1972	BOLIVIA (COCHABAMBA)	-	NEGATIVE	HEALTHY
H12	F	04/09/1978	BOLIVIA (SANTA CRUZ)	MIGRAINE WITH AURA, *Helicobacter Pilory* POSITIVE	NEGATIVE	HEALTHY
H13	F	03/07/1980	BOLIVIA (COCHABAMBA)	ALLERGIC ASTHMA	NEGATIVE	HEALTHY
H14	F	04/04/1965	BOLIVIA (LA PAZ)	-	NEGATIVE	HEALTHY
H15	F	05/11/1989	BOLIVIA (SANTA CRUZ)	-	NEGATIVE	HEALTHY
H16	F	23/11/1997	BOLIVIA	-	NEGATIVE	HEALTHY
H17	F	31/12/1988	BOLIVIA (LA PAZ)	-	NEGATIVE	HEALTHY
H18	F	28/08/1968	BOLIVIA (SUCRE)	-	NEGATIVE	HEALTHY
H19	M	05/02/1971	BOLIVIA	-	NEGATIVE	HEALTHY
H20	F	20/10/1972	BOLIVIA (SANTA CRUZ/LA PAZ)	HUMAN PAPILLOMA VIRUS POSITIVE	NEGATIVE	HEALTHY
H21	F	01/07/1980	-	-	NEGATIVE	HEALTHY

ANNEX 6. HUMAN PROTEINS IDENTIFIED IN PROTEOMIC ANALYSIS OF CD9, CD63 AND CD81 POSITIVE EVS ISOLATED BY DIRECT IMMUNOAFFINITY CAPTURE DIFFERENTIALLY EXPRESSED IN SAMPLES *T. cruzi* INFECTED PATIENTS COMPARED TO SERONEGATIVE *T. cruzi* INDIVIDUALS.

Protein Name	Accession Number	*T. cruzi* CARD PRE-TTM	*T. cruzi* CARD POST-TTM	*T. cruzi* INDET	HEALTHY	CARDIO NON *T. cruzi*
Group of Anion exchange protein	E2RVJ0	0	0	0	2.34 (N=5)	3.78 (N=2)
Complement component C8 alpha chain	P07357	0	0	0	2.34 (N=9)	1.51 (N=3)
Plasma protease C1 inhibitor	A0A7I2V2D2	0	0	0	2.34 (N=6)	4.54 (N=4)
Group of 59 kDa serine/threonine-protein kinase	V9HWF0	0	0	0	2.34 (N=6)	3.03 (N=3)
Proto-oncogene tyrosine-protein kinase Src	P12931	0	0	0	2.34 (N=6)	0.76 (N=4)
PGRMC1 protein	Q6IB11	0	0	0	2.34 (N=5)	0.76 (N=2)
cDNA FLJ58626, highly similar to Endoplasmin (Heat shock protein 90 kDa beta member 1)	B4DU71	0	0	0	2.34 (N=7)	1.51 (N=2)
Peroxiredoxin-6	P30041	0	0	0	2.34 (N=4)	1.51 (N=2)
Group of Chloride intracellular channel protein (Fragment)	Q53FB0	0	0	0	2.34 (N=3)	1.51 (N=2)
F-actin-capping protein subunit alpha	A8K0T9	0	0	0	2.34 (N=5)	0.76 (N=2)
Group of Prostaglandin G/H synthase 1	P23219	0	0	0	2.34 (N=5)	0.76 (N=1)
EH domain-containing protein 3	Q9NZN3	0	0	0	2.34 (N=5)	0.76 (N=1)
Syntaxin-binding protein 2	Q15833	0	0	0	2.34 (N=3)	0.76 (N=1)
NADH-cytochrome b5 reductase	A0A024R4X0	0	0	0	2.34 (N=3)	0.76 (N=1)
Phospholipid-transporting ATPase (Fragment)	Q59EX4	0	0	0	2.34 (N=2)	0.76 (N=1)
Aminopeptidase (Fragment)	Q59E93	0	0	0	1.87 (N=1)	11.35 (N=2)
Tropomyosin 1 (Alpha), isoform CRA_m	H7BYY1	0	0	0	1.87 (N=7)	3.03 (N=3)
Tropomyosin 1 (Alpha), isoform CRA_f	Q6ZN40	0	0	0	1.87 (N=7)	2.27 (N=3)
Group of Clathrin heavy chain 1	Q00610	0	0	0	1.87 (N=3)	2.27 (N=3)
Apolipoprotein E (Fragment)	Q8TCZ8	0	0	0	1.40 (N=4)	3.03 (N=6)
Carboxypeptidase N subunit 2	P22792	0	0	0	1.40 (N=8)	2.27 (N=3)
Immunoglobulin delta heavy chain	P0DOX3	0	0	0	0.93 (N=3)	2.27 (N=2)
Transferrin receptor protein 1	P02786	0	0	0	0.93 (N=1)	3.78 (N=1)
Annexin	B4DPJ2	0	0	0	2.80 (N=7)	2.27 (N=4)
Endonuclease domain-containing 1 protein	O94919	0	0	0	2.80 (N=6)	0.76 (N=4)
Tropomyosin 3 isoform 3 (Fragment)	A0A0S2Z4I4	0	0	0	2.80 (N=6)	2.27 (N=3)
B-cell receptor-associated protein	A0A2R8Y5M6	0	0	0	2.80 (N=1)	3.03 (N=3)
Malectin	Q14165	0	0	0	2.80 (N=3)	2.27 (N=2)
Group of L-lactate dehydrogenase A chain	P00338	0	0	0	2.80 (N=5)	1.51 (N=1)
Pigment epithelium-derived factor	P36955	0	0	0	3.27 (N=7)	1.51 (N=2)
Tumor rejection antigen (Gp96) 1 variant (Fragment)	Q59FC6	0	0	0	3.27 (N=7)	2.27 (N=3)
Group of Glycerol-3-phosphate dehydrogenase (Fragment)	Q53T76	0	0	0	3.27 (N=5)	1.51 (N=1)

Multimerin-1	Q13201	0	0	0	5.61 (N=8)	3.78 (N=4)
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 (Fragment)	Q53EP4	0	0	0	5.61 (N=5)	1.51 (N=3)
Alpha-actinin-1 (Fragment)	A0A804HLD0	0	0	0	5.14 (N=8)	3.78 (N=2)
60 kDa chaperonin	B3GQS7	0	0	0	5.14 (N=4)	0.76 (N=2)
Group of Adenylyl cyclase-associated protein	D3DPU2	0	0	0	4.21 (N=8)	3.03 (N=5)
Complement component C7	P10643	0	0	0	3.27 (N=9)	2.27 (N=3)
cDNA, FLJ93143, highly similar to Homo sapiens complement component 7 (C7), mRNA	B2R6W1	0	0	0	3.27 (N=9)	2.27 (N=3)
cDNA FLJ54622, highly similar to Prothrombin	B4DDT3	0	0	0	2.34 (N=10)	1.51 (N=4)
Epididymis secretory sperm binding protein	A0A024R240	0	0	0	3.74 (N=5)	3.03 (N=3)
Isocitrate dehydrogenase (NADP), mitochondrial	P48735	0	0	0	3.74 (N=6)	1.51 (N=4)
Tropomyosin alpha-4 chain	P67936	0	0	0	3.74 (N=8)	5.30 (N=5)
IGH + IGL c494_heavy_IGHV3-9_IGHD4-11_IGHJ3 (Fragment)	A0A5C2G9L4	2.09 (N=15)	1.53 (N=15)	2.47 (N=15)	0.47 (N=10)	0.76 (N=10)
IGH c311_heavy__IGHV3-49_IGHD6-19_IGHJ6 (Fragment)	A0A5C2GE80	2.09 (N=14)	1.53 (N=15)	2.47 (N=15)	0.47 (N=2)	0.76 (N=3)
HCG2039797 (Fragment)	A0N4V7	2.09 (N=11)	1.53 (N=12)	2.47 (N=4)	0.47 (N=10)	0.76 (N=8)
Apolipoprotein C-I	P02654	2.09 (N=11)	1.53 (N=12)	2.47 (N=1)	0.47 (N=10)	0.76 (N=10)
Protein CC2D2B	Q6DHV5	2.09 (N=10)	1.53 (N=11)	2.47 (N=7)	0.47 (N=9)	0.76 (N=3)
IGH c450_heavy__IGHV3-33_IGHD1-26_IGHJ4 (Fragment)	A0A5C2G648	2.09 (N=11)	1.53 (N=13)	2.47 (N=2)	0.47 (N=8)	0.76 (N=7)
Proteolipid protein 2	Q04941	2.09 (N=10)	1.53 (N=9)	2.47 (N=7)	0.47 (N=7)	0.76 (N=5)
NACHT, LRR and PYD domains-containing protein 10	Q86W26	2.09 (N=6)	1.53 (N=5)	2.47 (N=4)	0.47 (N=6)	0.76 (N=2)
IG c974_heavy_IGHV3-21_IGHD4-11_IGHJ3 (Fragment)	A0A5C2GQF6	2.09 (N=2)	1.53 (N=4)	2.47 (N=3)	0.47 (N=9)	0.76 (N=6)
LINE-1 type transposase domain-containing protein 1	Q5T7N2	2.09 (N=3)	1.53 (N=1)	2.47 (N=1)	0.47 (N=8)	0.76 (N=4)
DNA damage-induced apoptosis suppressor protein (Fragment)	E9PMA7	2.09 (N=3)	1.53 (N=4)	2.47 (N=8)	0.00	0.00
IGL c2094_light_IGKV4-1_IGKJ5 (Fragment)	A0A5C2FZH0	2.09 (N=3)	1.53 (N=6)	2.47 (N=8)	0.00	0.00
Calpain-8 (Fragment)	H0YEQ4	2.09 (N=2)	1.53 (N=1)	2.47 (N=1)	0.00	0.00
IGL c1252_light_IGKV3-20_IGKJ1 (Fragment)	A0A5C2FWQ9	2.09 (N=1)	1.53 (N=1)	2.47 (N=2)	0.00	0.00
F-actin-monooxygenase MICAL3	Q7RTP6	2.09 (N=3)	1.53 (N=5)	2.47 (N=5)	0.00	0.00

ANNEX 7. HUMAN PROTEINS IDENTIFIED IN PROTEOMIC ANALYSIS OF CD9, CD63 AND CD81 POSITIVE EVS ISOLATED BY DIRECT IMMUNOAFFINITY CAPTURE DIFFERENTIALLY EXPRESSED IN SAMPLES OF INDETERMINATE *T. cruzi* INFECTED PATIENTS.

Protein Name	Accession Number	*T. cruzi* CARD PRE-TTM	*T. cruzi* CARD POST-TTM	*T. cruzi* INDET	HEALTHY	CARDIO NON *T. cruzi*
Group of Gamma-crystallin S	A0A1B1PFW0	0	0	2.47 (N=2)	0	0
Protein unc-45 homolog A	A0A1W2PNX8	0	0	2.47 (N=1)	0	0
E3 ubiquitin-protein ligase ZSWIM2	Q8NEG5	0	0	2.47 (N=1)	0	0
IG c950_light_IGKV3-20_IGKJ2 (Fragment)	A0A5C2GYX6	0	0	2.47 (N=1)	0	0
Putative uncharacterized protein encoded by LINC00471	Q8N535	0	0	2.47 (N=1)	0	0
Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	B2R7M3	0	0	2.47 (N=1)	0	0
GAS2-like protein 2	Q8NHY3	0	0	2.47 (N=1)	0	0
La-related protein 1 (Fragment)	E5RHK4	0	0	2.47 (N=1)	0	0
Microbicidal oxidase (Fragment)	V9GZH6	0	0	2.47 (N=1)	0	0
Protein N-terminal asparagine amidohydrolase	H3BU50	0	0	2.47 (N=1)	0	0
Uncharacterized protein	A0A5F9ZHU2	0	0	2.47 (N=1)	0	0
Alternative protein C1orf124	L8E708	0	0	2.47 (N=1)	0	0
Alternative protein DNAH2	L8E8M4	0	0	2.47 (N=1)	0	0
Dapper homolog 3	G5E9H6	0	0	2.47 (N=1)	0	0
Pappalysin-2	Q9BXP8	0	0	2.47 (N=1)	0	0
IGH c856_heavy_IGHV4-39_IGHD4-17_IGHJ4 (Fragment)	A0A7S5ETP7	0	0	2.47 (N=1)	0	0
Phospholipid-transporting ATPase	A0A2R8YDI5	0	0	2.47 (N=1)	0	0
cDNA FLJ51186, highly similar to Homo sapiens ADP-ribosylation factor-like 6 interacting protein 2 (ARL6IP2), mRNA	B7Z8V5	0	0	2.47 (N=1)	0	0
Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	A0A590UJ46	0	0	2.47 (N=1)	0	0
Integrin alpha-4	P13612	0	0	2.47 (N=1)	0	0
Neuroblast differentiation-associated protein AHNAK	Q09666	0	0	2.47 (N=1)	0	0
Tumor protein 63	Q9H3D4	0	0	2.47 (N=1)	0	0
SAFB protein	B7ZLP5	0	0	2.47 (N=1)	0	0
Spermine oxidase	Q9NWM0	0	0	2.47 (N=2)	0	0
DENN domain-containing protein 4C	Q5VZ89	0	0	2.47 (N=3)	0	0
RB-associated KRAB zinc finger protein	Q9NYW8	0	0	2.47 (N=2)	0	0
Protein AKNAD1	Q5T1N1	0	0	2.47 (N=2)	0	0
Group of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4 variant (Fragment)	Q59FZ6	0	0	2.47 (N=3)	0	0
Exocyst complex component 3-like protein 2	A0A1C7CYX0	0	0	2.47 (N=2)	0	0

ANNEX 8. HUMAN PROTEINS IDENTIFIED IN PROTEOMIC ANALYSIS OF CD9, CD63 AND CD81 POSITIVE EVS ISOLATED BY DIRECT IMMUNOAFFINITY CAPTURE DIFFERENTIALLY EXPRESSED IN SAMPLES OF CARDIAC *T. cruzi* INFECTED PATIENTS.

Protein Name	Accession Number	*T. cruzi* CARD PRE-TTM	*T. cruzi* CARD POST-TTM	*T. cruzi* INDET	HEALTHY	CARDIO NON *T. cruzi*
Retrotransposon Gag-like protein 5	Q5HYW3	2.09 (N=1)	0	0	0	0
Group of Sodium-ascorbic acid transporter 2	A0MSJ5	2.09 (N=1)	0	0	0	0
Integrator complex subunit 6 (Fragment)	C9JAV7	2.09 (N=1)	0	0	0	0
Leucine-rich repeat-containing protein 63	A0A6Q8PFU4	2.09 (N=1)	0	0	0	0
Low-density lipoprotein receptor-related protein 1B	Q9NZR2	2.09 (N=1)	0	0	0	0
Scavenger receptor class F member 1	A0A0A0MR54	2.09 (N=1)	0	0	0	0
Nucleolar complex protein 4 homolog	Q9BVI4	2.09 (N=1)	0	0	0	0
Protein FAM71F1	F8WC62	2.09 (N=1)	0	0	0	0
Testis-specific Y-encoded-like protein 4	Q9UJ04	2.09 (N=1)	0	0	0	0
Group of IGH c651_heavy_IGHV3-23_IGHD3-22_IGHJ4 (Fragment)	A0A7S5BYL0	2.09 (N=1)	0	0	0	0.76 (N=1)
Protein SON	P18583	2.09 (N=1)	0	0	0	0
RING-type E3 ubiquitin transferase RAD18	A0A024R2I7	2.09 (N=1)	0	0	0	0
Zinc finger and BTB domain-containing protein 12	Q9Y330	2.09 (N=1)	0	0	0	0
Dynein axonemal heavy chain 9	E7EP17	2.09 (N=1)	0	0	0	0
Laminin subunit beta-2	P55268	2.09 (N=1)	0	0	0	0
Acrosomal protein KIAA1210	Q9ULL0	2.09 (N=1. less than 2 UP)	0	0	0	0
Death domain-containing protein 1	A0A1W2PR94	2.09 (N=1)	0	0	0	0
Ornithine decarboxylase (Fragment)	C9JG30	2.09 (N=1)	0	0	0	0
Nucleosome-remodeling factor subunit BPTF	Q12830	2.09 (N=1)	0	0	0	0.76 (N=1)
Soluble TIE1 variant 5	B5A952	2.09 (N=2)	0	0	0	0
IGL c2418_light_IGLV1-40_IGLJ3 (Fragment)	A0A5C2FZG9	2.09 (N=2)	0	0	0	0
Receptor-type tyrosine-protein phosphatase F	P10586	2.09 (N=2)	0	0	0	0
Myeloid leukemia factor 1	C9K0D4	2.09 (N=1)	0	0	0	0.76 (N=1)
Group of CP protein	A5PL27	0	21.38 (N=10)	4.95 (N=4)	10.75 (N=10)	13.62 (N=9)
Ceruloplasmin	E9PFZ2	0	21.38 (N=8)	4.95 (N=2)	10.28 (N=10)	13.62 (N=8)

IG c316_heavy_IGHV3-7_IGHD5-18_IGHJ4 (Fragment)	A0A5C2GLU4	0	1.53 (N=1)	2.47 (N=1)	0.93 (N=6)	1.513 (N=4)
Group of Beta-2-microglobulin	P61769	0	1.53 (N=3)	2.47 (N=1)	0.93 (N=8)	0.76 (N=2)
Filamin-C	Q14315	0	1.53 (N=1)	2.47 (N=1)	1.87 (n=3)	2.27 (N=5)
Thromboxane-A synthase	P24557	0	1.53 (N=1)	2.47 (N=1)	2.80 (N=6)	1.51 (N=3)
Hemoglobin subunit beta	P68871	0	1.53 (N=1)	2.47 (N=1)	4.21 (N=10)	6.81 (N=8)
Cytochrome c oxidase polypeptide Vb	A0A384NL93	0	1.53 (N=2)	4.95 (N=1)	0.93 (N=3)	0.76 (N=5)
Group of Pregnancy zone protein	P20742	0	4.58 (N=7)	2.47 (N=6)	8.88 (N=2)	6.05 (N=10)
Calcium-transporting ATPase	A8K9K1	0	1.53 (N=3)	2.47 (N=1)	8.88 (N=8)	7.57 (N=5)
Group of Protein-tyrosine-phosphatase	A0A087WTK0	0	3.05 (N=4)	2.47 (N=2)	6.07 (N=8)	6.05 (N=5)
ATP synthase subunit alpha, mitochondrial	P25705	0	3.05 (N=1)	4.95 (N=2)	5.14 (N=7)	6.81 (N=5)