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Chagas Disease

Basic Investigations and Challenges

Edited by Veeranoot Nissapatorn and Helieh S. Oz

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Contributors

Ulrike Kemmerling, Christian Castillo, Ana Liempi, Ileana Carrillo, Lisvaneth Medina, Guadalupe Garcia Liñares, Maria Elena Villagrán Herrera, José Antonio De Diego Cabrera, José Alejandro Martínez Ibarra, Manuel Sánchez Moreno, Hebert Luis Hernández-Montiel, Ricardo Francisco Mercado Curiel, Nicolás Camacho Calderón, Anabela Cordeiro-Da-Silva, Luis Gaspar, Terry Smith, Nilmar Moretti, Sergio Schenkman, Mercedes Gonzalez, Javier Varela, Hugo Cerecetto, Jesús Ortega-García, Edgar Alfonso Paredes-Gonzalez, Gerardo Álvarez Hernandez, Francisco Capani, Rodolfo Alberto Kölliker Frers, Justo Carbajales, Gabriela Razzitte, Roberto Pontarolo, Wilton Hideki Kawaguchi, Leticia Bonâncio Cerqueira, Michel Leandro Campos, Mariana Millan Fachi, Iara J Messias Reason, Rosa Lidia Solís-Oviedo, Víctor Monteón, Ángel De La Cruz Pech-Canul, Ruth López, Mauricio Canals, Andrea Canals, Salvador Ayala, Valentina Tapia-Garay, Dante D. Cáceres, Veeranoot Nissapatorn

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Meet the editors

Dr. Veeranoot Nissapatorn currently serves as an associate professor at the School of Allied Health Sciences and a coordinator of Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Thailand. She had more than 16-year working experiences at the University of Malaysia, a premier institution in Malaysia. She is actively involved in research and development with more than 100 publications, and her areas of interest are infectious parasitic diseases including epidemiology, clinically relevant, diagnostic challenges, natural products, and health awareness including human and animal aspects. She is an editorial board member of different journals and an active reviewer of over 30 different ISI and Scopus-indexed journals. Furthermore, she has published book chapters and also served as a guest editor, an international invited speaker in various conferences, and a visiting professor.

Dr. Helieh S. Oz has a DVM degree, MS degree (University of Illinois), PhD degree (University of Minnesota), and a clinical translational research certificate (University of Kentucky). She is an active member of the American Gastroenterological Association (AGA) and AGA fellow. Dr. Oz is a microbiologist with expertise in infectious, inflammatory diseases, drug discovery, pathogenesis, and micronutrients. Dr. Oz has several publications on inflammatory disorders and microbial and infectious diseases including Chagas disease and *Trypanosoma cruzi*. She served as an editor of books, e.g., *Nutrients, Infectious, and Inflammatory Diseases* (2018), special issues such as *Gut Inflammatory, Infectious Diseases, and Nutrition* (*Mediators of Inflammation*) (2018), and *Gastrointestinal Inflammation and Repair: Role of Microbiome, Infection, and Nutrition* (*Gastroenterology Research Practice*). Also, she is an editorial board member of different journals and an avid reviewer of journals.

Contents

Preface

Inasmuch as Carlos Justiniano Ribeiro Chagas had made American trypanosomiasis the centerpiece—the magnum opus of his life's work, the authors of this book have dedicated their time and efforts and have sacrificed much to heed his call with continuing the battle against this important neglected parasitic disease.

This book contains 11 chapters of indispensable materials significant to advance understanding further this neglected parasitic disease. Some of the chapters contained herein are as follows: "Slowed Development of Natural Products for Chagas Disease, how to Move Forward?" This chapter submits several concerns relative to drug development for Chagas disease. The lag in moving forward can mainly be traced from disinterest of the pharmaceutical industry. There is still no effective chemotherapeutic agent or vaccine for Chagas disease, and the authors present literatures relative to natural products for development as potential phytotherapeutics, which can be translated from the laboratory to the market and economically accessible to all. "Transmitter Insect of Chagas Disease in Northwest Mexico: A Comparative Study of the Cuticular Hydrocarbons Profile of Three Populations of Triatoma rubida - Peridomestic, Domestic, and Sylvatic" provides a deeper understanding on several species of vectors of Chagas disease, both well and rarely studied. This chapter presents factors involved in the transition of vectors from wild to domesticated types and evaluates these various species in terms of cuticular hydrocarbon profile.

Still, millions from different parts of the world are suffering from the debilitating and irreparable damage that Chagas disease inflicts upon the body. We hope that the readers of this book would one day, sooner, or later join in the efforts of eradicating this neglected parasitic disease.

I would like to extend my sincere thanks to Frederick R. Masangkay, associate professor, and Giovanni De Jesus Milanez, instructor IV from the Far Eastern University, Manila, Philippines, for their insights. Dearest readers though ordinary and simple individuals they may be, they have selflessly devoted their time and effort in whatever little personal way they can to help in the battle against Chagas disease, they did not neglect Carlos Chagas call.

Veeranoot Nissapatorn
School of Allied Health Sciences and
Research Excellence Center for Innovation and Health Products (RECIHP)
Walailak University, Thailand

Helieh S. Oz
Department of Internal Medicine
University of Kentucky, USA

Chapter 1

Introductory Chapter: Chagas Disease and Its Global Impacts

Frederick R. Masangkay, Giovanni DJ Milanez,
Helieh S. Oz and Veeranoot Nissapatorn

Additional information is available at the end of the chapter

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1. Introduction

Soft tissue specimens from mummified remains of members of the Chinchorro culture of the Andean coast in South America have been found to be positive for *Trypanosoma cruzi* (*T. cruzi)* DNA [1]. The Chinchorros were fishermen inhabiting the pacific coastal region of northern Chile and southern Peru and *T. cruzi* the causative agent of American trypanosomiasis or Chagas disease is presently listed as one of the several neglected tropical diseases (NTDs) and as one of the five neglected parasitic infections (NPIs) of the world focused by the World Health Organization (WHO) and the Center for Disease Control (CDC) for public health intervention [2]. This finding spells out that the causative agent of American trypanosomiasis has been around for 9000 years already as the samples which tested positive are dated back to 7050 B.C. [1]. There are several historical accounts that have also mentioned about the prevalence of Chagas disease but some of these are just speculative assessments as signs and symptoms of patients were not consistent with the current pathophysiology of the disease. There were even speculations that Charles Darwin himself was infected with Chagas disease but popular opinions disagree as there were no actual clinical evidences to support that Charles Darwin was indeed suffering from megacolon or heart disease even later on in his life [3].

2. *T. cruzi*: a primeval pathogen

It was only in 1908 during an anti-malaria campaign in supporting a railway construction in the North of Minas Gerais that Carlos Chagas, a Brazilian physician (hygienist and bacteriologist) during his observations made this disease the center piece of his novel findings.

A railroad engineer acquainted him with the presence of blood sucking insects in infested houses which preferentially were biting and taking blood meals on the face of occupancies in those mud huts during their sleep [4]. He identified the protozoan parasite *T. cruzi* in the bugs' gut and later confirmed the presence in the blood samples of individuals who had a history of being bitten by this blood sucking bug. For this, scientific minds from his time to the present are indebted for the collection of knowledge he made possible in the process of understanding the disease, its agents, and its course in both human and animal hosts.

3. Transmission: a wide spectrum of dissemination modes?

Chagas disease or American trypanosomiasis is caused by a protozoan parasite *T. cruzi* mainly identified in blood samples of patients who frequently had been bitten by a triatomine bug on the face hence its namesake as kissing bugs. An estimated 8 million people in Mexico, Central and South America are infected with Chagas disease [2]. This vector borne parasitic infection is not limited to rural areas but has also been identified in urban areas. In this modern time, diaspora has been an effective vehicle in spreading this infection to different parts of the world. While other modes of infections have also been identified from ingesting contaminated food or drinks with the feces of the kissing bug [5] to blood transfusions [6], and organ transplantation [7, 8] as being incidental causes of parasite transmission.

4. Chagas disease: debilitating health impacts

Signs and symptoms of a person having Chagas disease may include fever, fatigue, malaise, and sometimes rash or edema (chagoma) around the eye during the acute phase while cardiac and intestinal complication may present in less than 50% of those infected [2]. Infections persist throughout life and most infected individuals are not even aware that they carry the parasite. To date, there is no effective drug or vaccine that has been developed and vector control is still the main driving force for the parasite intervention.

The World Health Organization Fact sheet of 2017 outlines key information about Chagas disease. Approximately 6 to 7 million people worldwide are infected (mostly in Latin America) and some scientists even place estimates as high as 20 million. About 30% of chronic infections progress to cardiac implications and 10% to digestive tract abnormalities which are irreparable [9]. Chronic infections can lead to megaesophagus, megacolon, mega-small intestine, and mega gallbladder to name a few, other impairments of the gastrointestinal tract organs and tissues have been observed. A higher incidence of *Helicobacter pylori* infection, colon, and esophageal cancer has been demonstrated in association with trypanosomiasis as well [10]. Chronic chagasic cardiopathy on the other hand leads to cardiac lesions and the patient's immune status and initial parasitemia are significant determinants of the gravity and progression to organ failure [11].

The estimated annual cost of medical care for trypanosomiasis patients in Colombia in 2008 has been estimated to be 267 million US Dollars [9]. While a computational simulation model funded by the Bill and Melinda Gates Foundation has estimated the global economic burden to 7–19 billion US Dollars per year [10] and approximately 188 billion US Dollars per lifetime [11]. These figures are higher than the estimates for other prominent global diseases (rotavirus 2.0 billion US Dollars, cervical cancer 4.7 billion US Dollars, and Lyme disease 2.5 billion US Dollars) [12]. Meanwhile, these estimated figures alone raise large red flags which the scientific community and funding agencies should focus their attention again on trypanosomiasis. Although, naturally vector borne and localized in South America, this disease has found its way into several modes of transmissions and has spread and is continuously spreading in different parts of the world.

To date, there is still no vaccine against Chagas disease. Drug intervention with antiparasitic agents is only useful during the early phase of infection. Wherein, the signs and symptoms are usually misdiagnosed for other pathologic conditions or left undiagnosed altogether and if left undetected will consequently lead to debilitating conditions and mortality. For this reason, vector control and hygienic living conditions have been and should be a constant advocacy. Vector control cost estimates have been around 5 million US Dollars for spraying insecticides which is significantly less compared to risks in human well-being and accumulated medical care bills annually and through a lifetime. Maintaining clean living conditions in a household and using bednets in sleeping areas [13] cost even less as a reasonable means of intervention from preventing kissing bug bites.

5. Concluding remarks

This book is dedicated to the millions who have fallen victims and those presently and heavily burdened with Chagas disease. The contributors of the literatures herein contained for a significant time in their lives have become living witnesses to the ravage and devastation kissing bugs carrying *T. cruzi* can inflict upon an individual as well as a nation. These investigators have dedicated their time and being in so many different ways in their own personal and consolidated effort to provide the best, most comprehensive, and up to date knowledge to explore the American trypanosomiasis in all its available entirety. The chapters contained in this book will no doubt prove to be indispensable in getting up-close and personal into Chagas disease findings. Each chapter provides in-depth discussions of each specific area of historic, biologic, vector, and pathologic, along with clinical aspects, diagnostic challenges, natural products, drugs discovery, and various control measures as well as strategies relative to this very old yet still rampant and neglected parasitic infection. It is with great anticipation that this compilation of literature reviews and researches would find its way into the hands of experts as well as those novice who will soon be at the forefront of leading the fight against this debilitating infection. It is with great hope that this book will be a tool for the fruition of Chagas disease eradication.

Author details

Frederick R. Masangkay[1,2], Giovanni DJ Milanez[1,2], Helieh S. Oz[3] and Veeranoot Nissapatorn[4,5]*

*Address all correspondence to: nissapat@gmail.com

1 Biomedical Science Program, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, Thailand

2 Department of Medical Technology, Institute of Arts and Sciences, Far Eastern University, Manila, Philippines

3 Department of Internal Medicine, University of Kentucky Medical Center, USA

4 School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, Thailand

5 Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Nakhon Si Thammarat, Thailand

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Section 1

Biochemistry, Pathophysiology and Histo-immunological Study

Chapter 2

Biochemical, Cellular, and Immunologic Aspects during Early Interaction between *Trypanosoma cruzi* and Host Cell

Rosa Lidia Solís-Oviedo, Víctor Monteon,
Ruth López and Ángel de la Cruz Pech-Canul

Additional information is available at the end of the chapter

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Abstract

The close parasite-host relationship involves different aspects such as the biochemical, physiological, morphological, and immunological adaptations. Studies on parasite-host interaction have provided a myriad of information about its biology and have established the building blocks for the development of new drug therapies to control the parasite. Several mechanisms for the parasite invasion have been proposed through *in vivo* or *in vitro* experimental data. Since the first histological studies until the studies on the function/structure of the involved molecules, this complex interaction has been roughly depicted. However, new recent strategies as genetic and proteomic approaches have tuned knowledge on how the host reacts to the parasite and how the parasite avoids these host's reactions in order to survive.

Keywords: *Trypanosoma cruzi*, immune system, parasite interactions, animal model studies, *in vitro* models, phagocytic, non-phagocytic

1. Introduction

The life cycle of *Trypanosoma cruzi* comprises several morphological transformations involving both mammalian and vector hosts, where three different major developmental stages are identified: epimastigotes, trypomastigotes, and amastigotes (**Figure 1**). The developmental stages of *T. cruzi* alternate between non-infective and infective forms. Epimastigote and amastigote are non-infective but replicative stages in the gut of the triatomine vector and inside the

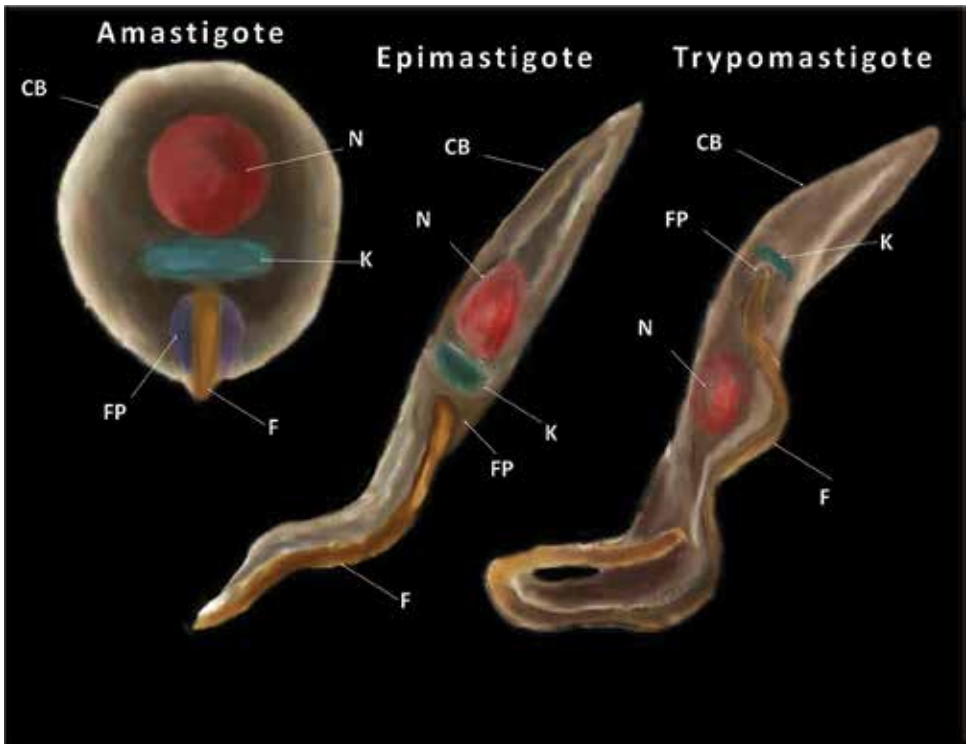

Figure 1. The different stages of *Trypanosoma cruzi*. The image depicted the amastigote, epimastigote, and trypomastigote stages from *T. cruzi* and their membrane domains: Nucleus (N), Kinetoplast (K), Flagellum (F), Flagellar Pocket (FP), and Cell Body (CB). Reprinted with permission from Ángel de la Cruz Pech-Canul et al. [1], Copyright © 2017.

mammalian cell, respectively. Trypomastigote stage is infective but non-replicative and can also be considered as two different developmental stages: the bloodstream trypomastigotes, found in the blood of the mammalian host, and the metacyclic trypomastigotes, found in the rectum of the triatomine vector .

T. cruzi is internalized by phagocytic and non-phagocytic nucleated host cells via multiple pathways. The first general steps through the interaction process of the *T. cruzi* and its mammalian host cell can be divided into three stages: (1) adhesion/recognition, (2) signalling, and (3) invasion [2, 3]. During the adhesion/recognition stage, diverse molecules with cell-adhesion properties are expressed on the membrane surface of the metacyclic trypomastigotes from of the parasite ; these molecules bind to receptors of the target host cells and are able to trigger signals pathway, toward the parasite invasion [4]. That invasive process allows *T. cruzi* internalization and involves the engulfment of the parasite, the formation of a *T. cruzi* parasitophorous vacuole (TcPV) [5], as well as the late disruption and the dispersion of the TcPV, thereby the parasite is released to the host cytoplasm where its replication and differentiation starts until the infective stage [6, 7]. The aim of this chapter is to discuss and to outline the interaction models during the early interaction between *T. cruzi* and its mammalian host cells.

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2. An overview of parasite interaction

One of the first barriers faced by *T. cruzi* during host cell invasion is the complexity of the host defence system. The skin and mucous membranes act as physical barriers which prevent penetration by microbes. Undoubtedly, they are the site for multiple and diverse types of chemical, physical, and biological contacts. Lipids and proteins are among the main components of the innate immune system in these tissues. Lipids comprise linoleic acid, oleic acid, squalene, ceramides, and sphingolipids, whereas proteins are more diverse, such as keratin on the surface of the skin or the cationic peptides *alpha*- and *beta*-defensins produced by neutrophils and mucosa tissue, respectively [8]. Furthermore, saliva produced by salivary glands of the vector contains a sort of proline-rich proteins and histidine-rich proteins both with antibiotic properties, lysozyme, peroxidase, lactoferrin, cystatins, and mucins [9]. Due to the rich protein content, both pH and salt concentration play a significant role as inhibitory factors during the parasite/host interaction.

The cellular composition of skin and mucous membranes is a fundamental barrier for permissive or refractory colonization/infection. In the skin, the epidermis is composed by 95% of keratinocytes and other cells present at low concentration, such as melanocytes, Langerhans cells, intra-epithelial lymphocyte, and Merkel cells. Keratinocytes express Toll-like receptors (TLRs) 1–6, 9, and 10 which are able to recognize basically all pathogen-associated molecular patterns (PAMPs) with exception of flagenin; as a consequence, they can secrete an array of mediators such as nitric oxide, leukotrienes, cyclooxygenase, metalloprotease 1 and 9, classical cytokines IL-1, IL-6, IL-8, TNF-alpha, and chemokines CXCL1 and CXCL8. Keratinocytes also express receptors for different cytokines (IL-1, IL-3, TNF-alpha, IL-17, IL-21, IL-22) and chemokines (CXCL9, CXCL10, CXCL11, and CCL20). Other skin cells present at low concentration have also a broad array of receptors that are able to respond to physical and chemical stimulus. In addition, a dense protein layer is found between epidermis and dermis which is composed by collagen type IV, laminin fibronectin, iodogen, and heparan sulfate; together, they structure the basement membrane [10]. The cellular composition of dermis is more complex and diverse. Fibroblast, myofibroblasts, macrophages, adipocytes, dendritic cells, mast cells, and mesenchymal stem cells are found among resident cells in the dermis (**Figure 2**), whereas transitory cells include lymphocytes, polymorphonuclear cells and monocytes. In addition, dermis presents an intricate network of nerves, lymph, and blood system. As skin, mucosal tissue has the property to react with a complex array of mediators required for immune surveillance and inflammatory response to tissue injury and infection. A remarkable differential feature between skin and mucosa tissue is the bias to immune tolerance and anti-inflammatory response in mucosal compartments [11, 12].

In natural conditions, *T. cruzi* infection is established when metacyclic trypomastigotes are deposited on injured skin or mucosa host tissue by blood feeding triatomine. Thus, metacyclic trypomastigotes has to face the above innate immune responses at the portal entry in order to survive (**Figure 2**). Since the pioneer work published by Romaña [13], where a histology description was done, limited information on this area of concern exists. It is very critical to take into account different factors in the relationship between parasite and host. For example, factors

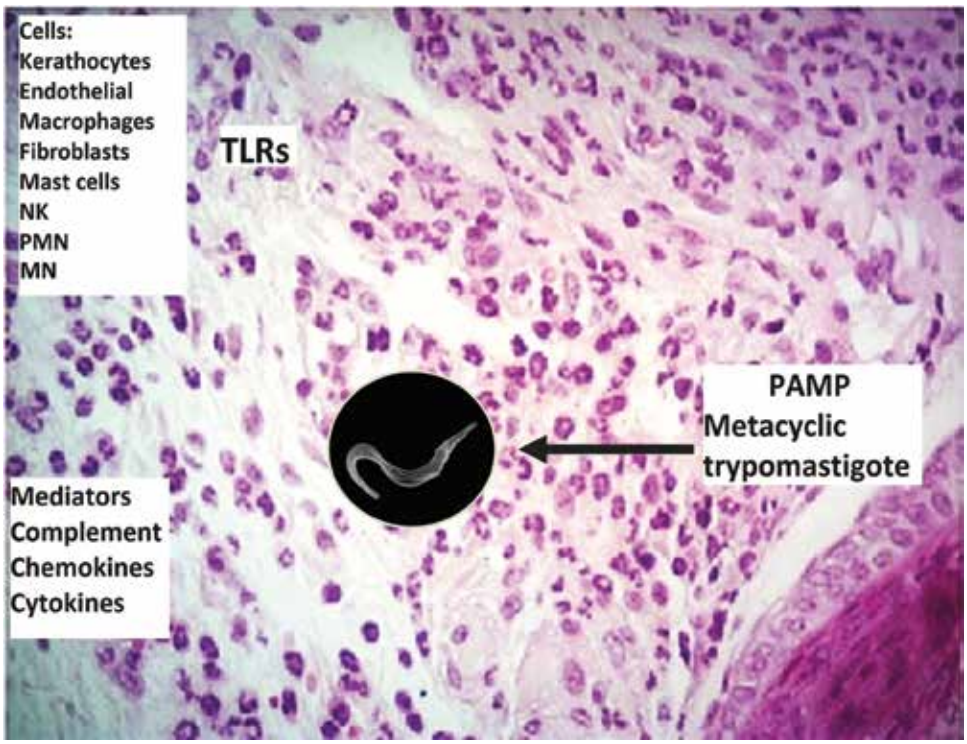

Figure 2. Skin cells of mouse and metacyclic trypomastigote parasite. Host cells were stained with hematoxylin-eosin. A *T. cruzi* trypomastigote is depicted inside the image. Common types of skin cells and some of the mediators for the inflammatory response are listed inside the image: pathogen-associated molecular patterns (PAMPs), natural killer (NK), polymorphonuclear (PMN), mononuclear (MN), and Toll-like receptors (TLRs).

as specie of vector are involved in the transmission, inoculum size, *T. cruzi* phase, portal of entry, *T. cruzi* strain, host immune responses, and microbiota presented in the vector.

3. Specie of vector and *Trypanosoma cruzi*

Firstly, there are many triatomine vector species that transmit the Chagas disease. Some of them have a wide geographical distribution and others are confined to restricted geographical areas. However, all of them can transmit *T. cruzi* infection with different efficacy, a feature that relies on biological behaviour and physiological condition itself. For example, metacyclogenesis involves the process of parasite transformation into the vector; this step is fundamental in order to accomplish the life cycle. The basic transformation that takes place inside the vector is from bloodstream trypomastigote phase to epimastigote and to metacyclic trypomastigotes. This last phase is essential for mammalian infection in as much as epimastigotes are vulnerable to innate immune mechanism. Thus, the metacyclogenesis that takes place into the vector is fundamental in order to switch to mammalian host. Perlowagora-Szumlewicks and Carvalhio-Moreira [14] described triatomine vector species influencing metacyclogenesis with remarkable observation.

They pointed out higher metacyclogenesis rates in *Rhodnius neglectus* and *R. prolixus* (50 and 37%, respectively), whereas in some *Triatoma* species, metacyclogenesis rates were dramatically lower in comparison (5% in *Triatoma sordida*, 7% in *T. brasiliensis*, and 1% in *T. pseudomaculata*). However *T. infestans* can reach up to 42%, in *T. rubrovaria* 27%, in *T. dimidiata* 26%, and *Panstrongylus megistus* metacyclogenesis rates can reach 27%. Other remarkable observation is that metacyclic trypomastigotes rate is not continuous along vector life span. In some cases, it can reach a plateau, but in other cases, it can reach several peaks before metacyclogenesis drops. In natural conditions, *T. barberi* can reach up 76%, in *T. pallidipennis* 15%, whereas in *T. dimidiata* 26% [15].

The metacyclogenesis of *T. dimidiata* in laboratory conditions is similar to natural conditions; in addition, metacyclogenesis is also influenced by the *T. cruzi* strain and the rate of metacyclic parasites change along the age of triatomine vectors [16]. Furthermore, *T. cruzi* strains can moderately influence the rate of metacyclogenesis that take place inside the same triatomine specie but have less impact when compared across triatomine specie [16, 17]. Altogether, the above data highlight the importance of triatomine species and *T. cruzi* strains in the development of metacyclic trypomastigotes: the natural parasite phase that will face mammalian host to complete its life cycle. Due to its importance, this variable should be taken into account for experimental design. Besides, the parasite strains show different virulence relying on virulence factors such as trans-sialidase activity, complement resistance, and cysteine protease cruzipain (TCC) [18]. Trans-sialidase removes and transfers sialic acid from host cells to parasite mucin-like glycoprotein. It is known that trans-sialidase activity is a virulence factor which allows parasite to invade and to escape from parasitophorous vacuole. This enzyme is more expressed in bloodstream and tissue-culture trypomastigotes than in metacyclic trypomastigotes. Trans-sialidase activity also depends on *T. cruzi* lineage and consequently its virulence [19].

Once metacyclic trypomastigotes have overcome the first nonspecific immune mechanical barrier (skin/mucosal tissues), they need to swing into the extracellular matrix proteins in order to find cells to invade for replication and then accomplish their life cycle. GP82, a surface glycoprotein found in both bloodstream and tissue-culture trypomastigotes, has the ability to bind to matrix extracellular proteins such as fibronectin, heparan sulfate, and laminin, serving as bridges for parasite-target cell association and leading to enhanced infection. However, this interaction inhibits cell invasion. The presence of the major cysteine proteinase cruzipain (TCC) helps to degrade these extracellular matrix proteins enabling cell invasion [20]. These surface glycoproteins are very polymorphic among *T. cruzi* strains resulting in different grades of virulence .

The complement system, another unspecific immune mechanism that is essential for inflammation and cellular lysis, can be activated by three pathways. The lectin triggered by mannose-binding lectins (mannose-binding proteins, ficolins, and CL-K1 proteins) that binds to pathogen-associated molecular pattern (PAMPs) rich in *D*-mannose, *L*-fucose, glucose, and *N*-acetyl-glucosamine, *O*-acetylated, and glycan compounds containing sialic acid which activate MASP-1 and MASP-2. The alternative pathway is triggered when the complex C3 (H_2O)-B factor is stabilized on a surface allowing the formation of C3 convertase (C3 (H_2O) Bb). Whereas the classical pathway activation depends on C1 complex interaction with antibodies or LPS and porins present in Gram-negative bacteria, but also with phosphatidylserine on apoptotic cells or via C-reactive proteins synthetized in liver as stress proteins [21].

The four phases of *T. cruzi* (amastigote, epimastigote, metacyclic, and bloodstream trypomastigote) can activate the complement system, but only epimastigotes are susceptible to lysis. However, some strains on metacyclic trypomastigote phase are more vulnerable [22, 23]. Some *T. cruzi* surface molecules enable parasite to evade innate and adaptive immune responses. There are other mechanisms to circumvent the action of complement system such as the presence of calreticulin (TcCRT), the complement regulatory protein (Gp160/TcCRP), the complement C2 receptor inhibitor trispanning (TcCRIT), and the presence of GP58/68 protein and T-DAF. For a comprehensive review, see [21].

Finally, it has been observed that in animal models, metacyclic trypomastigotes induce an inflammatory response at the site of inoculation, as early as 1 h, and it is composed basically of neutrophils while mononuclear infiltrate begins at 24 h with a maximum infiltration at day 15. Nonetheless, poor cytokine expression such as IL-2, Il-4, IL-10, IL-12, and IFN-*gamma* persists over a 2-week post-inoculation, whereas at the regional lymph node to the site of inoculation, it was evident as early as 1 h. The induced pattern of cytokine at the inoculation site is permissive to establishing infection, despite the appropriate immune response in other lymph secondary organs [24–26]. Our group recently reported that pre-exposure to faeces of triatomine decreases parasitemia in mice challenged with metacyclic trypomastigotes. This finding suggests that inflammatory reaction to bacteria faeces in immune individuals helps to control parasite load *in vivo* [27].

4. *In vitro* models

Diverse *in vitro* studies on the *T. cruzi* /host cell interaction process have been described through the years [28]. These studies have included a wide variety of eukaryotic cell lines and parasite strains, as well as the different parasite phases able to infect cells: amastigotes, metacyclic trypomastigotes, or both, bloodstream and tissue-culture trypomastigotes [2, 29]. *T. cruzi* is capable to invade phagocytic or non-phagocytic cells via endocytic mechanisms. Currently, three models for *T. cruzi* invasion have been proposed: lysosomal-dependent, lysosomal-independent, and actin-dependent [3, 6, 30].

Cortez and co-workers [30] recently showed that the participation of lysosomes in the parasite entry site depends on the source of the trypomastigote. They found that the metacyclic trypomastigotes invasion occurs mainly by the lysosome-dependent mechanism, whereas the tissue-culture trypomastigote invasion takes place mostly by the lysosome-independent mechanism. Interestingly, it has been reported that amastigotes are capable of invading host cells by the actin-dependent phagocytic mechanism probably due to their motionless nature [29, 31].

4.1. Lysosomal-dependent

The lysosomal-dependent model is also known as the lysosome exocytosis pathway. Tardieux et al. visualized the recruitment of lysosomes at the parasite entry site during the early event of internalization of tissue-culture trypomastigotes into their mammalian host cells, and they proposed that this process is required for parasite internalization [32]. PGTF is a soluble factor

proteolytically generated from trypomastigote which is capable to induce Ca^{2+} signaling in mammalian cells. The addition of PGTF during the host cell invasion of tissue-culture trypomastigotes showed that Ca^{2+} signalling plays a role in the parasite invasion through the reorganization of host cell microfilaments as well as in the migration and fusion of lysosomes [15, 33]. In addition, the increase of Ca^{2+} is required to trigger a form of endocytosis to repair the mechanically injured host cell membrane due to *T. cruzi* invasion [17]. The elevation of intracellular Ca^{2+} concentration triggers the exocytosis of lysosomes. The lysosomal enzyme acid sphingomyelinase (ASM) is released to the host plasma membrane where ASM converts sphingomyelin into ceramide: a lipid capable of forming ceramide-enriched endosomes [34, 35]. Ceramides are also capable to coalesce and to accumulate into the parasitophorous vacuoles, which suggest that this lipid plays an important role in the membrane deformation process required to allow the large trypomastigotes entry into the host cells [32, 36].

4.2. Lysosomal-independent

The lysosomal-independent mechanism depends on phosphatidylinositol-3 (PI 3)-kinase (PI3K) which is activated in the presence of *T. cruzi* bloodstream trypomastigotes. This mechanism is correlated to an efficient parasite invasion of non-phagocytes and phagocytic cells. *In vitro* analysis during *T. cruzi* infection of phagocytic cells has shown the presence of vacuoles enriched with lipids derived from the PI 3-kinase activities: phosphatidylinositol 3-phosphate (PI_3P), phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$), and phosphatidylinositol PI 3,4,5-triphosphate ($PI(3,4,5)P_3$) [37–39].

The inhibition of the class I and III PI 3-kinase activities abolishes the parasite entry into macrophages which suggests a prominent role of the host PI 3-kinase activities during the *T. cruzi* infection process [37]. A class III PI 3-kinase located in *T. cruzi* (TcVps34) is able to produce phosphatidylinositol 3-phosphate, and it has been shown that it plays an important role in vital processes for the parasite survival such as osmoregulation, acidification, and vesicular trafficking [40].

4.3. Actin-dependent

Amastigotes are also capable to penetrate host cell through its plasma membrane via the actin-dependent mechanism. This mechanism contrasts notably from the two models described previously in which trypomastigotes are involved [41, 42]. The invasion capability of amastigotes depends on the *T. cruzi* linage. Amastigotes from the *T. cruzi* I lineage (G strain) have a remarkable ability to invade non-phagocytic cells [29, 43], while the less-infective amastigotes belonging to *T. cruzi* II linage (such as the Y strain) are largely engulfed by phagocytic cells (macrophages) and occasionally by other cell types [43, 44].

Once inside the host cell, amastigotes show the same ability as trypomastigotes to disrupt the parasitophorous vacuole, to replicate in the cytosol, and to differentiate into the infective trypomastigote form. There is also evidence that trypomastigotes are able to differentiate into amastigotes extracellularly while circulating in the bloodstream [45]. This remarkable observation has unravelled an additional mechanism through which the parasite can move among intracellular compartments, elude the host immune system, and sustain the infection.

5. Conclusions

Chagas disease is a potentially life-threatening illness caused by *T. cruzi*. Currently, there are no vaccines which prevent the parasite infection; hence, vector control is still the most useful method to prevent such illness. Although the mammalian host has developed a fine battery of physical and biochemical defences, the parasite has adapted its metabolism to overcome the host defences. *T. cruzi* exhibits multiple strategies to evade the host defenses in order to survive, as summarized here; diverse studies have been conducted trying to unravel the basics of *T. cruzi* infection during the early interaction with its mammalian host. The different *in vivo* and *in vitro* experimental approaches showed a complex interaction depending on both, the parasite and the host characteristics. For example, the amastigote form was relatively recently described as a potentially infective form for host cells. Despite the fact that amastigote form is generally known as a replicative form in the mammalian host, it is capable to infect host cells within the host system in a completely different manner than the one described for the typical infective trypomastigote form. Despite the amount of studies on this topic, the comprehensive understanding of the parasite invasion mechanisms is still incomplete. More efforts should be followed for the elucidation of the early steps of parasite–host interaction as they are crucial for the development of future drugs to prevent the Chagas disease.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author details

Rosa Lidia Solís-Oviedo[1], Víctor Monteon[2], Ruth López[2] and Ángel de la Cruz Pech-Canul[3]*

*Address all correspondence to: angelpechcanul@gmail.com

1 Faculty of Chemical Sciences, Autonomous University of Chihuahua, Chihuahua, Mexico

2 Centre for Biomedical Research, Autonomous University of Campeche, Campeche, Mexico

3 CONACyT – Faculty of Chemical Sciences, Autonomous University of Chihuahua, Chihuahua, Mexico

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Chapter 3

Antiparasitic Mechanisms of the Human Placenta

Castillo Christian, Ana Liempi, Lisvaneth Medina,
Ileana Carrillo and Ulrike Kemmerling

Additional information is available at the end of the chapter

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Abstract

Trypanosoma cruzi, during vertical transmission, crosses the placental barrier. The trophoblast, a continuous renewing epithelium, is the first tissue of this anatomical barrier to have contact with the parasite. The epithelial turnover, including the trophoblast, is part of the innate immune response due to the fact that pathogens attach to the surface of cells prior invasion. Cellular processes such as proliferation, differentiation, and apoptotic cell death are part of the trophoblast turnover. Interestingly, *T. cruzi* induces all of them. In addition, the placenta expresses TLRs, whose activation leads to the secretion of pro-inflammatory and immunomodulating cytokines. *T. cruzi* is recognized by TLR-2, TLR-4, TLR-7, and TLR-9. In the present review, we analyze the current evidence about the trophoblast epithelial turnover, the induction of a specific cytokine profile as a local placental innate immune response, as well as other possible defense mechanisms against the parasite.

Keywords: *Trypanosoma cruzi*, placenta, epithelial turnover, TLRs, cytokine profile

1. Introduction

Congenital Chagas disease, caused by *Trypanosoma cruzi* (*T. cruzi*), is associated with premature labor, low birth weight, and stillbirths [1, 2]. The congenital transmission of pathogens is the consequence of complex interactions among the parasite, maternal and fetal/newborn immune responses, and placental factors. The placenta is the least-studied component of this "trilogy" [3, 4] but is essential in determining the probability of transmission since it forms the primary barrier between the maternal and fetal compartments throughout pregnancy [5].

Mother and developing fetus are protected against environmental challenges by the immune system; the placenta is able to modulate fetal as well as maternal immune responses. Maternal

immune system presents an enhanced capacity of cellular and molecular recognition and communication between each other. Therefore, during normal pregnancy, the maternal immune defenses assure the health of the mother and developing child. Moreover, the fetus during its development also acquires immune defenses that are able to modulate the maternal immune system. Considering this facts, the immune system responses are unique and particularly effective [6].

The maternal and the fetal developing innate and adaptative immune systems determine the probability of fetal/neonatal infection. Fetal infection is related to diverse pregnancy disorders such as abortion, preterm labor, intrauterine growth retardation, and preeclampsia [7]. Particularly, congenital *T. cruzi* infection can cause abortion, stillbirth, and intrauterine growth restriction [1, 2, 8].

The innate immune system presents a main role in protecting the developing child against *T. cruzi* infection. Thus, increase of pro-inflammatory cytokines is present in the sera of uninfected babies born to infected mothers [9]. However, in newborns who suffer from congenital infection, the levels of inflammation markers as well as active NK cells are low [10]. Therefore, the innate immune response is effective in uninfected newborn from chagasic mothers. The adaptive immune system is also relevant; for instance, maternal anti-*T. cruzi* antibodies are transferred through the placenta to the fetus and where they reduce the parasitemia [9].

Importantly, congenital transmission rates for *Trypanosoma cruzi* (*T. cruzi*) are relatively low (3.9–5.6%) [11, 12]. Moreover, the typical amastigote nests (intracellular parasites) cannot be observed in placentas from mothers with chronic Chagas disease [13] nor in human placental chorionic villi explants (HPCVE) infected in vitro with the parasite [14]. In the latter, only a few parasite antigens and DNA can be identified [14, 15]. In addition, other infections of the placenta are not commonly observed [11]. All these evidences suggest the presence of systemic and local defense mechanisms against pathogen and that the placenta is a key factor against *T. cruzi* infection.

2. Antiparasitic mechanisms of the placenta

Importantly, during congenital transmission, the parasites must cross the placental barrier [8, 16].

2.1. Placenta

The placenta is a temporary organ that provides nutrition and gas exchange for the developing fetus, ensuring normal embryo-fetal growth and development and supporting pregnancy-related changes in maternal physiological systems [17]. The human placenta is classified as discoidal, villous, and hemochorial and consists of a fetal portion, which originates from the *Chorion frondosum*, and a maternal portion, or basal decidua, which originates from the endometrium. The functional units are the floating chorionic villi, formed by the trophoblast, and

the villous stroma. The trophoblast comes into contact with maternal blood in the intervillous space (IVS) and is delimited by a basal lamina from the villous stroma, which is the fetal connective tissue containing the fetal capillaries. The placental barrier is formed by the trophoblast, basal laminae, villous stroma, and fetal capillary endothelium (**Figure 1**) [4, 8].

The placenta may contain as much as 500 mL of maternal blood, in the IVS, exposing the trophoblast to pathogens that might be present in it [5]. Therefore, the trophoblast is a key factor against congenital infection since it is the first fetal tissue that comes into contact with pathogens circulating in the maternal blood [11]. On the other hand, the placenta, as an immune regulatory organ, acts as a modulator of fetal as well as maternal immune responses [6]. The placenta, in particular the trophoblast, is also part of a local innate immune response. Three types of defense mechanisms in innate immunity have been described: (i) anatomical barriers, such as the placental barrier (**Figure 1**), (ii) cellular innate immune responses, and (iii) humoral innate immune responses. During tissue invasion, pathogen breaks the anatomical barriers, and innate immune cells are activated and secrete cytokines and chemokines to control pathogen replication [18, 19].

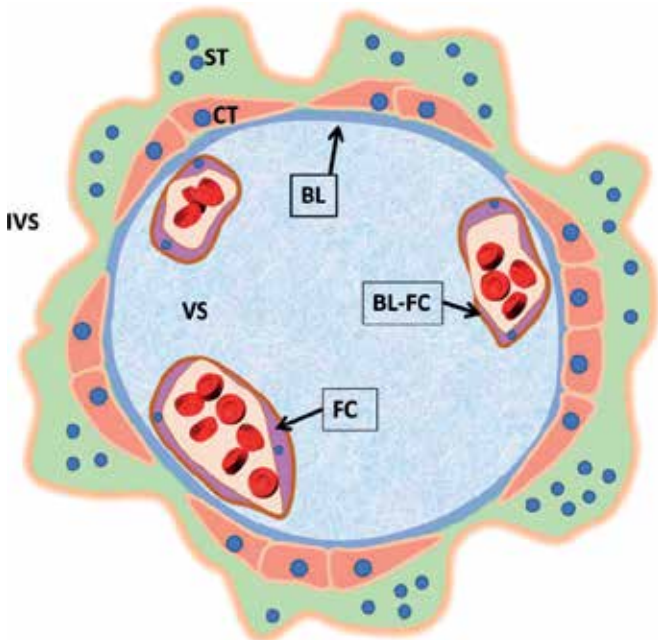

Figure 1. The placental barrier: the placental barrier is formed by the trophoblast composed by the superficial syncytiotrophoblast (ST) that contacts the maternal blood in the intervillous space (IVS) and the cytotrophoblast (CT) which corresponds to the germinative layer of the epithelium. The trophoblast is supported by the fetal connective tissue of the villous stroma (VS) that contains the fetal capillaries (FC). The placental barrier presents also two basal membranes (BM): (1) between villous stroma and trophoblast and (2) around fetal endothelium. The cells of the CT proliferate and afterward differentiate into the ST. The continuous incorporation of CT cells into the ST is counterbalanced by the formation of apoptotic ST knots, which are released into the IVS.

2.2. The trophoblast

The trophoblast is a bistratified epithelium composed of the superficial syncytiotrophoblast (ST) and the basal cytotrophoblast (CT). There is strong evidence that the ST layer is resistant to numerous pathogens, including *T. cruzi* [20, 21]. However, damage of the syncytium might allow the parasite access to the villous core, increasing parasite infection [5, 22].

2.3. The trophoblast epithelial turnover

The basal CT cells are the only ones of the trophoblast with proliferative capacity. The superficial multinucleated ST layer is highly differentiated and is unable to proliferate. Importantly, the ST contacts directly with the maternal blood [23–25], where in case of *T. cruzi* infection, the parasite circulates [11, 26]. The ST is a typical syncytium that is continuous and normally uninterrupted and covers all villous trees of the human placenta. The ST is formed and maintained by the continuous incorporation of CT cells through syncytial fusion, meaning that the CT cells suffer cellular differentiation. The continuous incorporation of CT cells into the ST is counterbalanced by the formation of apoptotic knots that are released into the maternal blood present in the IVS [24, 25]. The normal epithelial turnover assures the integrity of diverse anatomical barriers, including the placental one. The maintenance of the integrity of anatomical barriers is part of the innate immune system due to the fact that pathogens, prior to cell invasion, must attach to the surface of cells. As these cells are continuously eliminated, the attached pathogens are removed with them [3]. Thus, the trophoblast turnover should be considered as a defense mechanism against pathogens, including *T. cruzi*.

2.3.1. Cell proliferation

We have previously shown that the parasite induces, in the trophoblast, cellular proliferation. These experiments were performed in HPCVE and in the trophoblastic cell line BeWo; both models are commonly used in trophoblast studies [27]. In HPCVE *T. cruzi* increases DNA synthesis as well as the PCNA proliferation marker [11]. On the other hand, in BeWo cells the parasite also induces significant DNA synthesis (as determined by BrdU incorporation), increase of the percentage of cells in the ST and G_2/M cell cycle phases, and the expression of the widely used proliferation markers AgNORs, PCNA, and Ki67 [28]. Importantly, it should be taken into account that PCNA acts also as a molecular coordinator in multiple other cellular functions such as DNA damage repair, cell cycle control, cell survival, and gene expression [29]. Therefore, the increase of PCNA expression could also be a response to *T. cruzi*-induced cell and tissue damage. We have previously demonstrated that *T. cruzi* induces during ex vivo infection tissue disorganization of HPCVE [14] as well as apoptosis [30]. However, Ki67, a more specific proliferation marker that can be observed only during the active phases of the cycle [24], was significantly increased together with the other proliferation markers.

2.3.2. Cell differentiation

As described above, CT cells differentiate continuously and fuse with the ST [24, 25]. *T. cruzi* induces cell differentiation in the trophoblast in HPCVE and BeWo cells. Thus, the parasite

increases the protein expression of the major biochemical markers of trophoblast differentiation [31]: β-human chorionic gonadotropin (β-hCG) and syncytin [3]. Moreover, *T. cruzi* induces cell fusion in BeWo cells as demonstrated by a two-color fusion assay and by the analysis of the redistribution of the intercellular adhesion protein desmoplakin [3]. Previously, we have shown that *T. cruzi* activates the ERK1/ERK2 MAPK pathway [32]. Interestingly, the induction of trophoblast differentiation is mediated by the activation of the ERK1/ERK2 MAPK and other MAPK signal transduction pathways [33].

2.3.3. Apoptotic cell death

T. cruzi also induces apoptotic cell death in the trophoblast. The ST releases continuously apoptotic knots into the IVS [24]. In HPCVE, the induction of apoptosis has been demonstrated by the determination of the presence of pyknotic nuclei, induction of DNA fragmentation, caspase-3 like activity, and presence of caspase-3 and cleaved cytokeratin 18 [30]. Cellular processes related to apoptosis are also regulating cell differentiation (fusion) in the trophoblast. For instance, CT cell differentiation is regulated by caspases [34, 35]. Particularly, caspase-8, an apoptosis initiator caspase, regulates trophoblast differentiation and fusion. Caspase-8 is activated in highly differentiated CT cells just prior to fusion and escorts the fusing cell content including the nucleus into the ST, and it has not been found in proliferating CT cells [23, 36]. Moreover, the fusion of the trophoblast has been visualized by localizing caspase-8 [34]. *T. cruzi* induces in BeWo cells as well as HPCVE the expression and activation of caspase-8 [11, 37]. Moreover, the inhibition of caspase-8 increases the amount of parasite DNA and the number of intracellular parasites in BeWo cells [37]. The inhibition of caspase-8 decreases parasite-induced cellular differentiation and apoptotic cell death, but not cellular proliferation [11, 37].

2.4. The trophoblast and the innate immune cellular response against *T. cruzi*

The innate immune response against pathogens is initiated by pathogen pattern recognition receptors (PRRs), which include *Toll*-like receptors (TLRs) that recognize and bind highly conserved sequences known as pathogen-associated molecular patterns (PAMPs). The human trophoblast expresses all ten of the known functional TLRs [7], and *T. cruzi* is recognized by TLR-2, TLR-4, TLR-7, and TLR-9. Surface TLRs (TLR-2 and TLR-4) recognize glycosylphosphatidylinositol (GPI)-anchored mucin-like glycoproteins from *T. cruzi* surface [16, 38, 39]. We have shown that *T. cruzi* infection is related to TLR-2, but not to TLR-4 and TLR-9, expression, and activation [16]. The binding of TLR-2 to its ligands leads to activation of signaling pathways and upregulation of genes involved in the innate immune response including cytokines and chemokines [7, 16]. *T. cruzi* induces the secretion of IL-1β, IL-6, IL-8, IL-10, and TNF-α in HPCVE [16]. Interestingly, IL-1β, IL-6, and TNF-α secretions are also associated with cellular proliferation and differentiation in the trophoblast [40, 41], and inhibition of TLR-2 impairs trophoblast turnover (manuscript under review in "*Placenta*"). However, up to now, we do not know whether the activation of TLRs occurs mainly in the trophoblast or if other placental cells are also involved in this matter that should be addressed in the future. Importantly, as a consequence of our results, the TLR-2-initiated cytokine profile should also be considered as a local placental defense mechanism.

2.5. Other placental defense mechanisms against *T. cruzi*

The placenta, and particularly the trophoblast, expresses many noncoding RNAs including microRNAs (miRNAs) that regulate placental development function. Moreover, different miRNAs exhibit specialized functions during normal and pathological pregnancies. Placental miRNAs, packaged within exosomes and other vesicles or bound in protein complexes, are capable of communicating distinctive signals to maternal and fetal tissues [5]. Placenta-specific and trophoblast-derived miRNAs, encoded in the chromosome 19 miRNA cluster (C19MC), are released within exosomes and confer resistance to viral infection in other mammalian cells [42]. Preliminary results from our laboratory show that *T. cruzi* induces in HPCVE a specific C19MC-encoded miRNA profile. Some of those miRNAs are involved in the regulation of immune functions, particularly those of TLR-mediated pathways [43]. Studies on *T. cruzi*-induced miRNAs and exosomes are currently ongoing, being of particular interest since miRNA pathways are potential diagnostic tools and targets for therapeutic control of parasitic diseases [44] and other pathologies, including placenta-derived ones [5]. Targeting miRNAs constitute a promising possibility for the treatment of different diseases due to the facts that (i) miRNAs are regulators of gene expression, (ii) are relatively easy to manipulate, (iii) can be administrated in vivo, and (iv) present an apparent lack of adverse effects when administered intravenously. Moreover, *miRNAs are detectable in biological fluids, thus offering real potential as noninvasive biomarkers*, providing new diagnostic and therapeutic options during pregnancy and for several diseases as well [45, 46].

In summary, the studies about the placental defense mechanism that determines the probability of infection, together with parasite, maternal, and fetal/newborn factors, are of outstanding interest since they are potential diagnostic, prognostic, and therapeutic tools.

Author details

Castillo Christian, Ana Liempi, Lisvaneth Medina, Ileana Carrillo and Ulrike Kemmerling*

*Address all correspondence to: ukemmerling@uchile.cl

Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

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Chapter 4

The Mouse Model as a Tool for Histological, Immunological and Parasitological Studies of *Trypanosoma cruzi* Infection

María Elena Villagran-Herrera,
José Alejandro Martínez-Ibarra,
Manuel Sánchez-Moreno,
Hebert Luis Hernández-Montiel,
Ricardo Francisco Mercado-Curiel,
Nicolás Camacho-Calderón and
José Antonio de Diego-Cabrera

Additional information is available at the end of the chapter

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Abstract

The global expansion of Chagas disease is due to the constant migration of individuals from endemic countries with incidence of vector and nonvector transmission of *Trypanosoma cruzi*. The disease is present in its various stages: chronological characteristic signs and symptoms of the infection and its mechanism of immune system and cell and tissue damage. The first stage, which lasts 90 days approximately, is diagnosed by direct methods (blood smears stained with Giemsa, fresh and xenodiagnosis). The indeterminate-chronic stage is asymptomatic, but the growth and intracellular binary multiplication of the trypomastigotes continue promoting cell lysis and allowing parasites to infect other cells, with preferential tropism to organs producing mega syndromes such as cardiomyopathy, myocarditis, meningoencephalitis, megaesophagus and megacolon. Inadvertently, this process is repeated for several years leading to Chagas disease. The mouse inoculation allows checking the parasitemia in vivo and the development of the disease in short time (signs, behavior and tropism), histopathological alterations and detection of antibodies in serum. These parameters may vary when using different strains of *T. cruzi* from different geographical areas; Triatoma species due to their genetic variability are influenced by the environment, nutrition, reservoirs and habitat. The murine model ECA CD-1 has the ability to replicate human findings of Chagas disease.

Keywords: Trypomastigote, Chagas disease, murine model, amastigote, CD-1 strain

1. Introduction

In 1909, the Brazilian doctor and researcher Carlos Ribeiro Justiniano das Chagas discovered the etiological agent of the later called Chagas disease in the triatomine insect (family *Reduviidae*), a flagellate protozoan of the genus *Trypanosoma* and subgenus *Schizotrypanum* and designated the specie adding "cruzi" in honor of his teacher and mentor Oswaldo Cruz, hence the name *Trypanosoma (Schizotrypanum) cruzi*. Later, in 1926, another doctor of Argentine origin, Salvador Mazza described the magnitude of the endemy in Argentina, Bolivia and Paraguay, identifying the hemoflagellate parasite in blood samples, demonstrating in this way, the existence of the trypanosomatida infection, which was given the name of American Trypanosomiasis, since the vectors of *Trypanosoma cruzi* had been found only in America [1].

Chagas disease is a chronic debilitating affectation which impairs the health and the quality of life of infected people all around the world. The estimated number of infected people in the world arose from 30 million in 1990 to 6–8 million in 2010. In the past 20 years, the annual incidence decreased from 700,000 to 28,000 and the burden of Chagas disease decreased between 1990 and 2006 from 2.8 million disability-adjusted life to less than half a million [2]. Chagas disease is in close relation to the socioeconomic status of the population migration between Latin America and the rest of the world, and it currently represents one of the most important public health concerns [3]. The initiatives of the Americas have allowed achieving significant reductions in the number of acute cases and the presence of domiciliary Triatominae vectors in all endemic areas.

Trypanosoma cruzi belongs to the order of the kinetoplastid diseases, a group of parasites that has one or two flagella from a monophyletic group that diverged early from the branch common to all eukaryotic organisms. The morphological feature that distinguishes them is a prominent and paraflagellar structure known as kinetoplast, which corresponds to a condensation of DNA (DNAk), located on the inside of a single mitochondrion, which is branched across the cell. Within the family *Trypanosomatidae*, the *Trypanosoma* genus is most important because it includes a number of human diseases vectors such as *T. cruzi*, *T. brucei gambiense* and *T. brucei rhodesiense*, causal agents of Chagas and Sleeping sickness disease, respectively. Depending on the behavior of the parasite within the vector, the trypanosome genus has been divided into two groups. The first one called stercoraria, includes the trypanosomes that develop in the digestive tract of the vector, with the release of the infective forms in the stool (*T. cruzi* and *T. lewisi*). The second group called Salivaria includes trypanosomes that are initially developed in the digestive tube then passing through the epithelium and reaching the salivary glands, from where the infective forms are inoculated mechanically by bite or sting of the vector (*T. brucei*, *T. congolense* and *T. rangeli*) [4].

1.1. Life cycle

T. cruzi displays a digenetic life cycle alternating its multicellular life between the vertebrate host and its invertebrate vector. The cycle starts in the invertebrate arthropod when the

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insect sucks the blood of an animal carrying trypomastigotes in its blood, which gets to the stomach and are transformed into esferomastigotes and the replicative form epimastigotes. Subsequently, parasites migrate to the intestine where they multiply and eventually are transformed into the infective forms metacyclic trypomastigote, staying in the rectal ampulla until they are excreted with feces and urine. In this point, the life cycle continues in humans where the highly infective metacyclic forms aim to penetrate the skin or mucous membranes; although unable to pass through intact skin, they enter the body through skin or mucous membrane abrasions infecting macrophages, fibroblasts, smooth muscle and striated cells, Schwann cells, glial cells and neurons, excepting eosinophil and neutrophil cells. Once parasites have penetrated the cell, proliferation occurs and the trypomastigotes are released in the interior of a parasitophorous vacuole giving rise to amastigotes forms. The life cycle restarts with the insect feeding from an infected animal (**Figure 1**).

1.2. Routes of transmission

We can distinguish three cycles of vector transmission in *T. cruzi*. The primitive or wild cycle is zoonotic in nature. The protozoan parasite circulates between the insect vectors and the wild reservoirs (mammals of small and medium size). More than a hundred wild reservoirs of *T. cruzi* among marsupials, xenarthrans, bats, carnivores, lagomorphs, rodents and nonhuman primates have been described [6].

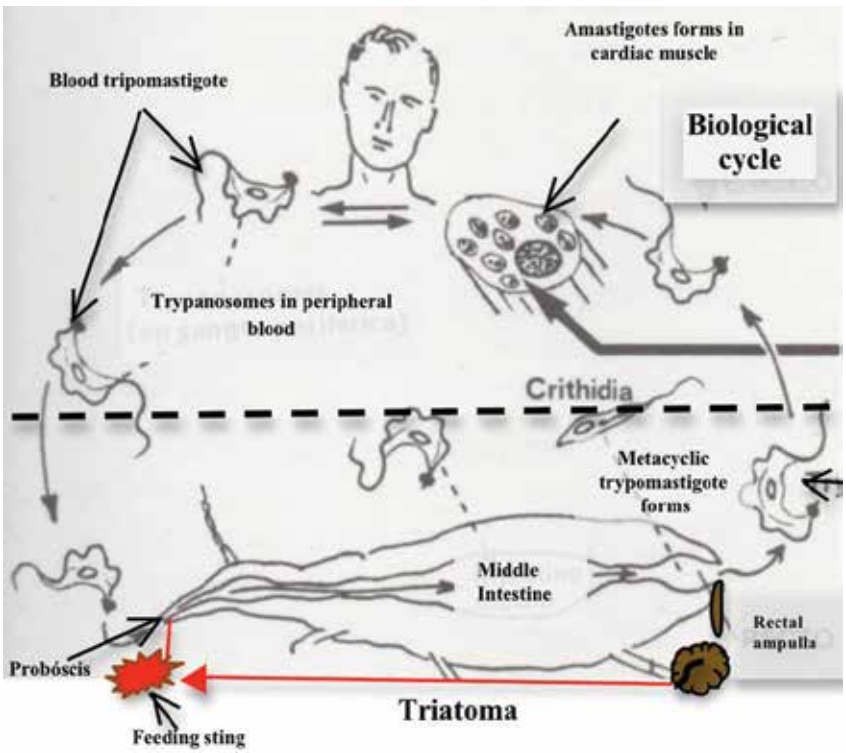

Figure 1. Digenetic biological cycle of *Trypanosoma cruzi*. Adapted by Federico-Mayer Rodolfo† and modified by de Diego-Cabrera José Antonio. Faculty of Medicine. Autonomous University of Madrid [5]. Spain, 1984 [5].

The domestic cycle comprises the infection of humans and the consequent Chagas disease. The domestic cycle is defined by factors in the anthroponotic foci, making people one of the last natural reservoirs of *T. cruzi*. Finally, the peridomestic cycle, comprising peridomestic mammals (rodents, marsupials, cats and dogs), which are in close contact with humans and their residences that have been built invading the habitat of wild triatomas that are attracted by the food and the lights of the houses.

Depending on the eco-epidemiological conditions of the place, both circles can overlap becoming an intradomiciliary cycle, especially when mankind invades the natural habitat of these vectors and builds houses fearing the entrance of reduvids (**Figure 2**).

On the other hand, the infection transmission by blood transfusion has become a serious complication in nonendemic countries, due to the migration of infected individuals from endemic regions [7]. This route is considered the second most important route of transmission in endemic areas [8]. *T. cruzi* resists processes of cryopreservation and thawing and can survive up to 18 days in total blood stored at 4°C. The vertical transmission is also known as a natal or congenital transmission, including prenatal, perinatal and postnatal care. This mechanism of transmission has a variable incidence between 0.1% and 18% according to geographical region [9], and has been regarded as the third in order of importance, next to vector-borne and transfusion transmission. An infected mother can transmit the parasite circulating in her blood during the second half of gestation. Among infected newborns, only 10–30% present symptoms [10].

The infection is not detected until adulthood in the course of the latent or indeterminate phase [11]. Spontaneous abortions have been reported, premature birth, intrauterine growth retardation, stillbirths and various clinical forms that can go from low birth weight, hepatomegaly,

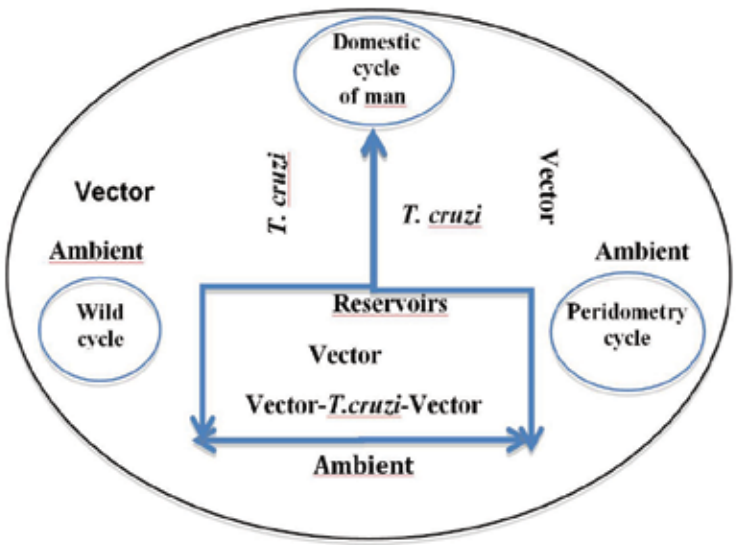

Figure 2. Exchanges between wild, peridomestic and domestic cycles of *T. cruzi* transmission. Adapted from Coura and Pinto Dias [6].

splenomegaly, acute respiratory symptoms, anemia, digestive disorders, Cardiac and Central Nervous System (CNS). The donation of organs has increased the number of infected people in urban areas. It has been informed about the transmission of infection to seronegative heart, bone marrow, liver, pancreas and kidney transplant recipients with variable transmission rates that reach 35% [12]. Patients infected with *T. cruzi* that should receive a transplant also represent a particular challenge, with risk of reactivation of the disease because of the immunosuppression after transplantation. The raw meat from infected rats and rabbit can be induced by the consumption of foods contaminated with triatomines or its feces, or by the ingestion of raw meat from infected mammalian hosts [13]. It must be confirmed by the detection of the parasites in a direct microscopic examination of a blood sample or other biological fluids of the patient. Many cases of numerous outbreaks of acute Chagas Disease are attributed to oral transmission; it has been detected in the Amazon region, due to the consumption of drinks or food contaminated with feces of infected triatomines [14]. It is also important to take into account the laboratory accidents that arise when research animals are handled mainly by postgraduate students, even though they occur in a smaller proportion [15].

1.3. Pathology and mechanism of injury

The disease presents three phases: the acute, chronic asymptomatic (intermediate or dormant) and the chronic symptomatic. The incubation period in the acute phase is 4–10 days and of shorter duration when the route of transmission is blood transfusion. This stage is generally asymptomatic, or it can occur with systemic manifestations that are common to other diseases such as fever, edema, lymphadenopathy, hepatomegaly and splenomegaly. It is accompanied by anorexia, fatigue, myalgia, headache and, occasionally, arthralgia. In some cases, there are signs of inoculation or entrance door, chagomas, lesions that are more frequent in the face and limbs of a forunculoid aspect, pink or violet and indurated borders. A typical sign in children is the bipalpebral edema (sign of Roman-Mazza). In this phase, the trypomastigotes are easily detected in the blood due to the high parasitemia.

In case the acute phase is overcome, there will be an extended period of chronic disease without clinical symptoms that lasts from 5 to 10 years, characterized by low parasitemia and by the presence of anti-*T. cruzi* IgG antibodies. About 30% of seropositive individuals reach the chronic phase, and in a span of 10–30 years, clinical manifestations such as heart disease and digestive megasyndromes show up, which may occur separately or coexist in the same patient. The chronic phase progresses slowly with a predominance of tissue damage. Digestive disorders consist in dilatation of viscera (mainly colon and esophagus, and in two-thirds of cases, the progressive myocarditis leads to the development of chronic Chagas disease heart (CChC). The relative prevalence of the various clinical manifestations varies according to geographic regions. In Argentina, as well as in Venezuela and Central America, the main clinical manifestation is the cardiomyopathy. On the contrary, in Chile and in the central region of Brazil, the mega syndromes are more frequent. This heterogeneous incidence of manifestations in different endemic areas could obey both biological and genetic differences of the circulating parasites [16] and host-related factors (age, gender, ethnicity, exposure to infections, family history of Chagas disease heart) [17], in addition to the introduction by migration of infected people from different countries around the world (**Figure 3**).

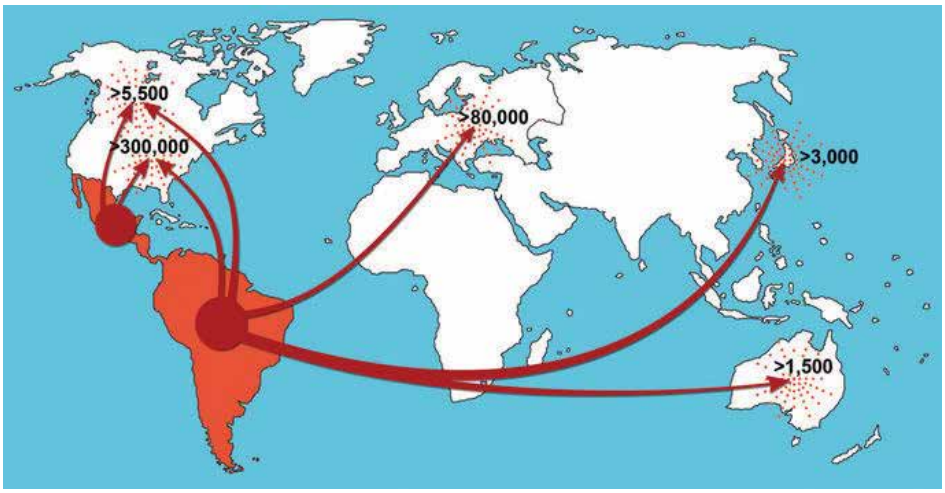

Figure 3. The estimated number of cases of Chagas disease in nonendemic countries, driven by constant migration. (ISGlobal, 2015).

In this last Chagas disease's phase, histological lesions are disseminated in the heart muscle, intestines and nervous system, inflammatory infiltrates composed mainly of CD8+ cells, in addition to nests (pseudocysts) full of parasites in their form of amastigotes [18].

Regarding the treatment, there are currently only two drugs available, benznidazole and nifurtimox. The therapeutic success is closely related to the stage of infection at the time of starting the treatment. Patients in acute phase (regardless of the route of infection), neonates and children, have better therapeutic prognosis [12, 15]. On the other hand, the success of such drugs is discussed in individuals with chronic infection and so far, there is no established therapeutic regimen [19]. The adverse effects are much more important among the adult patients; cases of photosensitivity and skin rashes, nausea, anorexia, weight loss and abdominal pain have been reported.

Recently, a group of biomedical and clinical scientists members of the network NHEPACHA (New Tools for the diagnosis and evaluation of the patient with Chagas disease), based on clinical and immunological evidence, have suggested new paradigms regarding the medicines for the Chagas disease in order to provide better treatment for patients in chronic phase [20].

On the other hand, the study of biochemical and biological characteristics of the hemoflagellate parasite has enabled the identification of new targets for chemotherapeutic agents; an example would be the drug trials with inhibitors of the biosynthesis of Ergosterol, Posaconazole and Ravuconazole, respectively, in patients with chronic Chagas disease.

1.4. Diagnosis of infection

Parasitological methods for detection of the acute phase have great sensitivity (direct methods) [21].

In the same way, these methods are used for the diagnosis of congenital infection in newborns and in children under the age of 6 months. The lack of maturation of the immune system and the presence of maternal IgG antibodies make, in the latter, the use of the serology for the infection diagnosis impossible [22]. The protozoan *Trypanosoma cruzi* is a powerful antigen and a few months after the initial inoculation, there is a humoral immune response that is effective is controlling the increase of parasitemia, which is mediated by antibodies and enzymes of the complement system. There are antibodies to various antigens of *T. cruzi* (surface, somatic and excretion), which belong to different classes (IgG, IgA, IgM) and subclasses [23–25]. The serologic test for the selection of blood donors must conform to the Official Mexican Standard for Epidemiological Surveillance, Prevention and Control of Vector-borne Diseases [26–28], citing that it must analyze the serum of each donor with two conventional immunological tests; if one of them is reactive, a third one will be carried on in such a way so as to qualify the donor as either positive or negative, with two reactive or two negative tests. In all cases, the laboratory diagnosis should be accompanied with epidemiological and clinical history of the patient, as the current and past source, the type of housing where it is found, the trips that could have been made to endemic areas and the history of blood transfusions and the infected mother.

1.5. Origin of the discrete units (UDTs) typing of *Trypanosoma cruzi*

The evolutionary history of *T. cruzi* infection is closely related to its vertebrate host [29]. The mammal fauna of South America in the cretaceous period mainly consisted of marsupials and

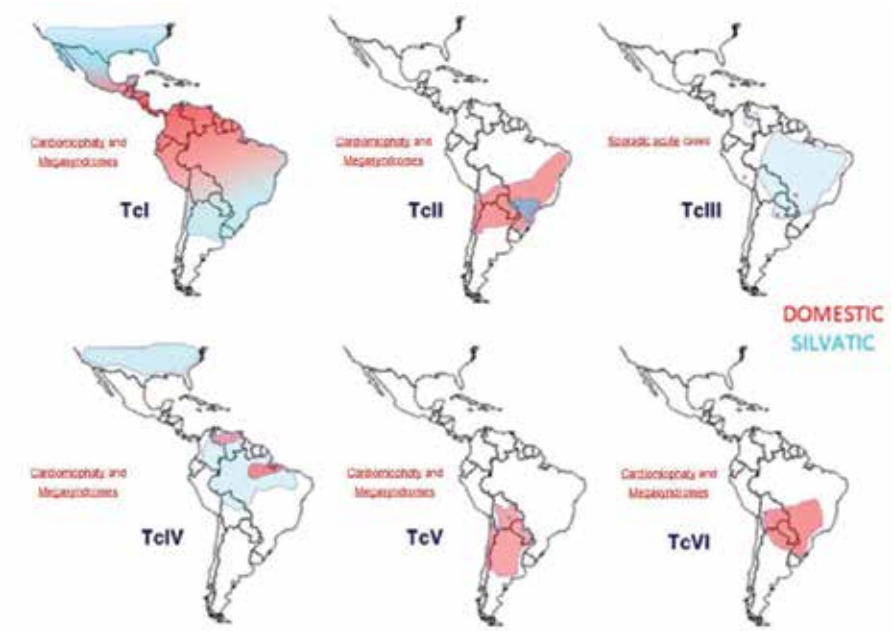

Figure 4. Geographic distribution of *Trypanosoma cruzi*, subpopulations disease phases with the corresponding primary clinical manifestations.

Figure 5. Distribution of *Trypanosoma cruzi* strains in México [35, 36].

placentals, the ancestral of the order Xenarthra (armadillos, anteaters and sloths), which were the natural reservoirs of the parasite at the time. The various ecotopes in whom were these two groups of hosts would have made possible the evolution by clonal propagation of two groups of parasites, which gave rise to the ancestral UDTs, TcI and TcII. It has been suggested that 1Cwi, evolved in association with the marsupial mammals of the genus *Didelphis* (weasels) and *T. cruzi* II, did in relation to the terrestrial mammals, such as armadillos [30]. *Trypanosoma cruzi* is a species composed of heterogeneous populations that circulate in nature between human beings, arthropod vectors, domestic animals, and wild reservoirs [31]. Currently, it is generally accepted that *T. cruzi* is a paradigmatic pattern of clonal evolution with low rate of gene recombination. A constant pattern of *T. cruzi*, behavior cannot be expected since different strains (subpopulations) circulate in nature. Extensive polymorphism promotes variation in infecting capacity, behavior in different hosts (virulence, histotropism, curves of parasitemia), adaptation to different vectors, immune response, stimulation, susceptibility to different chemical compounds, capacity of replication and differentiation, among others. Subpopulations are currently identified in the laboratory by biochemical, immunological and molecular biology assays [32, 33] (**Figure 4**).

Studies in murine experimental models have shown that both the parasite and host genotypes are crucial for tissue distribution and pathophysiology of infection by *T. cruzi* [34]. It has been previously reported that 81% of the Mexican strains of *T. cruzi* belong to lineage TcI and have different capabilities of infection, virulence and processing capacity in vitro, when compared to the other lineages [35, 36] (**Figure 5**).

2. Importance of the murine model in research American trypanosomiasis

2.1. The murine model in biomedical research

Animal models are very useful for studying human diseases because there are hundreds of pathogens that affect both humans and animals. The use of experimental animals in biomedical

research represents a key element for development of new prevention approaches and treatment of transmissible and nontransmissible diseases. Suffice it to recall the rabies vaccines, smallpox, tetanus, diphtheria, whooping cough and polio, the development of several antibiotics, insulin, and the knowledge of the genetic bases of inheritance [37]. No doubt that mice are the most commonly used animal for in vivo assays among experimental animal models in biology and medicine. The use of mice allows the study of mammal's reactions against aggressions like poisoning or infection (viral, bacterial, or parasitic), the study of immune responses and disorders and many others in several different fields like oncology, teratology and embryology [38] (**Figure 6**).

Herein, we present a comparative cross-sectional study involving four *Trypanosoma cruzi* strains obtained from three different species of triatomas captured in endemic areas of the states of Jalisco (*T. longipennis*), Morelos (*T. palidipennis*), Nayarit(*T. longipennis*) and Queretaro (T. Mexicana)

2.2. Collection sites

Several communities from different States of México were included in the present study: San Pablo, Tolimán in Querétaro State, Milpillas of Talpa de Allende in Jalisco State, Sant Catarina in Morelos State and Jala in Nayarit State (**Figure 7**).

2.3. Triatoma collection and maintenance

Cages covered with adhesive tape were used, with the glue facing outward. A live Wistar rat was placed inside the cage. Cages were placed at late night in strategic areas under the loose stones of poultry and farm animal fences, fallen leaves and wooden logs. Cages were collected the next day, early in the morning (**Figure 8**).

Triatomas glued to the surface of the gummed paper were carefully detached, with the aid of entomological tweezers and placed in jars covered with mosquito mesh. A piece of filter paper in accordion shape was placed inside the bottle to facilitate the movement of the triatomas and the collection of urine or feces deposited on its surface.

The triatomas are maintained inside the bottles at 25–26°C and 60% humidity (RH) in bacteriological incubator. Triatomas were blood fed directly from a shaved rabbit every 2 weeks allowing them to feed for 20–25 m and then they were placed back in the incubator (**Figure 9**).

2.4. Study of the intestinal content in the triatomine

We use two techniques for collecting intestinal content from triatomine after blood feeding. In the first one, the triatomine is introduced in a 10 x 20 mm tube; normally the bug deposits

Figure 6. The laboratory animal is "any specie of animal that is kept under certain conditions and is used for scientific purposes" [37, 39].

Figure 7. Map of the Mexican Republic. The black stars indicate the capture zones of the triatomas, used in our investigation.

stool or urine in the bottom of the tube and then it is collected with saline solution. The second technique consists in pressing gently the triatomine abdomen, inducing that the rectal blister freely releases the stool (semi-separated blood). Intestinal content is collected in a watch glass and saline solution is added at 37°C. In both techniques, the metacyclic trypomastigote and epimastigote forms are observed fresh, using a microscope with 400 magnifications. The trypomastigotes are counted in a Neubauer chamber. If the count is above 10,000 parasites per cubic centimeter, mice are inoculated as mentioned below.

The same procedure is performed with each one of the strains of species of triatomas captured (**Figure 10**).

2.5. *Trypanosoma cruzi*, inoculation in mice

Male mice of CD-1 strain are used since estrogen in females can stimulate the activity of macrophage phagocytes and, the localized immune response [38].

Using an insulin syringe four groups of 10 mice were inoculated intraperitoneally with $3x10^3$ epimastigote and/or trypomastigote forms of *Trypanosoma cruzi*, isolated from four species of

Figure 8. Traps are placed in the collection site (A). Cage with triatomas stuck to the adhesive tape (B) Photos. Villagrán-Herrera.

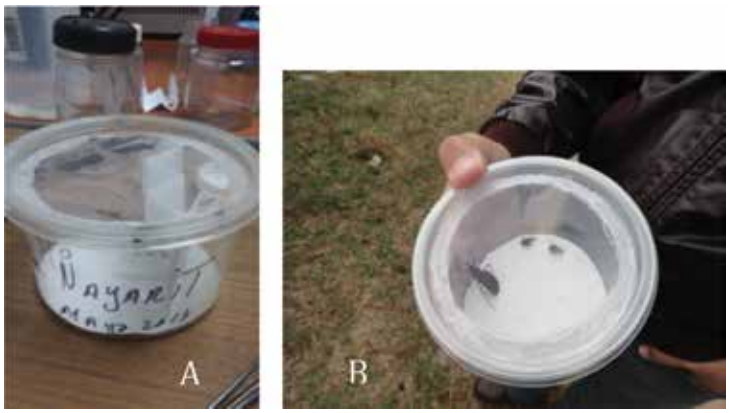

Figure 9. A and B. Transportation and storage of collected triatomas. Photos Villagrán Herrera.

triatomas (*T. mexicana, T. pallidipennis, T. longipennis* and *T. dimidiata*). Mice from the control group were inoculated with saline solution.

2.6. Study of behavior (signs)

After the first day of inoculation, the behavior of the infected murine model was observed, comparing it with an uninfected control.

2.7. Study of the parasitemia

Parasitemia levels are determined in infected mice 5,10,15,20,25 and 30 days post *T. cruzi* inoculation. Blood is obtained from the distal part of the queue 1:4000 EDTA is used as anti-coagulant, in a pipette of leukocyte count. Numbers of parasites per milliliter are calculated from sample observations in Neubauer chamber [37, 38].

2.8. Histopathological analysis

Histological sections of 10 microns are obtained from mouse dissected organs (brain, heart, intestines and skeletal muscle) and stained with H/E. Microscope slide preparations are

Figure 10. Tube techniques to obtain stool from the Triatoma (10A). Fourth stage Triatoma nymphs feeding on a rabbit. Photos Villagrán-Herrera.

observed at 40X [39]. Tissues from infected mice and the respective control animals are included in the analysis.

2.9. Detection of antibodies in serum (detection of anti-*Trypanosoma cruzi* antibodies in serum)

Blood is obtained by cardiac puncture and centrifuged at 5000 rpm to separate the clot from the serum and maintained at −20°C until it is used to carry out an ELISA (Accutrack Chagas Microelisa Test) in the search of anti-*Trypanosoma cruzi* antibodies.

3. Results

The mice inoculated with all the studied *T. cruzi* strains showed an altered behavior when compared to control animals. The signs presented 24 h post *T. cruzi* inoculation, the mice exhibited hyperactivity, the hair was dull and bristled, the hind legs became intertwined, and it began to drag them away, with great difficulty in moving forward.

However, it was possible to observe some differences in the virulence of each strain according to the geographic area geographic area where they came from (**Figure 11**).

The inoculant obtained from triatomines from Talpa de Allende in Jalisco state and Santa Catarina in Morelos state generated in the corresponding mice a parasitemia of 3: 4 trypanosomes per field, at 14 and 16 days postinoculation, respectively. In both cases, altered movements and physical shape of the mice began at about the same time. By day 20 and 23, respectively, the parasitemia reached the peak, so it was proceeded to sample fresh blood and dissect organs in order to perform the serological and histopathological assays

Parasitemia in mice inoculated with the *Trypanosoma cruzi* from triatomines caught in Jala, in Nayarit State reached the peak 30 days post inoculation. It was possible to detect anti-*T. cruzi* antibodies in 2 mice out of 10 by conventional ELISA test.

Blood parasitemia was undetectable in mice inoculated with *T. cruzi* strains obtained from triatomines collected in San Pablo and Tolimán in Querétaro State. The animal behavior was

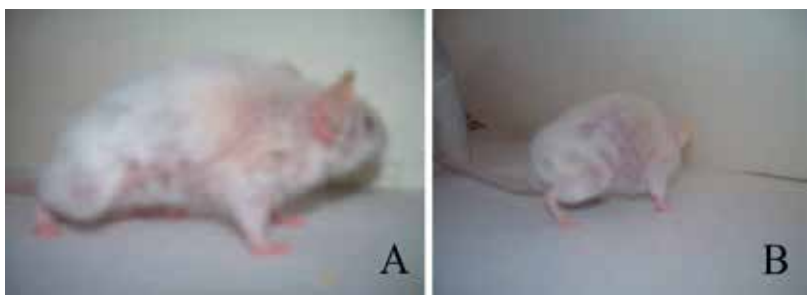

Figure 11. *T. cruzi* infected mice exhibiting hyperactivity, dull and bristled hair and intertwined hind legs. (11A and 11B). Photos Villagran Herrera.

Geographic area	Species of Triatoma	Days in which presents parasitemia	Frizz hair	Difficulty walking	ELISA test	Brain	Heart	Skeletal muscle	Intestine
Talpa de Allende. Jalisco	*T. longipennis*	14	Positive	Positive	Reactive	Negative	Positive	Positive +++	Negative
Jala Nayarit	*T. dimidiata*	30	Positive	Negative	Reactive	Negative	Negative	Positive ++	Negative
San Pablo Tolimán Qro.	*T. mexicana*	It was presented in 90 days	Negative	Negative	Reactive	Negative	Negative	Negative	Negative
Sta. Catarina Morelos	*T. pallidipennis*	16	Positive	Positive	Reactive	Negative	Positive	Positive++	Negative

Table 1. Results of the behavior, parasitemia, serology and histology in the murine model, infected with inoculum of four strains of *Trypanosoma cruzi*, isolated from triatomas captured in different geographical areas.

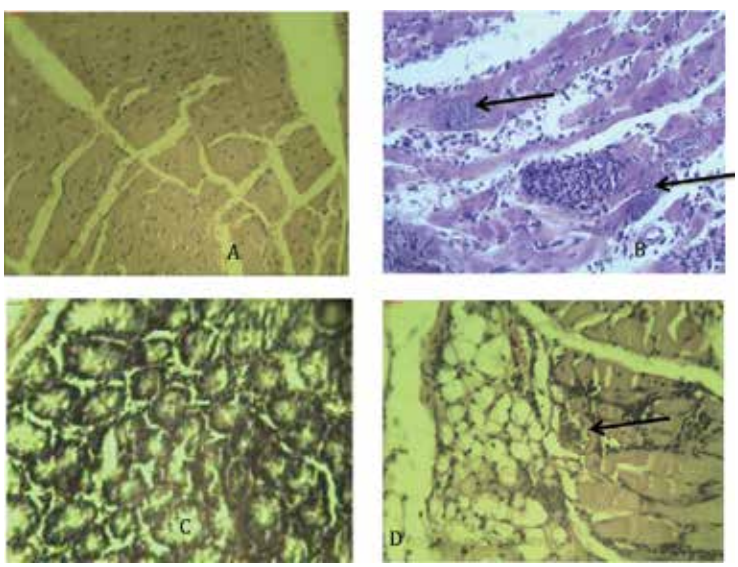

Figure 12. Histopathological analysis of cardiac (A, B) and intestine muscle (C, D) tissues. A and C, control groups. B, cardiac tissue with pseudocysts with high parasitemia and formation of new agglomerations of amastigotes, Jalisco strain. D, intestine muscle tissue with a mild *T. cruzi* parasitemia, Nayarit strain. Photos Villagran-Herrera.

completely normal when comparing to control group. Organs looked slightly bigger, mainly the intestines and heart. It was possible to detect anti-*T. cruzi* antibodies in the serum. Results are summarized in **Table 1**.

Presence of amastigote nests and histopathological damage in heart and intestine muscle showed direct relationship with parasitemia level, which indicates that trypanosomes are installed and recognize different tissues where they reproduce rapidly intracellularly, resulting in a greater number of parasites in blood after they differentiate into trypomastigotes. This sequence is only observed in the most virulent strains such as those from the States of Jalisco, Morelos and Nayarit (**Figure 12**).

4. Conclusions

In the present study, assessment of clinical manifestations, parasitemia levels, histological changes and seropositivity in murine model allowed us to know the behavior of different *T. cruzi* strains found in triatomas from different geographic areas in Mexico. This confirms the existence of genetically different strains that produce a complex called "cruzi" from the pathognomonic and morphophysiological point of view, as previously reported [40].

T. cruzi infection depends on genotype of both the parasite and the mammalian host, which in turn influences tissue tropism and the pathophysiology of infection.

We were able to identify two different *T. cruzi* strains (Tc I) from triatomines from two communities from Queretaro State that exhibited mild virulence when compared to other three strains from triatomines from three different States in México. Isozyme characterization waits to be carried out in order to explain if those observed differences might be attributable to the fact that different species of blood-sucking triatomine insects were used [40].

5. Discussion

Mitie-Nisimura et al., in 2014, were able to induce acute phase inoculating mice by intraperitoneal injection trypomastigote forms of *T. cruzi*. Authors observed the microvascular alterations and oxidative stress in the brain and the formation of pseudocysts full of amastigotes in the heart muscle [41]. Espinoza et al., in 2010 inoculated Balb/c mice with two strains of *T. cruzi* I (Tc I) isolated from patients in Mexico in order to study the immune response [35]. The first case of clinical infection with *T. cruzi* was reported in a horse in South Texas in 2015, observing forms of amastigotes in the spinal cord and cardiac tissue.

Espinoza et al., in 2010, observed contrasting differences between two *T. cruzi* strains isolated from *Triatoma barberi* and *Triatoma mexicana*. In the first case, virulence was clearly observed while in the second one, productive infection and morphological alterations were not observed, and only the anti-*T. cruzi* antibodies were detected.

Author details

María Elena Villagran-Herrera[1]*, José Alejandro Martínez-Ibarra[2], Manuel Sánchez-Moreno[3], Hebert Luis Hernández-Montiel[1], Ricardo Francisco Mercado-Curiel[1], Nicolás Camacho-Calderón[1] and José Antonio de Diego-Cabrerade[4]

*Address all correspondence to: mevh@uaq.mx

1 Department of Biomedical Research, Faculty of Medicine, Autonomous University of Queretaro, Santiago de Querétaro, Queretaro, Mexico

2 Area of Medical Entomology, University Center of the South, University of Guadalajara, Guadalajara, Jalisco, Mexico

3 Laboratory of Molecular Biology of Parasites, Faculty of Science, University of Granada, Granada, Spain

4 Department of Preventive Medicine, Public Health and Microbiology, Faculty of Medicine, Autonomous University of Madrid, Madrid, Spain

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Section 2

Vector, Epidemiology and Clinical Aspect

Chapter 5

Transmitter Insect of Chagas Disease in Northwest Mexico: A Comparative Study of the Cuticular Hydrocarbons Profile of Three Populations of *Triatoma rubida*: Peridomestic, Domestic and Sylvatic

Edgar Alfonso Paredes González,
Gerardo Álvarez Hernandez and
Jesús Ortega-García

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77169

Abstract

In México, biogeography data are available for species of triatomas called *Trypanosoma cruzi* transmitters; for example, the phyllosoma complex is distributed in several states of the south-central southeast of the country. In contrast, Northwestern Mexico species such as *Triatoma rubida* are considered sylvatic and in the process of domestication. The lack of research of these northern species of the country has generated an ignorance that contrasts with a growing number of alleged new cases of Chagas disease registered in health institutions in states such as Sonora. From the six species of triatomas that are potential transmitters of the *trypanosoma* in the state of Sonora, *Triatoma rubida* is the only one that has recent studies of distribution and transmission capacity. It is important then to know the degree of domesticity of the native species with the capacity of transmission of *Trypanosoma cruzi* and to define areas of risk. The process of adaptation of the sylvatic triatomines to the peridomestic and the domestic habitat has been understood in terms of environmental and biological variables. In this research, the profile of cuticular hydrocarbons of a peridomestic, domestic and sylvatic population of *Triatoma rubida* was analyzed and compared.

Keywords: *Triatoma rubida*, triatomines, cuticular hydrocarbons

1. Introduction

Chagas disease (CD) is caused in humans and animals by the parasite *Trypanosoma cruzi* (*T. cruzi*) and it is a major cause of mortality in the Americas. It is estimated that about 100 million people are at risk of infection from 6 million people who are infected, generating 56,000 new cases per year for all forms of transmission and 12,000 deaths annually [1–3]. In Mexico, the actual prevalence of CD is unknown and several epidemiological studies have demonstrated the presence of the disease in large urban and rural regions of the country [4]. Even so, it is estimated that there were approximately 1,100,000 infected individuals and 29,500,000 at risk of infection [5, 6]. The most important factors for this to happen are: (1) adaptability of triatomines to human dwellings and the circulation of *T. cruzi* among them and sylvatic and domestic animals; (2) the poverty situation in communities with poor housing and (3) the migration of people between communities and even distant countries where it did not exist [2, 7–10].

In Mexico 32 species are reported; 19 belong to the gender *Triatoma*, 6 to the gender Meccus, 2 to the gender *Panstrongylus* and 1 species of the genders *Belminus, Dipetalogaster, Eratyrus, Paratriatoma and Rhodnius. Triatoma barberi (Usinger), T. dimidiata (Latreille), T. pallidipennis, T. longipennis and T. mazzotti (Usinger)* are the main species found in our country, considered good transmitters of *Trypanosoma cruzi*, *T. barberi* (usinger), *T. dimidiata* (castreille), *T. pallidipennis*, *T. longipennis*, *T. mazzotii* (usinger), *P. picturata, T. mexicana (Herrich-Schaeffer) and T. gerstaeckeri (Stal) Rhodnius prolixus, Dipetalogaster maxima* and *Panstrongylus* spp. [11, 12]. Many of them are described and studied in the central and southern part of the country. However, to date, the factors that predispose the northern part of the country to CD are unknown despite the presence of transmitters in this part of Mexico [13]. The northern arid zones of Baja California Norte (BCN), Baja California Sur (BCS), Chihuahua, Sonora, Durango and Coahuila report a limited presence of domestic triatomines [6, 7]. In the state of Sonora, six species of triatomines have been described: *Triatoma rubida*, they belong to the subgroup *Rubrofasciata*, with five subspecies (*cochimiensis, jaegeri, rubida, sonoriana and uhleri), Triatoma protracta*, *Triatoma recurva*, *Paratriatoma hirsuta papagoensis*, *Triatoma sinaloenses* and *Triatoma incrassata* [14]. All these species are considered sylvatic with little epidemiological value. However, *T. rubida* and *T. recurve* have been associated with human dwellings and with high infection rates (90%) [15–17].

2. Bibliographic revision

2.1. *Triatoma rubida*

This insect has a wide geographic distribution in the Northwest of Mexico, Nayarit, Sinaloa and Sonora, and has been found in the Southwest of the United States in the states of Arizona, California, New Mexico and Texas. It is an established species throughout its range, and there is no information available on its dispersion [18, 19]. The populations of *T. rubida* have been divided into several subspecies based mainly on differences in the pattern and color of transverse spots on the connective border surrounding the abdomen (**Table 1**) [14]. Under laboratory conditions, *T. rubida* completes its life cycle in 130 days, developing 2 generations with

very low mortality during their shedding, so that more than 98% of their eggs hatch and 94% complete their development until adulthood. *T. rubida* is distinguished because the female manages to eat in less than 10 min and her time of defecation can be immediate or between 5 and 20 s after her blood intake. The insect behaves very persistently during feeding and has the ability to hang firmly onto the host until it completes its feeding. This is consistent with the information collected during the fieldwork, where people report having seen their pet such as dogs carrying the bugs attached to the body [20, 21]. According to Tropical Disease Research (TDR) and the World Health Organization (WHO), studies on the mobility of sylvatic and domesticated populations in endemic areas of types can potentially be adaptable to the human habitat [22, 23]. The process of adaptation is considered a dynamic and continuous phenomenon that varies from one species to another according to its degree of ecological adaptation to eco-modified man-made ecosystems. It should also be considered that transmission (which becomes important in some areas) can occur without necessarily occurring true habitat events but only cases of invasion in human environments by adult triatomines or human-vector contact in the sylvatic environment [24–26]. The destruction of the ecotopes can cause changes, and eventually the disappearance of sylvatic animals as natural blood resources for triatomines, resulting in the invasion of houses by the vector in the search for human blood and exposure of the population to the risk of contracting Chagas disease [27–29].

Currently in the anti-vectorial fight of CD, in endemic countries like Brazil, Argentina, Bolivia and Peru, morphometric, biochemical, molecular and genetic studies of vector species are being developed, that contribute in the decision-making for the eradication in their houses. One of these lines of work is the analysis of the cuticular hydrocarbons (CH) of the triatomines [30].

2.2. Cuticle of insects

The cuticle of the insect is secreted by a double layer of epidermal and hypodermic cells. The hypodermis is described as a functional syncytium and is formed as a base membrane particularly during deposition and expansion of the old cuticle. The cuticle is formed by an inner procuticle composed of chitin (*N*-acetylglucosamine) and protein and a thinner chitinous outer layer the epicuticle. The pro-cuticle in turn is divided into an inner layer and an outer layer called exocuticle (pre-mutated cuticle) which is formed by sclerosed protein. The inner endocutaneous layer (cuticle after shedding) has remnants of the same sclerosed protein [31, 32].

Subspecies	Geographical Distribution
Triatoma rubida (Uhler)	Baja California Sur, Sonora
Triatoma rubida cochomiensis Ryckman	Baja California
Triatoma rubida jaegeri Ryckman	Isla Estanque BC., Sonora
Triatoma rubida sonoriana Usinger	Sonora, Sinaloa y Nayarit
Triatoma rubida uhleri Usinger	Suroeste de USA, Sonora y Veracruz

*Lent et al., 1979 [14].

Table 1. Geographic distribution of sub-species of ***Triatoma rubida****.

2.3. Insects' cuticular hydrocarbons

The insect's cuticular lipids consist of aliphatic material, which forms a thin layer in its integument. These lipids or surface waxes are presented as highly stable complex mixtures with unique structural characteristics. Among its main compounds hydrocarbons (HCs), fatty alcohols and waxes of high molecular weight predominate. The main function of these lipids in the insect is to restrict water loss and avoid lethal drying. It has been shown that they also participate in the absorption of chemical substances that can affect the activity of microorganisms and intervene in various chemical communication processes [33–35].

Cuticular hydrocarbons (CH) are continuously synthesized in the insect's intrategumenal tissue, through the enzymatic action of fatty acid synthetase (FAS), an acetyl CoA for elongation, a reductase and a decarboxylase that produces hydrocarbons and CO_2. The epidermal cells responsible for its production are the oenocytes that lie beneath the hypodermis. Oenocytes transport hydrocarbons through tissues through a hemolytic lipoprotein called liporin. This lipid synthesis is considered dynamic and changes as the insect passes through its nymphal stages, stopping at the adult stage. De Renobales et al. [36], proposed that the hydrocarbons synthesized by the insect are stored inside their tissues until the next shedding. The insect needs a new layer of lipids as regulators of its permeability [35, 37].

Based on studies conducted in particular on nine species of triatomines of the genus *Triatoma*, *Pastrongylus* and *Rhodnius*, it has been found that such HCs are alkanes of 27–33 carbon atoms and chains of branched alkanes with 1–3 methyl groups inserted along a carbon skeleton from 29 to 41 carbon atoms [35]. The predominant linear components are nC27, nC29, nC31 and nC33, while in the branched fraction predominate isomers of dimethyl- C37, trimethyl-C37, trimethyl-C35 and trimethyl-C39 as reported by Juárez et al. [38] (**Figure 1**). Williams and Jackson [45] suggested that hydrocarbon differentiation may be an early evolution of specialization; in addition, geographic differentiation also led these authors to suggest that the phenotype may be differentiated prior to species divergence. Similarly, Juárez and Brenner [39, 40], considered that the composition of triatomine HC can be used as a taxonomic criterion to separate individual populations and

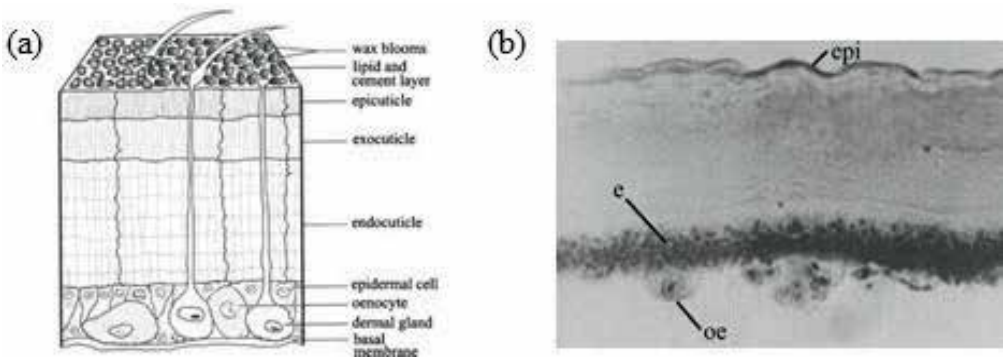

Figure 1. (a) Diagram of a cross-sectional area of the insect integument, illustrating the major layers of the cuticle and (b) cross-sectional view of *T. infestans* integument. Epi, epicuticle; e, epidermal cell layer; oe, oenocytes. Source: Juarez, M.P. 2007.

specimens, based on the graphical comparison of their corresponding profiles (fingerprints) or through the quantitative calculation of numerical indicators such as the determination of HC [41].

Finally, understanding how insect HCs, together with other surface lipids, are involved in the absorption of chemicals is essential for the timely and adequate vector control measures to be applied in the future [42, 43].

This research analyzes and compares the profile of cuticular hydrocarbons of a peridomestic, domestic and a sylvatic population of *Triatoma rubida*; this is an insect that transmits the Chagas disease in the state of Sonora. The rationale for this proposal was to define the hydrocarbon profiles of *T. rubida* peridomestic, domestic and sylvatic, in order to obtain differences between each of the swarms and to be able to differentiate the three populations of insects by their HC profile. Having the knowledge that *T. rubida* participates in the vectorial transmission of *T. cruzi*, the study is very helpful and of high epidemiological value, to take measures to eradicate and/or control it in human dwellings.

3. Materials and methods

3.1. Sampling area

The city of Guaymas Sonora was chosen because it is considered, in this study, to be an endemic area of the CD. The port is situated at 110°53′34″ North latitude and 27°55′30″ West Greenwich, at a height of 15 m. It has a desert or hot climate, with a maximum monthly temperature of 30–35°C in the months from July to August and a minimum average monthly temperature of 18°C. Its average annual temperature is 28°C. Its vegetation is xerophytic type, where mesquite (*Prosopis velutina*), pitahaya (*Stenocereus thurberi*), palo fierro (*Olneya tesota*), palo verde (*Parkinsonia aculeata*), jito (*Forchammeria* watsonii) and scrub subinerme abound [44].

3.2. Sampling and capture of insects

Three districts of the City of Guaymas were monitored to collect the batch of peridomestic and domestic insects, where the epidemic was known: El Rastro, Cerro Gandareño and Yucatán. In these areas, the existence of triatomines was known, particularly *Triatoma rubida sonoriana*, which was the subspecies chosen for this research and had entomological indices indicating infestation in 63%, colonization of 68.4% and density of 8.5% [15].

The sylvatic insects were collected from the surrounding hills, a hill in the northern part was chosen 1400 m away from the city where domestic and peridomestic insects were collected. *Neotoma* spp. were observed in this area and confirmed during visits. Four nests of *Neotoma* were distributed in a radius of 60 m all placed at the base of pitahayas (*Stenocereus thurberi*).

Once collected, the two pairs of main and secondary wings were extracted from the adult insects which were wrapped in foil and transported to the laboratory of parasitology at the University of Sonora, North Caborca Unit. For the identification of the morphological characteristics that define *rubida*, the keys described by Lent and Wygodzinsky [14] were used.

3.3. Analysis and extraction of hydrocarbons

Cuticular hydrocarbons (CHs) were extracted following the methods of Juárez and Blomquist [45], and Juárez et al. [46]. Each pair of specimen wings were given a washing treatment with 2 mL of distilled water twice to remove any contaminants such as feces or soil particles. They were then transferred to a 4 mL glass vial with a screw cap, Teflon septum and properly labeled. A 1 mL of high-performance liquid chromatography (HPLC) grade hexane was added with 99% purity (Sigma-Aldrich, México). With this solvent they were kept for 1 day, at a temperature of 28°C for the extraction of the cuticular lipids.

3.4. Isolation of hydrocarbons

The next step consisted of separating the hydrocarbons from the other cuticular components (lipids, waxes, etc.) present in the extract. For this purpose, the lipid mixture was reconstituted from each vial with hot hexane and then applied to a mini glass column (10 × 5 mm ID) with 1.74 g of silica gel, 60% pore and 70-230 (Sigma-Aldrich, México; cat. 288,624). Previously equilibrated with hexane, the elution was carried out with 4 mL of hexane for each sample (4 mL/mg). The silica from the column was renewed every three samples (**Figure 2**).

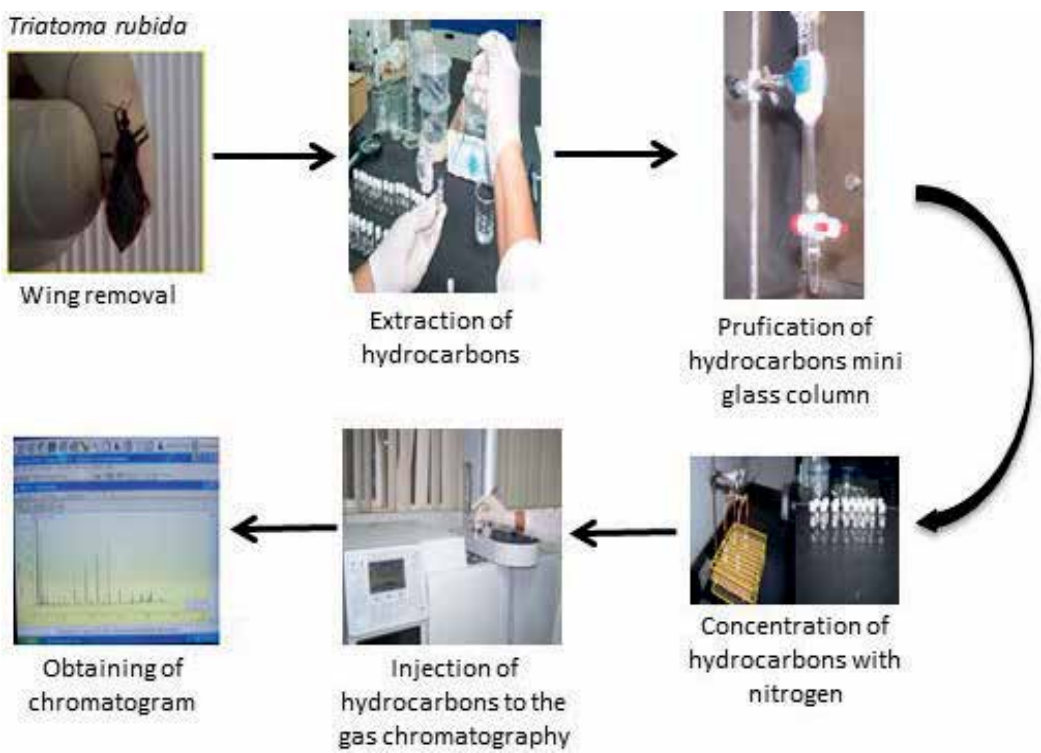

Figure 2. Diagram for the extraction of cuticular hydrocarbons.

3.5. Obtaining the chromatographic profiles of each population

Once the methodology was adjusted, 3 μl of each of the hydrocarbon samples extracted from *Triatoma rubida* was injected into the chromatograph. To do this, 7 μL of hot hexane was taken and poured into the vial containing the hydrocarbons of each specimen, until the largest amount possible from the sample was mixed by circular movements in the bottom of the container, where the required amount was injected in the GC.

3.6. Identification of HC by GC-MS

For the identification of the linear hydrocarbons, an HP 6890 chromatograph coupled to an Agilent 5975C VL mass spectrometer was used. GC conditions were HP-5MS nonpolar column of 30 × 0.25 mm ID × 0.25 μm film; helium carrier gas at 1.5 mL/min constant flow; oven temperature programmed 50°C (1 min) to 200–50°C /min, then to 320–3°C/min (25 min) and the injector was operated in split-less mode at 320°C. The conditions of the MS detector were ionization energy of 70 eV; transfer line at 320°C; the ionization chamber at 230°C and the quadrupole at 150°C. For the analysis of the collected data, an MSD ChemStation Agilent Technologies Inc. was used.

4. Statistical analysis

We estimated central tendency measures and compared the relative means of abundance of hydrocarbons between genera; the significance was tested by a nonpaired t (Excel 2006 package), after normalization of the data with arcosene. Relative means (Tukey's post hoc test) were compared between the three populations through a one-way analysis of variance (ANOVA). The data were presented in tables and graphs. All tests were estimated at one tail and values of $p < 0.05$ were considered as statistically significant. For these analyses the statistical package BioStat 2007 was used.

5. Results

5.1. Collection of insects

A total of 120 peridomestic, 50 domestic and 50 sylvatic specimens were collected. Of the 220 insects, there were nymphs of second stage (NII; 1.4%), nymphs of the third stage (NIII; 11.4%), nymphs of the fourth instar (NIV; 17.3%), 53.6% were nymphs of the fifth instar (NV; 53.6%), 6.8% were adult females (AF) and 9.5% were adult males (AM). No specimens of the first nymphal period were found in all three populations.

5.2. Analysis of the hydrocarbons: Retention times obtained

The gas chromatographic standardization process allowed to obtain the retention times of 14 commercial hydrocarbons standards, AccuStandard Brand, Inc. USA (purity 99%), which were used to estimate Kovats indexes for each sample analyzed (**Table 2**).

Standard	Name	Retention Time (Minutes)
C19	Nonadecane	6.31
C20	Eicosane	7.18
C21	Heneicosane	8.23
C22	Docosane	9.51
C23	Tricosane	10.99
C24	Tetracosane	12.67
C25	Pentacosane	14.49
C26	Hexacosane	16.44
C28	Octacosane	20.45
C30	Triacontane	24.46
C32	Dotriacontane	26.36
C36	Hexatriacontane	35.71
C38	Octatriacontane	38.78
C40	Tetracontane	41.25

Table 2. Retention time of injected standards.

5.3. Identification of the cuticular hydrocarbons profile of *Triatoma rubida*

The 35 components of *T. rubida* were detected (**Figure 3**); however, for this study, only 14 major peaks were considered (**Figure 4**). Five linear hydrocarbons were identified in the three populations of *T. rubida* in both females and males, corresponding to the first five selected peaks of the chromatogram: pentacosano, heptacosano, nonacosano, hentriacontano and tritriacontano. It is important to note that these five hydrocarbons are present in all three populations (**Figure 5**).

The chromatographic profile was similar for the three populations, and the hydrocarbons corresponded to n-alkanes with a continuous series of C25, C27, C29, C31, C33 and C35. In addition, the Kovats indices identified C35.52, C36.00, C37.74, C37.75, C38.00, C39.41, C39.60 and C39.83, which are likely representations of branched isomers of alkanes. The location of the methyl branches of these hydrocarbons, by the proposal of Katritzky et al. [47], was estimated. The Kovats indexes: IK3552, IK3600, IK3774, IK3775, IK3800, IK3941, IK3960 and IK3983 therefore correspond to the hydrocarbons described in **Table 3**.

5.4. Quantification in percent of area of hydrocarbons analyzed

The relative amounts in the percent of area of each linear and branched hydrocarbon analyzed for each population were obtained. In **Table 4**, the data for the peridomestic, domestic and sylvatic *Triatoma rubida* population are presented.

In **Table 5**, the total amounts for linear and branched hydrocarbons are presented for each of the three populations of *rubida;* also the relative percentage of the majority hydrocarbon is

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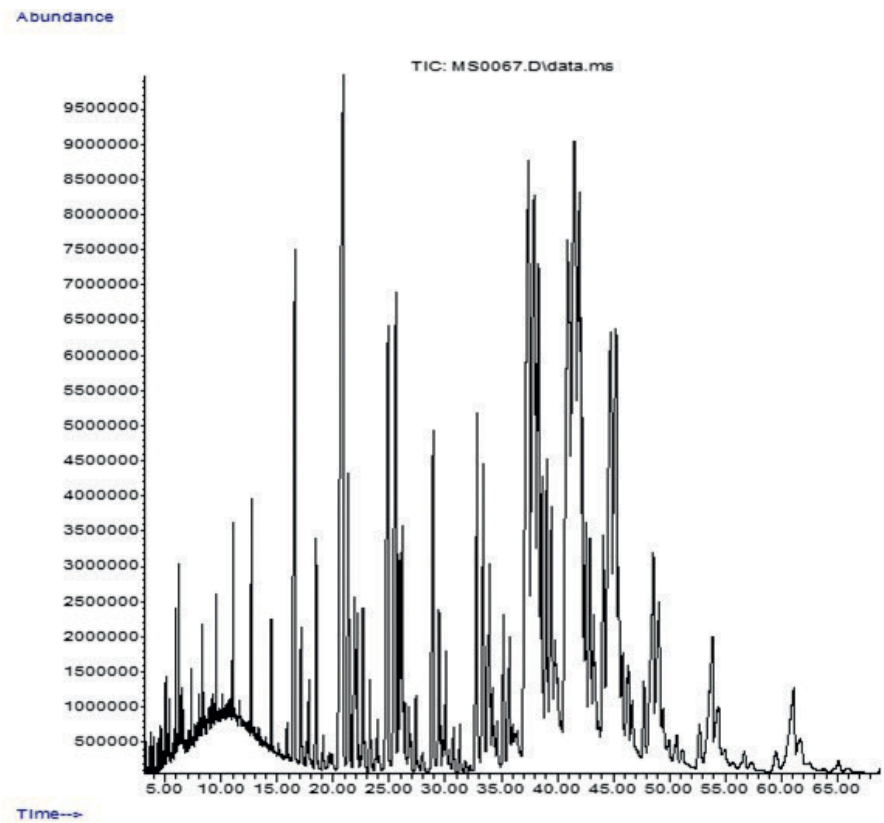

Figure 3. Ion chromatogram total of *Triatoma rubida*.

presented. Using statistical analysis and using the unpaired "t" test, when comparing females and males for each population, a significant difference was found between males and domestic females for the IK2700 peak. When comparing peridomestic males and females, significant differences were found for the IK3100 peak. Regarding the comparison of sylvatic males and females, significant differences were observed for the IK3100 and IK3300 peaks, in addition to the peaks IK2500 and IK2700 (**Figure 6**).

5.5. Comparison of the three populations of *Triatoma rubida*

To analyze the differences between *rubida* species, the one-way parametric analysis of variance (ANOVA) was used. When comparing the three populations of females, significance was found for the relative average abundance of the C27 hydrocarbon. Tukey's post hoc test estimated the differences among the three female populations, finding a significant difference between domestic and sylvatic ones ($p = 0.00001$).

In the comparison of groups of males, significant differences were also found between domestic males and peridomestic males in HC27 ($p = 0.01$) and HC29 ($p = 0.03$), whereas when comparing domestic with sylvatic males, there were significant differences in HC33 ($p = 0.002$). On the other hand, when comparing the populations of females with males, significant differences were observed in the population of domestic females compared to that of peridomestic and sylvatic

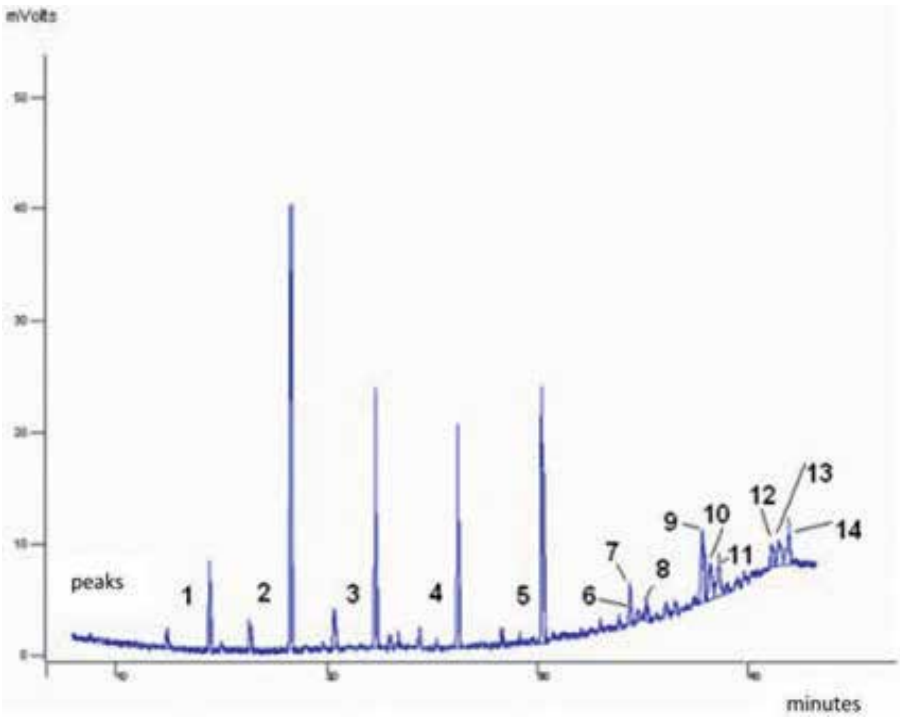

Figure 4. Selection of 14 hydrocarbon peaks, majority in *Triatoma rubida*.

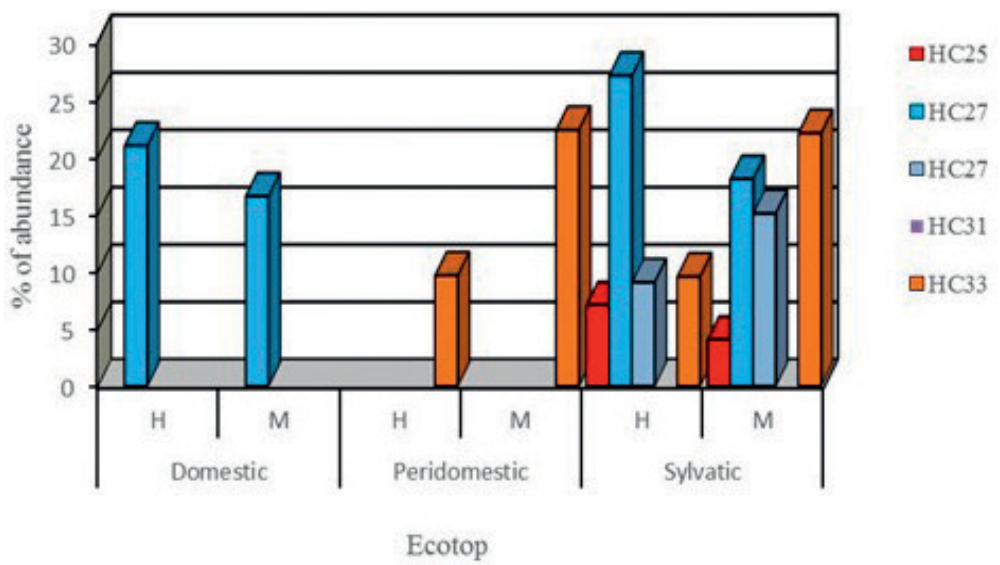

Figure 5. Quantitative variation of cuticular hydrocarbons of *T. rubida* considering its generous.

males in the HC 29 ($p = 0.01$), 31 ($p = 0.03$) and 33 ($p = 0.001$). Meanwhile, the population of peridomestic females, when compared to domestic males and sylvatic males, had significant differences in HC27 ($p = 0.007$), HC29 ($p = 0.01$) and HC33 ($p = 0.0009$). Finally, in the comparison

Retention rate	Type of hydrocarbon
3574	03 Methyl Pentacontane
3600	3x Dimethyl Pentacontane
3752	13, 23 Dimethyl Heptatriacontane
3775	15,19,23 Dimethyl Heptatriacontane
3800	3x Dimethyl Octariacontane
3941	x Trimethyl Nonatriacontane
3960	xx Dimethyl Nonatriacontane
3983	15.19, 23 Trimethyl Nonatriacontane

*Katritzky et al., 2000 [47].

Table 3. Qualitatively identified branched hydrocarbons.

Peak	Hydrocarbon*	Kovats Index	Domestic[1]		Peridomestic[2]		Sylvatic[3]	
			Male	Female	Male	Female	Male	Female
1	n-25	2500	6.59 ± 2.3	5.50 ± 2.3	4.50[a] ± 0.09	7.56[b] ± 0.9	5.54 ± 1.3	6.18 ± 1.0
2	n-27	2700	16.50[a] ± 1.3	20.90[b] ± 2.0	19.00 [a] ± 3.2	27.88[b] ± 0.8	23.00 ± 1.5	26.64 ± 2.6
3	n-29	2900	9.33 ± 1.2	10.80 ± 2.0	12.82 ± 1.6	13.83 ± 0.8	15.29 ± 0.9	16.10 ± 1.9
4	n-31	3100	11.86 ± 1.8	10.00 ± 1.4	15.69[a] ± 1.2	9.41[b] ± 0.9	14.08 ± 0.7	12.17 ± 2.3
5	n-33	3300	14.06 ± 2.7	12.00 ± 1.8	22.29[a] ± 1.7	9.61[b] ± 0.6	15.72[a] ± 1.3	12.29[b] ± 1.7
6	n-35	3500	5.19 ± 1.7	5.20 ± 0.4	2.98 ± 0.3	3.82 ± 0.5	2.59 ± 1.3	3.03 ± 0.5
7	03 Methyl Pentacontane	3574	2.74 ± 0.3	2.90 ± 0.2	1.55 ± 0.3	1.81 ± 0.3	1.84 ± 1.0	2.00 ± 0.3
8	3x Dimethyl Pentacontane	3600	2.76 ± 0.6	2.00 ± 0.4	1.53 ± 0.1	1.47 ± 0.3	1.70 ± 0.5	1.40 ± 0.3
9	13, 23 Dimethyl Heptatriacontane	3572	6.55 ± 0.2	7.40 ± 1.0	5.61 ± 0.9	7.73 ± 1.2	5.77 ± 0.3	6.00 ± 0.3
10	15,19,23 Dimethyl Heptatriacontane	3775	7.07 ± 0.8	6.80 ± 1.3	4.84 ± 2.7	4.73 ± 1.2	4.13 ± 1.0	4.04 ± 1.4
11	3x Dimethyl Octariacontane	3800	5.63 ± 1.1	4.90 ± 0.5	3.02 ± 1.3	4.00 ± 0.2	3.38 ± 0.6	3.00 ± 1.6
12	x Trimethyl Nonatriacontane	3941	2.14 ± 0.3	1.90 ± 0.3	1.96 ± 0.3	2.22 ± 0.6	1.90 ± 0.2	1.70 ± 1.2
13	xx Dimethyl Nonatriacontane	3960	4.79 ± 0.8	4.50 ± 0.9	3.16 ± 0.9	3.57 ± 0.8	3.18 ± 0.6	3.00 ± 1.1
14	15.19, 23 Trimethyl Nonatriacontane	3983	4.79 ± 1.9	5.20 ± 0.9	2.56 ± 2.2	3.50 ± 0.9	3.07 ± 0.7	3.00 ± 2.2

*The hydrocarbons and peak number are the same as reported in **Figures 6-7**. The means were compared with the unpaired *t* test. [a, b] The differences between males and females were significant among the hydrocarbons in each row. Population analyzed: [1,2] n = 12 females and n = 9 males; [3] n = 9 females and n = 12 males.

Table 4. Relative percent of majority hydrocarbons of ***Triatoma rubida*** peridomestic, domestic and sulvatic (%).

Population /Hydrocarbon	*Triatoma rubida*					
	Domestic		Peridomestics		Sylvatic	
	Female	Male	Female	Male	Female	Male
Linear	56.76	51.75	76.41	76.22	72.11	77.28
Branched	43.24	48.25	23.59	23.78	27.89	22.72
2700*	37.40		49.64		46.88	

*Major component of hydrocarbon detected.

Table 5. Content of cuticle hydrocarbons in ***Triatoma rubida*** (%).

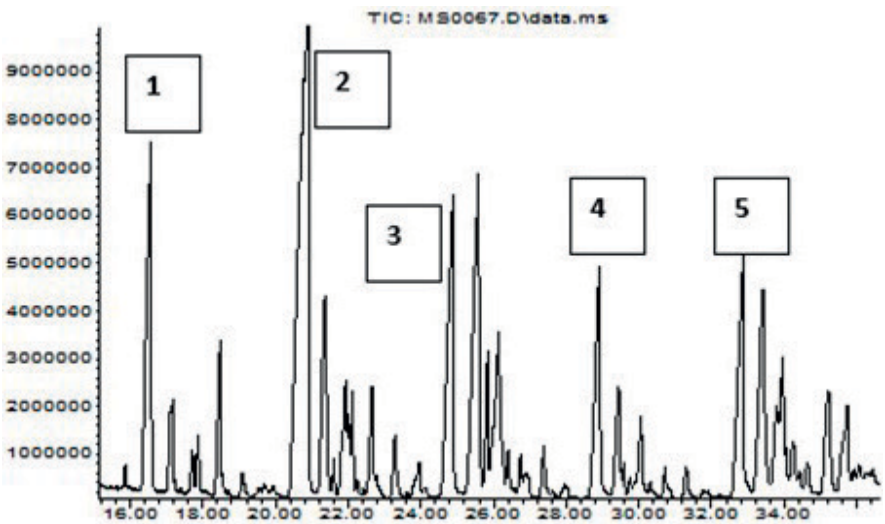

Figure 6. Confirmed linear hydrocarbon mass spectra.

of populations of sylvatic versus domestic males and peridomestic males, significant differences were observed for HC27 ($p = 0.0001$) and HC29 ($p = 0.002$).

6. Discussion

6.1. Domestic population

The *GC–MS* analysis showed that in the population of *Triatoma rubida*, domestic, odd chains of HC prevailed, and C27, C29, C31 and C33 predominated, which together represented 56.8% of HC in females and 51.8% for the males. The branched hydrocarbons represented 32.4% in males and 36.5% in females, of total hydrocarbons. The relative amount of pentacosane hydrocarbon was 37.40%. The characteristics of the typical chromatogram of females and males showed that the chromatographic profiles of the cuticular hydrocarbons in domesticated *Triatoma rubida* were qualitatively very similar for both genera. However, their relative amounts were different, as demonstrated in the pentacosane hydrocarbon, where the female had 20.9% and the male had 16.50%.

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Based on the graphical representation of the chromatographic profiles, the identification of five of their linear hydrocarbons by mass spectrometry and the statistical analysis when comparing the relative means between genders, we suggest how the typical profile of domestic Triatoma rubida is described in **Figure 7**.

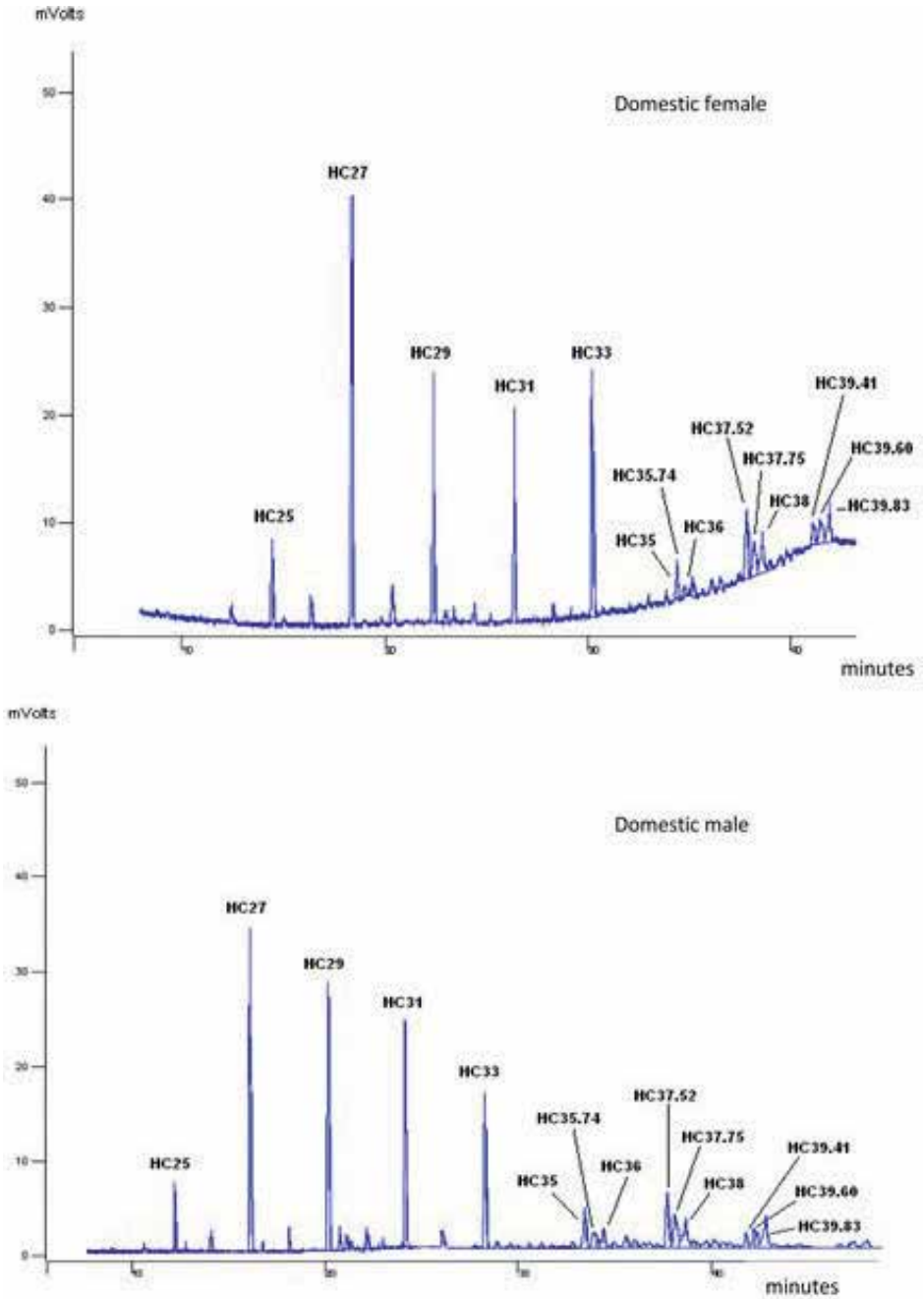

Figure 7. Typical chromatogram of female and domestic male of *Triatoma rubida*.

When comparing this profile of hydrocarbons obtained with studies of other triatomines, a clear differentiation of species can be seen. For example, the literature reports that *T. barberi*, one of the habitat triatomines, is considered to be a transmitter of CD in Mexico, has major alkanes such as C29, C31 along with C33 and C27, respectively and most of its branched components correspond to mono-, di- and trimethyls of C33, C35 and C37 [43]. On the other hand, *T. dimidiata*, considered one of the most important domestic triatomines in Mexico, presents a profile of cuticular hydrocarbons, formed by saturated hydrocarbon chains ranging from C22 to C35. Of these, the odd one strands like C31 followed by C29, C27 and C33 and small amounts of the C22 and C30 hydrocarbons prevail. It also has branched alkanes, most of them mixtures of different isomers: mono-, di- and trimethyl in their internal chains [43]. *Triatoma longipennis* insect belonging to the phyllosoma complex, widely distributed particularly in Central and Southern Mexico, is considered to be a *Triatoma* with a high degree of habitation [48] and has a saturated hydrocarbon profile ranging from C23, C25, C27, C29, C31 to C33, with the majority being C29 (16%) [49].

6.2. Peridomestic population

The GC–MS analysis showed that the chromatographic profiles of the HC in *Triatoma rubida* peridomestic are qualitatively very similar for both genders. As in the domestic population, they corresponded to n-alkanes with a continuous series of C25, C27, C29, C31, C33 C35. In addition, C35.52, C36.00, C37.74, C37.75, C38.00, C39.41, C39.60 and C39.83 were identified, were likely to be representative of branched isomers of alkanes. The location of the methyl branches of these HCs, by the proposal of Katritzky et al. [47], was estimated. The proposal made it possible to estimate that the hydrocarbons IK3552, IK3600, IK3774, IK3775, IK3800, IK3941, IK3960 and IK3983 correspond to the same hydrocarbons identified in the domestic population.

For this population, odd strands prevailed predominantly, C27, C29, C31 and C33, which together represented 76.41% in females and 76.22% in males. The total relative amount of branched hydrocarbons in males and females was 23.59% and 23.78%, respectively. The relative amount of the pentacosane hydrocarbon was 49.64%. Based on the graphical representation of the peridomestic rubida species chromatographic profiles, in their identification by mass spectrophotometry and in the statistical analysis when comparing the relative means between genders. We can suggest as typical profile of Triatoma rubida peridomestic described in **Figure 8**.

6.3. Sylvatic population

The analysis of *GC–MS* showed that sylvatic-type *Triatoma rubida* HC is qualitatively very similar for both sexes. These HCs corresponded to n-alkanes with a continuous series of C25, C27, C29, C31, C33 and C35. In addition, C35.52, C36.00, C37.74, C37.75, C38.00, C39.41, C39.60 and C39.83 were identified, which are likely representations of branched isomers of alkanes. Through the proposal of Katritzky et al. [47], the location of the methyl branches for the identified hydrocarbons was estimated, resulting as qualitatively equal to the two previous populations. Odd numbers, predominantly C27, C29, C31 and C33, chains prevailed in this population, which together represented 72.1% of the relative percentage in females and 72.3% for males. The total relative amount of branched hydrocarbons in males and females

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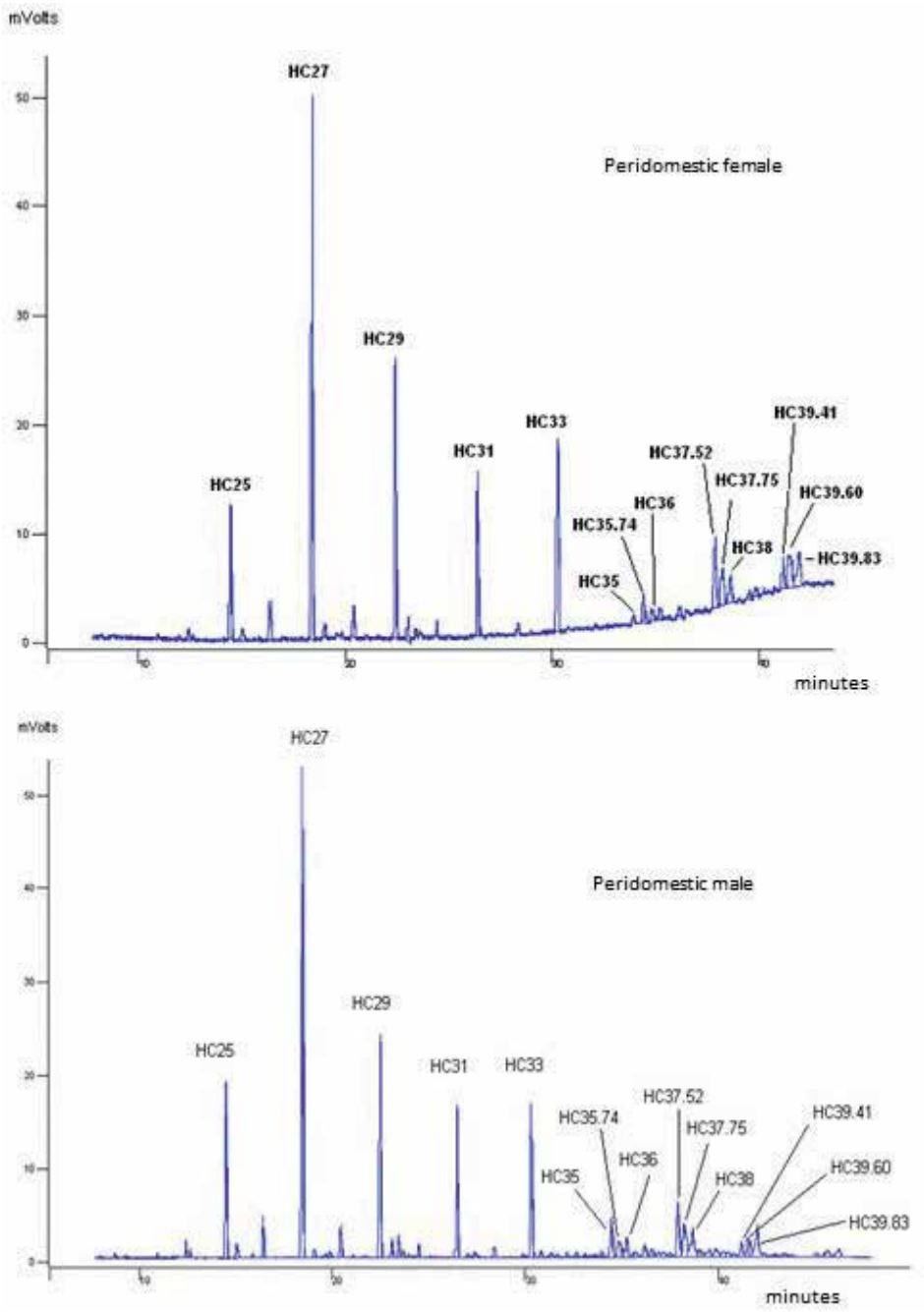

Figure 8. Typical chromatogram of female and peridomestic male of *Triatoma rubida*.

was 27.9 and 22.7%, respectively. The relative amount of the pentacosane hydrocarbon was 46.88%. The pentacosane hydrocarbon of females was present in 27.88% while in the males they presented 19%, similarly the hentriacontano hydrocarbon was 15.59% in males with

respect to 9.41% of the females. Likewise, there were differences in the tritriacontano hydrocarbon of females, 9.61% with respect to 22.29% in males.

Based on the graphical representation of the chromatograms of sylvatic *rubida* species, their identification by mass spectrophotometry and statistical analysis when comparing the relative means between sexes, the typical profile of sylvatic Triatoma rubida can be suggested as described in **Figure 9**.

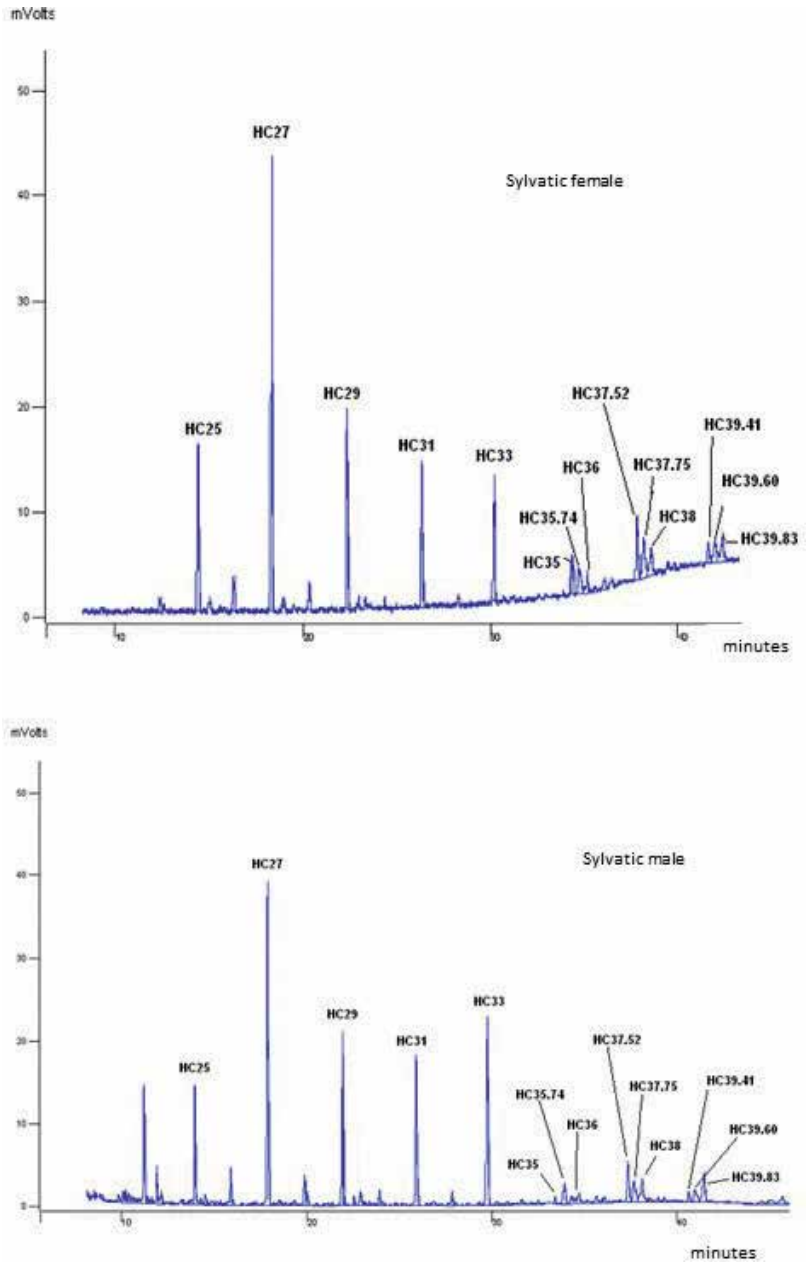

Figure 9. Typical chromatogram of female and sylvatic male of *Triatoma rubida*.

Juárez et al. [38], suggested that quantitative rather than qualitative differences support the idea that cuticular hydrocarbons represent primitive characteristics for *Triatomas*. For example, the study of the tribes *Rhodnius* and *Triatomini* found few common traits among them, however, they converge in their hematophagous habits, even though they come from very different habitats. This led them to suggest that the HC profile obeys an ancestral base set by the selection, favoring the presence of certain hydrocarbons associated with conditions of dry habitats and humid ecotypes such as that of the City of Guaymas.

Therefore, species of triatomines of dry regions present their cuticular profiles as more abundant and complex than their congeners of humid regions. Among Triatoma, *T. brasilinesis* and *T. pseudomaculata* from arid regions of Northeastern Brazil present a more complex profile than *T. infestans* from less dry regions of Central Brazil and Argentina, and in turn the three species mentioned present larger complexity when compared to *T. bimaculata* and *T. vitticeps* from coastal regions [50].

In Mexico, *T. barberi* that lives in dry regions presents a more complex HC model. The population area of *Dipetalogaster maximus* that has been collected from Baja California Sur presents abundant and longer saturated chains and constitutes 60–67.8% of the total hydrocarbon mixture for females and males, respectively. Thus, the relative abundance of n-alkanes may be related to the exposure of adverse conditions [51].

7. Conclusions

This research provides basic knowledge on the cuticular lipids of Triatoma rubida. A unique and very different profile of cuticula hydrocarbons was obtained from *Triatoma barberi*, *Triatoma dimidiata* and *Dipetalogaster maximus*, species are considered as transmitters of *Trypanosoma cruzi* in Mexico. No characteristic profile in the cuticular hydrocarbons was found in the collected population of females and males of *Triatoma rubida*. However, the cuticular hydrocarbons profile of the peridomestic, domestic and sylvatic populations of the insect is qualitatively similar, but were identified by significant quantitative differences, so it is possible to state that distinctions can be made between populations. The profile of cuticular hydrocarbons, identified in this study, can be used as a reliable chemotaxonomic tool to identify the populations of *T. rubida*, considering the expression of hydrocarbons as the chemical phenotype of the vector that responds to environmental and biological factors of the insect.

Author details

Edgar Alfonso Paredes González[1], Gerardo Álvarez Hernandez[2] and Jesús Ortega-García[1]*

*Address all correspondence to: jortega@guayacan.uson.mx

1 Universidad de Sonora, Unidad regional Norte Caborca México, Mexico

2 Universidad de Sonora, Departamento de Ciencias Químicas, Unidad Centro Hermosillo Sonora, Mexico

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Chapter 6

Eco-Epidemiology of Chagas Disease in Chile

Mauricio Canals Lambarri, Andrea Canals Cifuentes, Salvador Ayala, Valentina Tapia-Garay and Dante Cáceres Lillo

Additional information is available at the end of the chapter

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Abstract

There are four vector species of Chagas disease in Chile: *Triatoma infestans*, responsible for the domestic cycle; *Mepraia spinolai*, the main wild vector; and *M. gajardoi* and *M. parapatrica*, two coastal wild species whose importance as vectors is not well known. They are species of dry environments of the central-north region of Chile, whose best predictors of distribution are warm average temperatures and low rainfall. They are found in rock quarries, nests of birds, and small mammals, and *T. infestans* has sylvatic foci associated with a Bromeliaceae species. While human blood represents 70% of the diet of *T. infestans*, in *M. spinolai* this value is 7%, which means that a large part of Chagas disease in Chile is due to *T. infestans*. However, all species have high percentages of *T. cruzi* infection. Chagas disease in Chile follows the distribution of *T. infestans*, and although the cycle of domestic transmission by this vector is interrupted, there is still a constant prevalence and mortality and ascending incidences. Models predict that although climate change will not vary greatly the north-south distribution of vectors, it could increase the reproductive number of the disease, increasing risk areas of Chagas disease.

Keywords: Chagas disease, ecology, vectors, epidemiology, Chile

1. Introduction

American trypanosomiasis or Chagas disease is caused by the flagellated protozoan *Trypanosoma cruzi*, transmitted by several hematophagous insect species (Hemiptera, Reduviidae, and Triatominae) which in Chile are known as vinchucas. This protozoan species undergoes part of its development cycle (epimastigotes and trypomastigotes) in the insect vector, and when it ingests blood of a vertebrate host, the infectious trypomastigotes are eliminated with the

dejections on the host; these are able to enter the bloodstream through the small wound or scratch lesions. Inside the *T. cruzi* continues its development with intracellular forms (amastigotes) and extracellular forms (trypomastigotes), producing damage which may be manifested in an acute form, although it generally does so after many years as a chronic form that compromises the digestive tract (megacolon or megaesophagus) and/or heart disease.

Two cycles of vector transmission in Chagas disease are described: (1) the domestic cycle maintained by domestic vectors, in Chile *Triatoma infestans*, which includes a man and an animal reservoir constituted by domestic and peridomestic animals such as cats, dogs, cows, horses, and others, and (2) the wild cycle maintained by wild vectors (such as *Mepraia spinolai*) and the wild animal reservoir composed of rodents, small marsupials, rabbits, etc. These cycles are not completely separated, since there are species that transit between the peridomestic and wild environments such as dogs, cats, goats, and other farm animals (**Figure 1**) [1–3]. In addition, wild vectors can penetrate into domestic environments and feed on humans [4], and domestic vectors can form wild colonies [5–7].

Its discovery is due to the Brazilian doctor Dr. Carlos R. J. Chagas, who in 1907 was the director of the Oswaldo Cruz Institute and director of the National Malaria Control Program. Dr. Chagas examined huts, finding a large number of insects that he named *Conorrhinus sanguessuga* (now *Panstrongylus megistus*) that contained "*Crithidia*." After experiments of inoculation

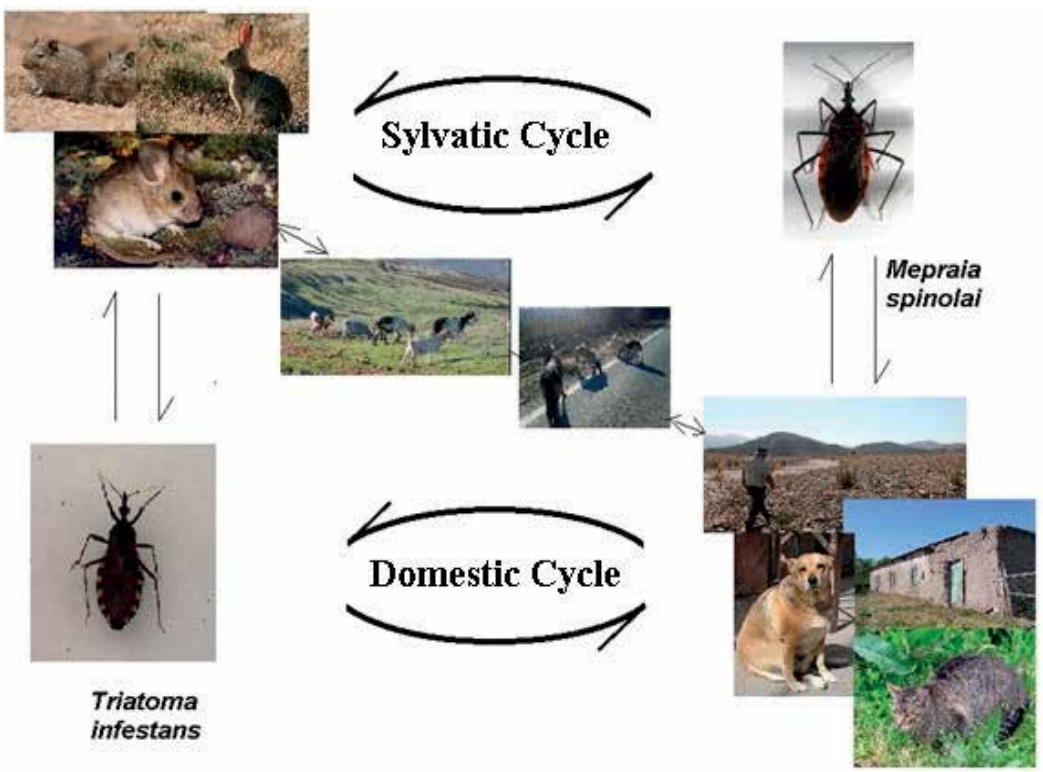

Figure 1. Domestic and wild cycles of Chagas disease.

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in monkeys, he found a great quantity of flagellates, calling the new species *Shyzotrypanum cruzi* (today *Trypanosoma cruzi*) in honor of Dr. Oswaldo Cruz. Later, in 1909 he found a naturally infected cat and on April 14 found in the village of Santa Rita a 2-year-old girl named Berenice who lived in a house infested with *P. megistus* with a feverish condition and found in her blood the protozoan *T. cruzi*, describing the disease in 1909 [8]. Later, Carlos Chagas wrote about acute and chronic forms of the disease (1911) and the life cycle of *T. cruzi* (1931) [9], among other topics.

The first studies of Chagas disease in Chile were carried out by Dr. Juan Noé in 1921, with appreciation of *T. cruzi* in the vector *Triatoma infestans* around Santiago. Subsequently, the Argentine Dr. Salvador Massa, led by Noé, demonstrated the specificity of *T. cruzi* in cardiac fibers in experimental animals. With the creation of the Department of Parasitology of the State Health Office in 1937, systematic investigations led to the demonstration of the first case in Chile [10].

Knowledge of this disease has been progressively increasing in Chile thanks to the contribution of numerous researchers. Schenone, in 1980, [11] recognized three stages in the investigation of this disease. In the first stage, from 1937 to 1943, research focused on detecting the disease and tried to determine the endemic areas, detecting vector species and reservoirs. Important studies in this stage included those of Drs. G. Gasic, V. Bertín, *S. Massa*, and R. Gajardo Tobar. In the second stage, from 1944 to 1952, there was a more systematic study, determining the magnitude of the problem and conducting systematic surveys to determine the epidemiology better. The remarkable work of Dr. Amador Neghme was published in this period. The third stage can be established from 1953 to 1994 where there was a fruitful work of many researchers who have helped to clarify clinical aspects, congenital transmission, blood banks, epidemiology, distribution of endemic areas, the reservoir, natural history, ecology, physiology, and behavior of vectors. In this stage, the contributions of Drs. H. Schenone, W. Apt, and A. Atías and their teams stand out in epidemiological and clinical aspects. The fourth stage subsequent to 1995 had been added in which the investigation has focused more on specifying aspects not revealed in the previous periods and that has been marked by the acknowledgment of the interruption of the home transmission chain by *Triatoma infestans* which occurred in 1999. Research was focused mainly on laboratory and diagnostic improvement, on congenital and transfusion treatment and transmission, and on the study of the ecology of Chagas disease vectors and *T. cruzi*. There were important improvements in the detection of the disease, the control of blood banks and the success of domiciliary disinfestation carried out by the vector control area of the Ministry of Health. A negative effect of the declaration of the interruption of the vector transmission chain has been less interesting and resources for the study and control of this disease contributes to perpetuate it as an unattended disease.

2. Eco-epidemiology of vectors

Four species of insect vectors of Chagas disease have been described in Chile. The species responsible for the domestic cycle is *Triatoma infestans*, and the species involved in the wild cycle are *Mepraia spinolai* (**Figure 2**), *M. gajardoi*, and *M. parapatrica*.

Figure 2. *Triatoma infestans* and *Mepraia spinolai*, the main vectors of Chagas disease in Chile.

2.1. Distribution and niche

The vector *T. infestans*, a bug with characteristic yellow spots in the connexivum, has the widest distribution in Chile, between 18 and 34°S (**Figure 3**). It is found in domestic habitats, but foci of reproductive populations have also been detected in wild habitats [5–7]. In the domestic habitats, it is found in cracks of adobe walls or in "quincha" (wooden branches covered with mud), roofs of branches, and dwellings and chicken coops of the peridomiciliary zone. In sylvatic habitats it has been found forming colonies associated with plants of *Puya chilensis* (Bromeliaceae).

Mepraia spinolai is a wild species endemic to Chile with high polymorphism, with apterous females, and apterous, winged, and brachypterous males with red spots in the connexivum [12–14]. It is found between 26 and 34°S, from the sea level to 3000 m (**Figure 3**) [11]. Their habitat is made up of rocky areas such as quarries and cracks, bird nests, and animal caves [15] and sometimes peridomiciliary pens and walls and in human dwellings [16, 17].

Mepraia gajardoi is a coastal, wild species that lives between latitudes 18 and 26°S, feeding on small reptiles and mammals in the area. The males have generally blackish coloration with small reddish lateral spots in the dorsal part of connexivum. Male brachypterous and female micropterous.

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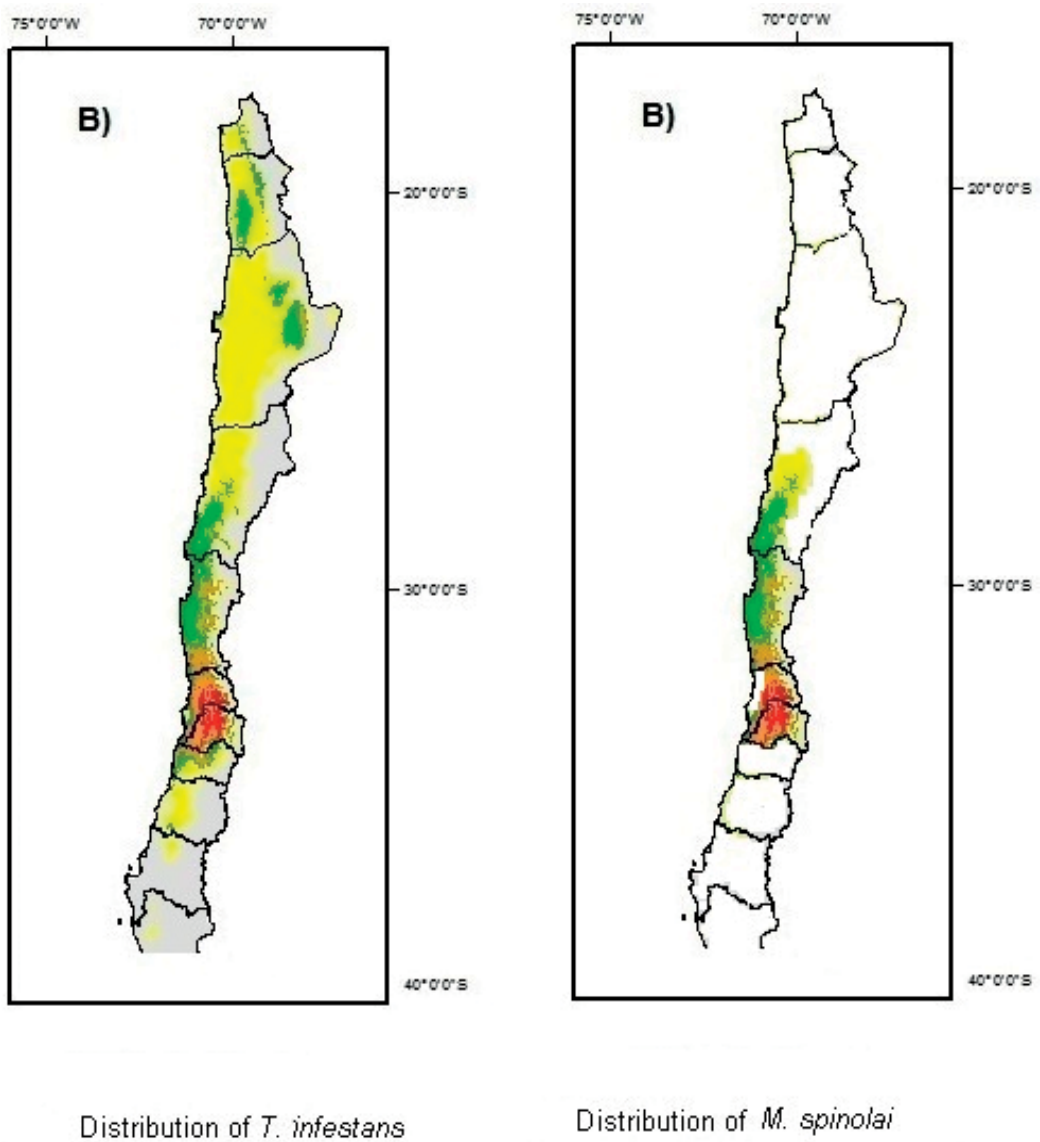

Figure 3. Approximate distribution of the two main vectors of Chagas disease (modified from [18] and [19]).

Mepraia parapatrica is a blackish species with red-orange spots in the connexivum, which only lives at 25°S. The females are micropterous, while the males are brachypterous and macropterous. It also lives on the coast, feeding on rodents and reptiles in the area.

Climate change predictions in Chile include an increase in temperature, with a gradient of higher to lower temperatures from north to south and from the Andes to the Pacific Ocean. During the 2011–2030 period, the temperature increase would be about 0.5°C in the southern

zone and 1.5°C for the north and the Chilean altiplano. A decrease in precipitation of 5–15% is expected during the same period. The distribution of *T. infestans* and Chagas disease is associated with maximum temperature and the precipitation during the driest month [18]; thus, the distribution of *T. infestans* would be little affected by climate change. This is consistent with the decrease in suitable areas proposed for *M. spinolai* in the same area and is in contrast to the high impact on the distribution of *M. gajardoi*, a species with a small distribution on the coast of northern Chile [19]. Under the assumption of niche conservatism, the latter species would suffer disappearance of its habitat, while *M. spinolai*, a species with distribution similar to *T. infestans* from 25°S southward and with similar preferred environmental conditions, would decrease its distribution area in the interior valleys while increasing its distribution on the coast [19]. Since *T. infestans* is a species residing in arid and semiarid habitats, its distribution area would not be affected significantly. However, the transmission risk of Chagas disease in this zone could be increased because of changes in metabolism, survival, reproduction, biting rates, or densities of the insects.

2.2. Ecology and population dynamics

There are very few attempts to estimate the population density of vectors. A useful measure is the so-called triatomine index used for domestic vectors such as *T. infestans*, which is defined as the number of insects captured by a person in a house in 1 hour. Schenone estimated in these species indices of 54.5 for the Antofagasta Region in 1980, 19.8 for the Atacama Region, 26.7 for the Coquimbo Region, 37.0 for Valparaíso Region, 50.5 for the Metropolitan Region, and 17.2 for the O'Higgins Region. Later, Canals et al. [20] estimated the densities of vinchucas per human in high endemic rural areas, calculating for these zones an average of 25.3 individuals/humans, with a maximum in the Coquimbo Region of 39.1 individuals/human. For the case of *M. spinolai*, the maximum triatomine index in peridomiciliary zones was 64 vinchucas/person/hour, and the estimated maximum density was 4.59 vinchucas/human [21]. The density per square meter has been estimated in this species between 15 and 364 vinchucas/m^2, with an average of 79 vinchucas/m^2 in areas of rock walls in Colina [22]. This species can be found in high densities [22–26], even in mixed colonies with *T. infestans*, and has been found in human habitations [14, 17, 27, 28]. For example, Schenone et al. [27] reported the presence of 288 specimens in 50 rural dwellings in regions III, IV, V, and Metropolitan. In this sense *M. spinolai* is a potentially dangerous species, especially in areas where the usual contact with humans occurs, as in quarry areas, and in some areas around Santiago where it is currently being urbanized, such as Colina, Lampa, and Til-Til. Also, from the perspective of its food sources, *M. spinolai* is in the phase of domiciliation [4, 29, 30], with 7.4% human blood in its diet [29, 31].

Another approximation to get an idea of the density can be obtained from the number of specimens referred to the Institute of Public Health of Chile; these come in greater proportion from the regions of Antofagasta, Atacama, Coquimbo, Valparaiso, and Metropolitan (Santiago). A recent study [32] reported that 8331 triatomines have been received between 2005 and 2016. A 73.7% correspond to *T. infestans*, 24.0% to *M. spinolai*, and 2.3% to *M. gajardoi*. Their numbers increased over this period. This high proportion of *T. infestans* could be explained because this

species, once eliminated from human habitations, has been able to recolonize wild habitats with several foci whose magnitude has not been completely clarified. From the first description of a wild focus in Chile in 2006 [5] in Calera de Tango, and in Til-Til, the new outbreaks in Sahondé, Putaendo [6] to the reports of wild foci in the cities of Valparaiso 2009, Atacama 2014, and Coquimbo 2015 [7].

The data reported by the Ministry of Health show the proportion of infested dwellings (home infestation) in 1999 and annual data since 2010 and divide the information into the percentage of colonized houses, that is, colonies with evidence of active reproduction, the individual visits to houses that receive adult individuals but without colonization, and the total (the sum of both). Household infestation has clearly decreased in this period, but the proportion of houses with individual visitors has increased. Currently, domiciliary infestation is estimated at 0.05% [32].

The vinchucas in Chile are long-lived species, surviving around 18 months in the laboratory [12, 13, 24, 33]. Their development is strongly affected by temperature and relative humidity like all triatomines [34]. The development of *T. infestans* completely ceases at temperatures below 16°C, and temperatures above 40°C are lethal [35]. The effect of relative humidity (RH) and its importance in molt periods has been discussed [36]. Combinations of variable ranges of low temperature and RH affect the maturation of *T. infestans* and *M. spinolai* (**Table 1**). While at constant temperature and humidity, the pre-imago period consisting of five nymph states (N1–N5) is 6.1 months in *T. infestans* [37]; this increases to 14.3 months in variable

	T. infestans	**EC**	***M. spinolai***	**EC**
Fecundity (eggs/female/week)	1.9	(15–32/40–90)	0.25	(28/70)
	1.0	(25/75)		
	5.4	(26/60)		
Egg viability (%)	74.6	(17/70)	27.7	(24/73)
	93.7	(24/73)		
Mortality rate (death/individual/day)	0.0083	(25/75)	0.0055	(24/73)
	0.0070	(15–32/40–90)		
Reproductive number (Ro)	1.36	(15–32/40–90)	22.9	(28/70)
	25.04	(26/60)		
Generation time (months)	14.7	(15–32/40–90)	13.2	(28/70)
	7.1	(26/60)		
Intrinsic rate of growth (r)	0.021	(15–32/40–90)	0.24	(28/70)
	0.45	(26/60)		

(EC: Temperature (°C)/Relative humidity (%)). References. Canals et al. [20].

Table 1. Some population parameters of *T. infestans* and *M. spinolai* in different environmental conditions.

environments [24, 38, 39]. In the main wild vector, *M. spinolai*, this period is between 9 and 10 months under constant conditions [12, 33], while in environments with RH and T variability, only nymphs are obtained up to N5 status. With greater restriction at low temperature and humidity, they only reach the N2 status in 12 months [24]. The arrest of secondary development in unfavorable conditions (induced diapause) has been observed in both species [24, 33, 40]. Also, some parameters like the net reproductive rate (R_0) are strongly affected. Both species present survival curves with exponential decays and similar mortality rates [24, 28, 33, 41]. The mortality and fertility rates in *T. infestans* present periodic variations during the year even under relatively constant environmental conditions. For example, under laboratory conditions the mean fecundity of *T. infestans* varied from 0.39 eggs/female/week in autumn to 1.63 eggs/female/week in spring, with maximum values of 7.59 eggs/female/week in spring and 5.83 eggs/female/week in summer. Maximum mean mortality and fertility rates were in spring and summer [42].

2.3. Ecophysiology and behavior

Triatoma infestans is a diurnal insect, while all species of the genus *Mepraia* are diurnal. In the laboratory *M. spinolai* moves between 15 and 42°C with a preferred temperature of 24.8°C [43]. *T. infestans* moves between 18 and 42°C with a preferred temperature of 24.2°C [43]. Other authors have found that *T. infestans* preferred temperatures between 26 and 27°C [44] and variations from 28 to 29°C at the beginning of the night, immediately after the ingestion of blood, but 25°C 12 days later [45, 46]. The synchronization of activity patterns to the L/D cycle is similar to other triatomines [47–51]. Other activities synchronous to the L/D cycles are the rhythms of oviposition [52], molt [53], hatching [46], and aggregation [54]. The stimulus for orientation and approach to a potential prey is temperature, displaying the classic pattern of antennal movements-locomotor movement and extension of the proboscis [55, 56]. *T. infestans* decreases the frequency of antennal movements when it is within 30 to 15 cm of its prey, whereas *M. spinolai* always maintains a low frequency, which may be due to a greater distance of perception in this species [57]. Once in contact with its prey, *T. infestans* introduces its proboscis, sucking blood, being able to increase its weight from four to six times in a single ingestion [38, 39, 57]. The volume of blood ingested is estimated between 30 and 70 ml when fed on a species such as *Mesocricetus auratus* [58]. The bite frequency in *T. infestans* has been estimated at 0.0754 bites/day [21]. The bite frequency of *M. spinolai* has been estimated at 0.155 bites/day, with volumes of blood ingestion between 20 and 160 mg. The intake volume has been found to be inversely correlated with the weight prior to intake, which is an indicator of nutritional status. Thus, the volume of the intake is related to the time elapsed since the last feeding and to the degree of distension of the abdomen [59].

T. infestans emits its dejections during the feeding act [60], which takes on average 19.5 min [38, 39, 57], usually between 3 and 4 min from the start of feeding, while in *M. spinolai* this latency is 24.4 min and not necessarily on the prey, which decreases the probability of transmission [38, 39, 57]. The bite rate and the latency between the start of the bite and the dejection are affected by the presence of the *T. cruzi* parasite in the vector; *M. spinolai* showed a higher frequency of bite and a lower latency of dejection in infected individuals [59].

The feeding spectrum of *T. infestans* in Chile was obtained by means of ag-ac reactions and double gel diffusion [60]. The diet of this species is made up of humans 68.4%, cats 6.1%, dogs 3.2%, rabbits 5.9%, rodents 1.6%, Artiodactyla 0.3%, birds 7.2%, and amphibians 0.5%. The proportions for *M. spinolai* are humans 7.4%, cats 3.7%, dogs 12.3%, rabbits 53.1%, rodents 9.9%, Artiodactyla 12.3%, and birds 1.2% [29]. The amplitude of the trophic niche of *M. spinolai* is greater than that of *T. infestans*, and they have a niche overlap of 0.229 [29]. An increase in the amplitude of the niche in times of greater heat has also been demonstrated [29]. Introducing the information of the food profile of 15 species of triatomines with multivariate analysis techniques has shown a clear separation between species of domiciliary habits and peridomiciliary, wild, and stenophagous specialists; *M. spinolai* is among the peridomiciliary species [4]. In a later study, this species was located between wild and peridomiciliary [30].

The average percentages of *T. cruzi* infection in the vectors (trypano-triatomine indices) are *M. gajardoi* 16.92 ± 17.77%, *M. spinolai* 20.88 ± 11.51%, and *T. infestans* 41.83 ± 13.23% [32]. The trypano-triatomine indices of *M. gajardoi* have remained stable over time, although based on a small number of individuals. The same occurs with the *M. spinolai* indices, but *T. infestans* shows an increasing trend.

The high trypano-triatomine indices in *T. infestans* are interesting, since a decrease of these indices was expected once these insects were removed from human habitats. Thus, for example, in Uruguay, the indices were drastically reduced as a result of vector control actions [61]. An explanation for the lack of a decline in these indices in Chile could be that despite the change in diet that involves the elimination of the human environment, *T. infestans* can find rodents and other species with high infection rates that make high trypano-triatomine indices persist. This may be true, since there is a great diversity of infected wild, domestic, and peridomestic mammals, some reaching levels of infection higher than 10% [15, 62], but this is also probably true in Uruguay.

3. *Trypanosoma cruzi* lineages

Lineages of *T. cruzi* have been recognized for a long time. Initially, isoenzymes that differ in gel electrophoresis were classified as zymodemes Z1 and Z2, the first one mainly associated with the wild cycle and the second one with the domestic cycle [63]. Subsequently, biochemical and genetic differentiation was carried out, and two lineages, TcI corresponding to Z1 and TcII corresponding to Z2, were proposed. However, the first TcII was divided into five subgroups a, b, c, d, and e, where TcIIb corresponded to Z2, TcIIa to the new zymodeme Z3 and TcIIc, and d and e to hybrids [64]. Subsequently, subgroups were also recognized in TcI: a, b, c, and d [63, 65]. Currently, it has been simplified into six subgroups, from TcI to TcVI, where TcI corresponds to Z1, TcII to TcIIb (Z2), TcIII to TcIIc, TcIV to TcIIa (Z3), TcV to TcIId, and TcVI to TcIIe.

Only TcII and TcVI have been found in *M. gajardoi* in Chile [66, 67], TcI and TcII in *M. spinolai* [66] and subsequently TcI, TcII, TcV, and TcVI [68], while in *T. infestans*, the most important

lineage is TcI circulating in wild (93.3%) and domiciliary (100%) individuals. TcII, TcV, and TcVI have also been detected mainly in nymphs, suggesting differential adaptation of *T. cruzi* lineages between nymph states [69].

It has been proposed that in small wild mammals TcI and TcII would be associated with marsupials and placental mammals, respectively. However, the TcI, TcII, TcV, and TcVI lineages have been detected in the rodent *Octodon degus* in Chile [26, 70]. The same lineages have been detected in wild *Oryctolagus cuniculus* [71], and in 117 individuals of different infected species, TcI, TcII (TcIIb), TcV (TcIId), and TcVI (TcIIe) have been detected with frequencies of 38, 41, 26 and 9%, respectively, in wild mammals. In peridomestic mammals the frequencies of these lineages were 29, 33, 43, and 14%, respectively. More than one lineage was found in 31% of the individuals analyzed, without specific association with marsupials [72, 73]. Thus, it seems that the information on the wild mammal reservoir and the vectors *T. infestans* and *M. spinolai* is quite consistent in pointing to TcI, TcII, TcV, and TcVI as the main circulating lineages, although more studies are still missing in the other wild vectors of the genus *Mepraia*.

4. Reservoir

The animal reservoir is very extensive; it is constituted by mammals of the peridomestic environment such as dogs, cats, goats, rabbits, sheep, horses, donkeys, and cattle, some carnivores, numerous rodents, and some small marsupials. Infection in mammals by *T. cruzi* has been described in around 150 species in America [74] and is widespread in numerous orders; the most important reservoirs are dogs, cats, and goats, due both to high infection rates and to their mobility which establishes a bridge between domestic and wild cycles. Proportions of infection by *T. cruzi* have been reported 14.5% in dogs, 10.7% in cats, 9.4% in goats, 12.1% in rabbits, and 4.8% in sheep [75]. Current studies with PCR in wild mammals have reported percentages of infection between 26 and 59% in the species *Capra hircus* (goat), *Thylamys elegans* (marsupial), *O. degus*, *Phyllotis darwini*, and *Abrothrix olivaceus* (rodents) [72] and large increases in interannual infection rates from 300–400%, which could be explained by a delayed response to the El Niño weather phenomenon after the emergence of small rodents [25].

5. Distribution of the disease

Chagas disease extends in America from the Southern United States at parallel 35°N to southern South America at parallel 34°S on the Chilean side and 45°S on the Argentine side. In this zone, 21 countries report active transmission, with current prevalence very difficult to assess since most of the studies refer to the prevalence in endemic rural areas [15]. The median prevalence in these areas is approximately 8.15%. The lower extreme values probably represent Chagas disease in controlled or urban areas and the maximum Chagas disease prevalence in hyperendemic areas without protection of health systems. Currently, emergence of Chagas disease in Europe has been reported through immigrants and subsequent congenital

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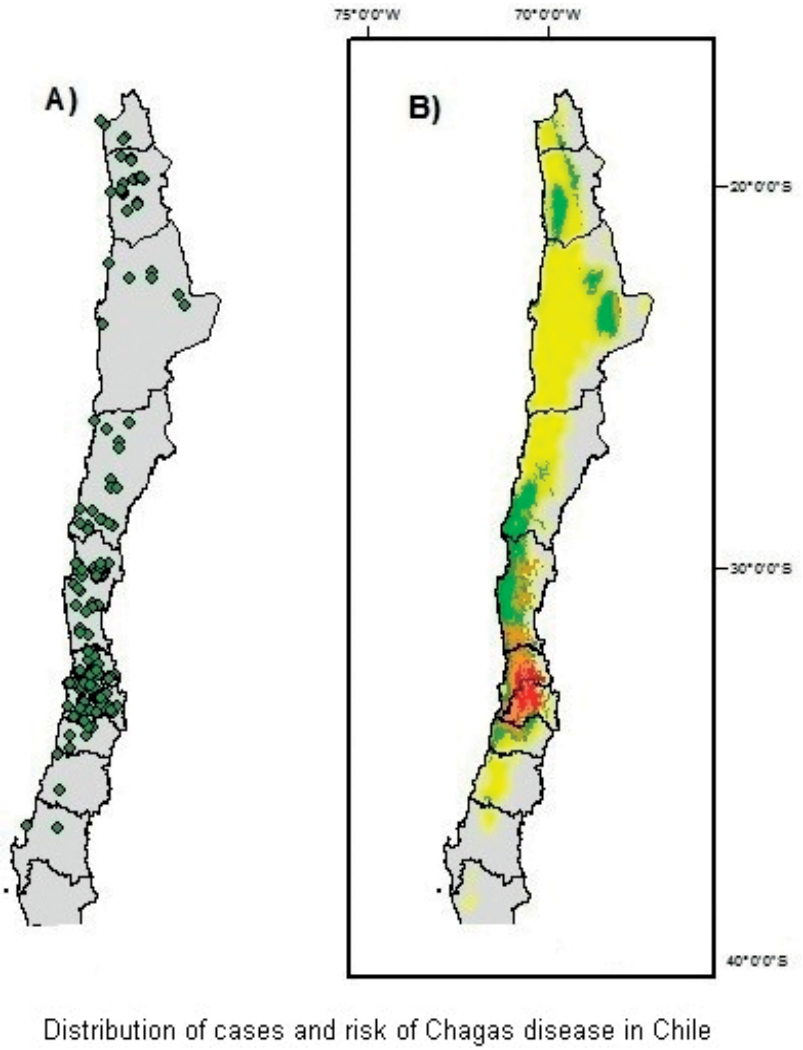

Figure 4. Distribution of cases and the risk of Chagas disease in Chile (modified from [18]).

transmission. For example, Basile et al. [76] reported more than 4000 cases diagnosed in different countries, with the highest number of cases in Spain, but Strasen et al. [77] proposed around 95% underreporting. These latter authors indicated that there could be between 14,000 and 180,000 cases in this continent.

In Chile, Chagas disease is distributed between the Arica and Parinacota Region (18°30'S) and the O'Higgins Region (34°36'S) (**Figure 4**), coinciding in large part with the distribution of the vector domestic *T. infestans* [18]. The highest incidence rates are recorded between Antofagasta and Coquimbo, with Antofagasta, Coquimbo, and Metropolitan Regions concentrating approximately two-thirds of the reported cases (**Table 2**).

Administrative region	Cases	Incidence rate (cases × 10^{-5})
Arica and Parinacota	57	23.8
Tarapacá	64	19.0
Antofagasta	223	35.8
Atacama	67	21.4
Coquimbo	292	37.9
Valparaiso	210	11.5
Metropolitan	313	4.3
O'Higgins	20	2.2
Maule	2	0.2
Bío Bío	11	0.5
Araucanía	1	0.1
Los Ríos	2	0.5
Los Lagos	0	0.0
Aisén	0	0.0
Magallanes	0	0.0
Total country	**1262**	**7.0**

Table 2. Cases and incidence rates of Chagas disease in Chile (2015) (health ministry).

6. Prevalence and incidence

Chagas disease is one of the main neglected diseases that affects the Americas and has now become an emerging disease in some parts of America and Europe [78–81], which has even been compared to the early stage of the HIV/AIDS epidemic [79]. The annual incidence varies between 28,000 and 56,000 people and between 10,000 and 14,000 deaths per year [79], affecting 6–11 million individuals [82] with 65–100 million people at risk in the world [78–80, 83]. The population at risk in Chile is 873,415 people [80, 81]. The latest national health survey (ENS) reports a prevalence of *T. cruzi* infection of 0.7% of the population, with a prevalence of 1.5% in rural areas and 0.6% in urban areas [81], and ministerial reports indicate that home infestation by *T. infestans* is practically nonexistent [80], which contrasts sharply with the data reported in the 1980s and 1990s. For example, between 1937 and 1980, a general prevalence of 16.7% was reported in endemic areas, with a maximum of 43.6% in the Coquimbo Region [11], which did not vary significantly between 1982 and 1985 [84], whereas between 1982 and1989 differences were already reported between rural areas with prevalence of 16.7% and urban areas with prevalence of 1.9% [16]. The same occurs with domiciliary infestation, in which previous reports indicated infestations between 26.8 and 33.2% of dwellings in endemic areas between Arica and the O'Higgins Regions [11, 80].

A study covering 60 years of the disease in Chile of patients referred to the University of Chile and studied through xenodiagnosis revealed an average prevalence of 9.35 ± 0.1% [32]. This prevalence value of 9.35% should be considered as the level for highly endemic zones. It is in the range between 8 and 12% reported by Apt and Reyes in 1986, also based on xenodiagnosis,

and is similar to the estimated value for Latin America. This value does not present any variation in the 65 years of study, regardless of the changes in the health systems or interruption of the chain of transmission. Accurate estimation of the prevalence of Chagas disease is a difficult task since it depends not only on the sampling characteristics (random, stratified, etc.), sample size, and bias but also on the screening method used, which in the case of Chagas disease can be by xenodiagnosis, ELISA, immunofluorescence, and/or Western blot.

The values reported by the 2009–2010 ENS based on 4650 volunteers using IgG ELISA show 0.7% average prevalence, with 1.5% in the rural population and 0.6% in the urban population. These values are surprisingly similar to those reported for the urban population in 1982–1989 [85]. In this sense, the prevalence shows an evolution toward the values of an urbanized population.

The incidence in Chile shows a progressive rise from 1985 onward (**Figure 5**), without a slope change attributable to the interruption of the vector transmission chain. On the other hand, there is no appreciable effect attributable to the improvement in detection. For example, supreme decree 158 of 2004 in Chile declares Chagas disease as a notifiable disease, which is not reflected in the trend curve; later in 2008 circular four instructs blood banks to investigate the presence of *T cruzi*; law 1839 of 2009 stipulates the national policy of blood services; and in 2011 in circular B51, number 17 stipulates the surveillance of Chagas disease and establishes ways to record and inform the health authority [80, 81]. The incidence before 2009 had an average value of 2.7 ± 1.29 per 100,000 inhabitants, whereas after 2009 the average is 7.3 ± 2.02

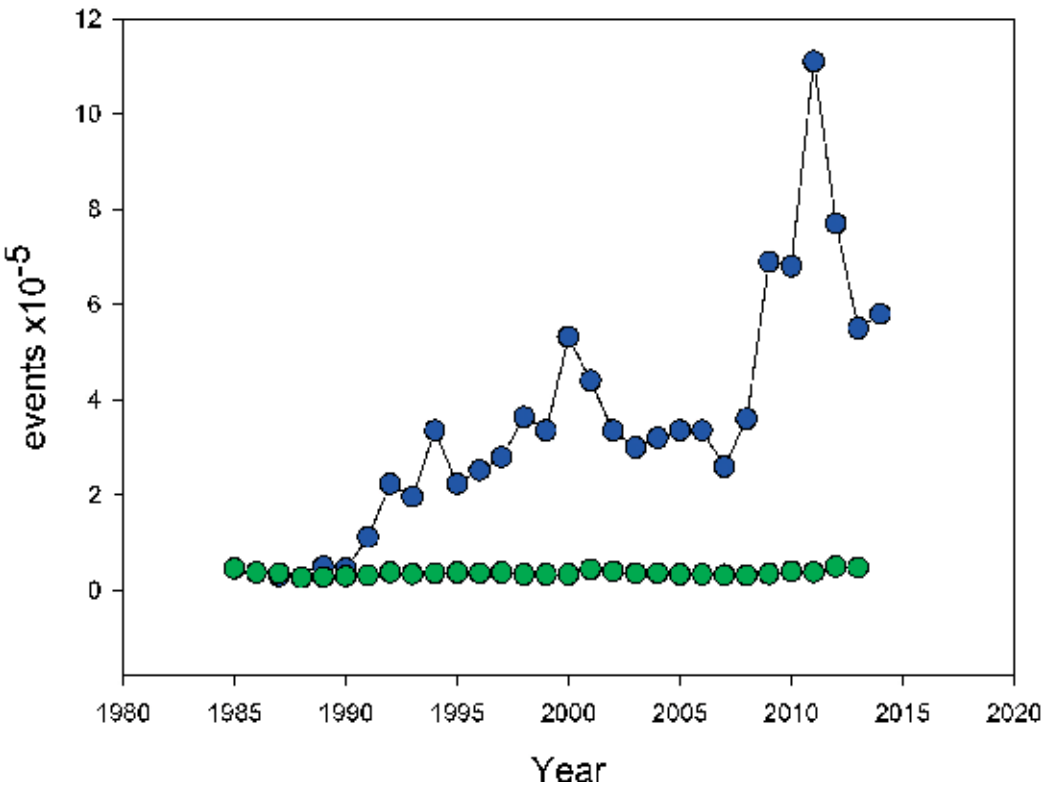

Figure 5. Incidence (blue) and mortality rates (green) of Chagas disease in Chile (Health Ministry).

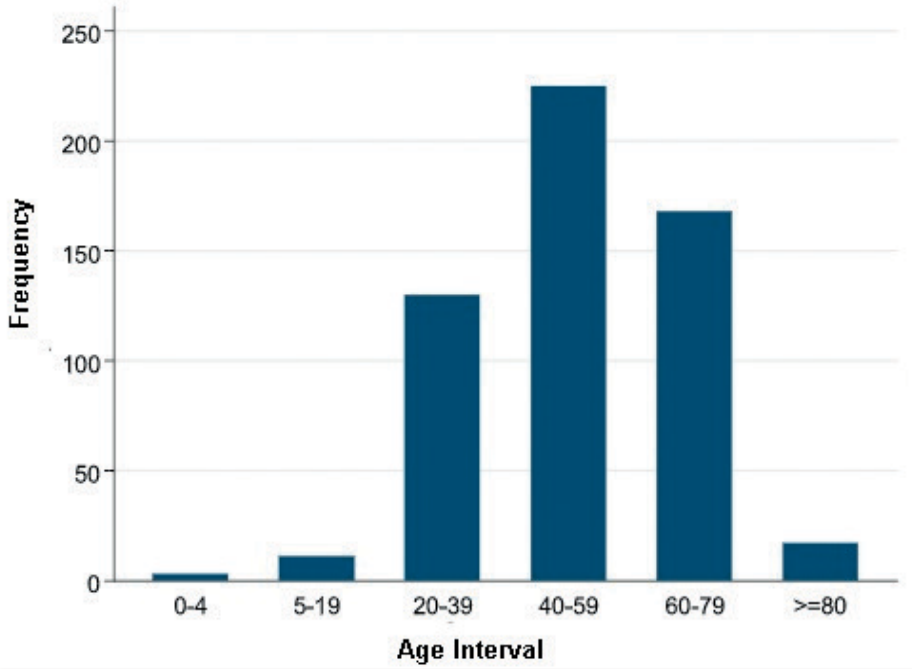

Figure 6. Age distribution of Chagas disease in 2010 (Health inistry).

per 100,000 inhabitants [32], staying relatively stable. One explanation for this temporal dynamics is that the progressive increase was not explained by a particular milestone but by a progressive improvement in the notification attributable to better staff preparation and a better notification system. Another possibility that cannot be ruled out is that this increase is real, and in this case, it should have an impact on mortality rates in the long run, a fact that is not yet evident. If the relative stability detected since 2009 persists, it would indicate that it would be reaching an adequate estimation of the endemic equilibrium, as the models predict [23, 86].

Mortality shows stability over the years (**Figure 5**). The average mortality rate is 0.36 ± 0.55 per 100,000 inhabitants [32]. Chagas disease affects the entire population in Chile, especially people of working age, with the highest rates between 30 and 65 years of age (**Figure 6**) [81].

7. Transfusion and congenital Chagas disease

The seroprevalence of *T. cruzi* in blood donors has been studied in Chile since the 1960s, with a national average of 3.7% reported between 1962 and 1988 [15]. Subsequently, this value has decreased; it was estimated between 0.5 and 1.6% according to information from the ISP between 2000 and 2005, estimated with the ELISA IgG method, and confirmed with IFI [87]. Currently, the seroprevalence of *T. cruzi* in blood donors is estimated at approximately 0.6%. The latest studies in the blood bank of the Clinical Hospital of the University of Chile were 0.4% positive out of a total of 24,568 [87].

A meta-analytic study that considered 13 case studies and 51 observational studies in ten countries estimated the rate of congenital infection was 4.7% with a CI of 3.4–5.7%. This means that in a population of mothers infected with *T. cruzi*, 4.7% of newborns are congenitally infected [88]. In this study it is proposed that in endemic areas the rate is 5% in endemic countries but lower in non-endemic countries (2.7%) and proposes a rate of 2.5% for Chile. In the same year, in a study conducted in Choapa, a highly endemic area, this rate was estimated at 4.7% [89].

8. The reproductive number (R_0)

The transmission of Chagas disease depends mainly on vector and congenital transmission. Accidental and transfusion transmissions are very rare and oral transmission only occurs in particular areas where the contact between man and nature is very close. The reproductive number of an infectious disease (R_0) corresponds to the average number of secondary infections produced by an infected individual. Thus, if R_0 is greater than 1, the disease is established in the population, and if R_0 is less than 1, the disease disappears. In the case of Chagas disease, the main component of the reproductive number is given by vector transmission, and when this is cut off, congenital transmission alone is incapable of perpetuating the disease [86, 90]. There are very few attempts to estimate the R_0 of this disease, an exception being the study by Massad [91], who estimated it at $R_0 = 1.25$ for a region of Brazil. Other estimates based on plausible approximations of many parameters obtained values of $R_0 = 7$ in Colombia [92] and $R_0 = 2.86$ [23] and $R_0 = 1.52$ in Chile [86]. However, recently in a spatial approach, a median value of $R_0 = 1.02$ has been proposed, with an approximate spatial variability between 0 and 5. The reproductive number could vary in different climate change scenarios as consequence of changes in entomological parameters such as biting rate, density, and mortality rate, increasing the Chagas risk area between 13 and 18% [93].

9. Conclusion and the way forward

Chile is in a privileged situation. It has only one domestic vector and probably only one wild vector of epidemiological importance, and the chain of domestic vector transmission by *T. infestans* is cut off. The wild reservoir is made up of small rodents, and the circulating lineages of *T. cruzi* are similar to the rest of America. However, there are some problems that persist: (1) The peri-anthropic reservoir is diverse and has bridging animals between the wild and domestic cycles that facilitate the spread of Chagas disease, such as dogs and goats; (2) housing and education conditions in Northern Chile are limited; (3) there are no control or education campaigns on wild vectors, and human dwellings are built on their territory. In addition there are wild foci of *T. infestans* and vector home intrusion; (4) prevalence, incidence, and trypano-triatomine indices still do not decrease; and (5) climate could change the epidemiological situation.

Given the obvious opportunity to eradicate Chagas disease in two generations, as predicted by the models, the way forward should be focused on (1) strengthening education campaigns of the population; (2) strengthening the monitoring of housing and peridomestic animals; (3) detection, study, and control of wild foci; and (4) reinforcing the study of the effect of climate change on the epidemiology of Chagas disease.

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Author details

Mauricio Canals Lambarri[1,2]*, Andrea Canals Cifuentes[3,4], Salvador Ayala[5], Valentina Tapia-Garay[1] and Dante Cáceres Lillo[1]

*Address all correspondence to: mcanals@uchile.cl

1 Programa de Salud Ambiental, Escuela de Salud Pública, Facultad de Medicina, Universidad de Chile, Chile

2 Departamento de Medicina, Facultad de Medicina, Universidad de Chile, Chile

3 Programa de Bioestadística, Escuela de Salud Pública, Facultad de Medicina, Universidad de Chile, Chile

4 Dirección Académica, Clínica santa María, Chile

5 Departamento de Asuntos Científicos, Instituto de Salud Pública, Chile

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Chapter 7

Chagas Cardiomyopathy: Role of Sustained Host-Parasite Interaction in Systemic Inflammatory Burden

Rodolfo A Kölliker-Frers, Matilde Otero-Losada,
Gabriela Razzitte, Mariela Calvo,
Justo Carbajales and Francisco Capani

Additional information is available at the end of the chapter

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Abstract

The economic and social burden associated with Chagas disease morbidity and mortality is regrettably large in Latin America causing more deaths than does any other parasitic disease. Inflammatory dilated cardiomyopathy is, by far, the most important clinical consequence of *Trypanosoma cruzi* infection. The insidious persistence of this parasite determines chronic myocarditis progression. The clinical outcome is multifactorial and depends on the particular parasite strain and virulence factors, the infective load and route of infection, the parasite ability to by-pass the protective immune response, the intensity and type of immune response during the acute infective phase, and the host genetic background. From the immunological viewpoint, host control of *T. cruzi* has been shown to depend on both humoral and cell-mediated adaptive responses and from the innate immune system. In this review, we discuss the most relevant literature conveying information on the relevance of identifying a subset of systemic inflammatory molecules as potential markers of cardiovascular risk morbidity and mortality in patients with Chagas disease. Concurrently, a direct role for the parasite in the perpetuation of myocardial inflammation is substantiated. Ultimately, host-parasite interactions determine the course of the ongoing systemic inflammation and the perpetuation of myocardial inflammation in genetically predisposed patients.

Keywords: Chagas disease, myocarditis, cardiomyopathy, inflammation, immune system, cardiovascular risk

1. Introduction

Chagas disease is a parasitic disease caused by the *Trypanosoma cruzi* that affects over 12 million people in Central and South America, causing more deaths than any other disease of its kind. Large migrations of infected people from the endemic areas are usually observed mainly in the United States of America and Europe. The most frequent cardiac complications of chronic Chagas disease are left ventricular dilation and dysfunction, aneurysm, congestive heart failure, thromboembolism, ventricular arrhythmias, and sudden cardiac death. Chagas disease diagnosis is based on serology, namely immunopositivity for immunoglobulin G antibodies to *T. cruzi.*

Inflammatory dilated cardiomyopathy is, by far, the most important clinical consequence of *T. cruzi* infection. The chronic chagasic cardiomyopathy (CCC) is roughly progressive, and its treatment does not differ from that of any other non-chagasic cardiomyopathy in the absence of strong evidence. Clinical symptoms usually include dyspnea, palpitations, precordial pain, syncope and eventually, sudden death.

Epidemiological data show high mortality and morbidity resulting from the cardiovascular disease in chagasic patients. However, there are no hints to suspect cardiovascular risk in the silent period of the disease (asymptomatic form). Noticeably, inflammatory factors are upraised during the silent period of the Chagas disease. Like atherogenesis, immune-inflammatory-mediated effector mechanisms commanded by Th1/Th17 cells are involved in the pathophysiology of Chagas disease, having a similar histological hallmark which includes Th1/Th17 cells, macrophages, and a characteristic cytokine profile.

Host control of the *T. cruzi* appears to depend on both humoral and cell-mediated adaptive responses, and on the innate immune system as well [1]. The cytokines strongly activate multiple functions relevant to cardiovascular homeostasis. According to the literature on the subject, there is robust evidence of a systemic upraised level of inflammatory mediators in patients with Chagas disease suggesting that the interplay between the parasite aggressiveness and the host immune response might have a key role in the perpetuation of myocardial inflammation.

The role of the parasitemia is more controversial associated with immunosuppression, disease reactivation, and disease severity. Due to the arousal of a strong and specific immune response against the parasite, nearly two-thirds infected people become protected and may stay in an indeterminate stage of the infection characterized by low parasitemia level for 10 or even up to 40 years after the prime infection. The other one-third infected people, however, develop symptoms, entering the symptomatic chronic stage of infection typically characterized by cardiomyopathy.

The antigenic stimulation though persists all over the chronic stage. Then, the clinical outcome depends on multiple factors like parasite persistence, the particular *T. cruzi* strain, the infective load and virulence factors, the route of infection and sidestepping the host immune response by the parasite, the strength of the immune response at any time, and of course, genetic predisposition [2]. In fact, the sustained parasite-host immunity interactions induce systemic inflammatory mediators' upregulation and fibrosis, both crucially involved in myocardial tissue damage and the resulting disturbances in the cardiac conduction system, mainly affecting the autonomic ganglia, nerves, and the microvasculature.

Unlike the study of the classical risk factors, research studies on how inflammatory status affects the development and determines the progression of cardiomyopathy have not yet identified or clinically validated relevant biomarkers [3]. Interestingly, secluded evidence suggests that the inflammatory status might be associated with increased morbidity and mortality. Deepening our understanding of the pathophysiology of Chagas disease, it will make way to identifying new molecular targets for the design of CCC prophylactic vaccines and therapeutic drugs.

2. Cardiomyopathy genes

Dilated cardiomyopathies (DCM) are characteristically defined by the presence of left ventricular dilatation, and contractile dysfunction [4]. Genetic mutations that involve genes encoding the cytoskeleton sarcomere, nuclear envelope proteins and others account for up to 35% of the total cases. Hypertrophic cardiomyopathies (HCM) and dilated (DCM) cardiomyopathies are heart muscle diseases related to genes variants encoding sarcomere proteins [5]. Among these proteins, the most common are the β-myosin heavy chain (MYH7) [6], the cardiac myosin-binding protein C (MYPBC3) while the myosin light chain (MYL3) and the regulatory myosin light chain (MYL2) are rare [7]. Certain variants in sarcomere genes also cause DCM, albeit less frequently. Of note, variant location does not absolutely predict whether it will trigger HCM or DCM.

Heart failure associated with cardiomyopathies is often caused by mutations in sarcomeric genes, resulting in contractile dysfunction and cellular damage. This may stimulate the production of a robust proinflammatory response. Intriguingly, flow cytometry analysis revealed a significant increase in total macrophages and classically activated proinflammatory (M1) macrophages in DCM hearts as compared with normal hearts. Serum cytokine analysis in dilated cardiomyopathy hearts showed a striking increase in interleukin IL-6 in rodents. Furthermore, RNA-seq analysis revealed the upregulation of inflammatory pathways in DCM hearts. Altogether, these data indicate a robust proinflammatory response in DCM hearts, likely in response to cellular damage triggered by an MYBPC3 mutation and the resultant contractile dysfunction [8]. In addition, other genes have been implicated in DCM, particularly the monocyte chemoattractant protein-1 gene polymorphism [9].

The epigenetic factors that contribute to myocarditis include consumption of alcohol or drugs, exposure to toxins, and metabolic and endocrine disturbances. The typically presenting symptoms are related to congestive heart failure, and can also include circulatory collapse, arrhythmias, and thromboembolic events [10].

3. Anatomopathological findings of Chagas cardiomyopathy

Chagas disease is typified in the WHO classification within the group of specific diseases of the myocardium. Alternatively, the denomination of cardioneuropathy has been proposed to express the frequent and severe importance of the autonomic affectation and the consequent dysautonomia associated with the functional and clinical alterations. The observed

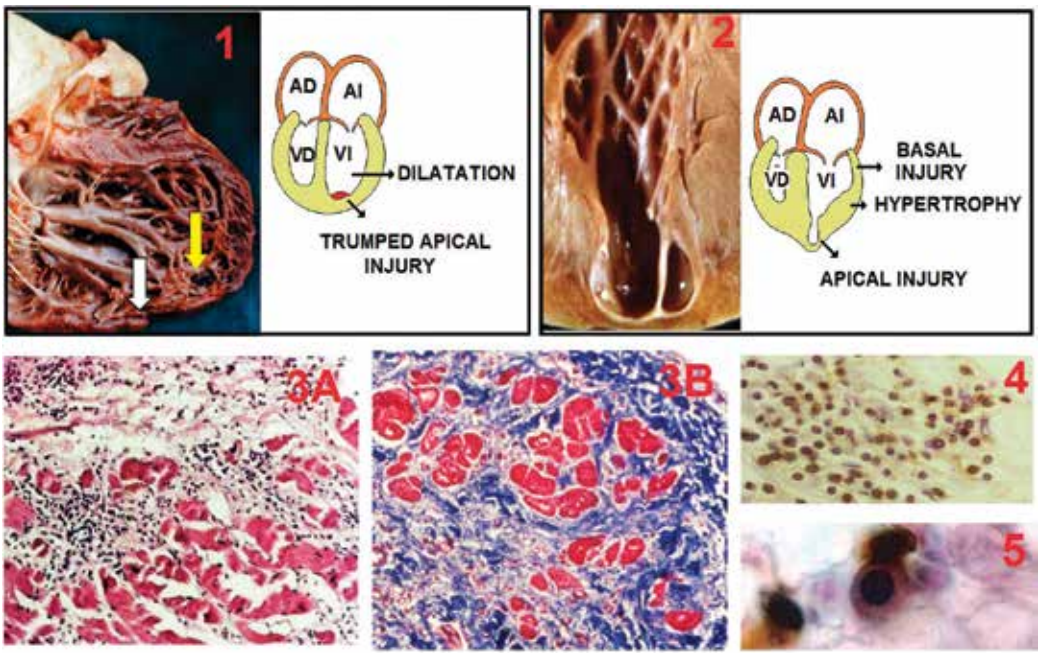

Figure 1. Anatomopathological findings. Figure courtesy of Dr. José Milei. Panel 1, left. High-grade heart dilatation. Thining of the apical wall of the left ventricle (white arrow) and cavitary thrombus (yellow arrow). Panel 1, right. Schematic representation. Panel 2, left. Characteristic apical aneurysm. Panel 2, right. Schematic representation. Panels 3–5. Histological features. Panels 3A and 3B. Microscopically, myocardial lesions consisted of a chronic inflammatory process with fibrotic scars and extensive mononuclear infiltrates. Panel 4. Immunostaining for T lymphocyte. Positive cells express CD45R0 antigen (brown); specialized myocardial cells have almost disappeared. Extensive mononuclear infiltrate, the majority of them being T lymphocytes. X20. Panel 5. Double immunostaining for the simultaneous demonstration of T lymphocytes (CD45RO) and macrophages (CD68). T lymphocytes (brown) in close contact with a macrophage (pinky cytoplasm). X1000.

macroscopic alterations are: (a) cardiomegaly (more than 500 g weight) and (b) characteristic apical lesion associated with localized parietal thinning, rarely presenting as posterior basal or parietal, or an apex aneurysm. Some authors propose two characteristic morphological types of chronic chagasic cardiopathy: "type I" or concentric, is characterized by a predominance of left ventricular hypertrophy with little dilatation or none, and circumscribed closed apical lesion and "type II" or eccentric, characterized by a wide dilatation and opening of the apical zone and frequently associated with thrombosis. Pathological evolution can progress from the concentric to eccentric type (**Figure 1**).

4. Clinical expression of the acute phase of Chagas disease

The Chagas disease presents two phases [10]. The initial acute phase, lasting for about 2 months after the infection and characterized by high parasitemia and parasite invasion to the tissues, is asymptomatic or shows mild unspecific symptoms. In this phase, symptomatic patients usually develop characteristic skin lesions or unilateral purplish swelling of the lids,

usually known as the Romana's sign, and can present fever, headache, difficulty in breathing, lymphadenitis, vague myalgia, and abdominal or chest pain. In this phase too, immunosuppression, and the decreased inflammatory response result in increased parasite load in locally infected tissues upon generalized tissue invasion [11].

5. Clinical expression of the chronic phase of Chagas disease

To date, regardless the chronic phase be asymptomatic or symptomatic with cardiological alterations, the prognosis on the eventual development of heart disease in a given patient is not feasible.

5.1. Clinical asymptomatic expression of the chronic phase

The reason most patients with Chagas do not develop heart disease is uncertain. The first studies suggested that asymptomatic patients without clinical and cardiological alterations and the general population had a similar cardiovascular risk, but over time, epidemiological data suggest a higher risk than presumed.

Collectively, the studies highlight the importance of studying the early inflammatory parameters indicative of early cardiovascular damage, evaluating potential implication on morbidity and mortality, and prognostic and therapeutic relevance.

5.2. Clinical symptomatic expression of the chronic phase

The clinical expression of the chronic phase usually manifests as alterations in the cardiac conduction system, mainly arrhythmogenesis and dysrhythmias derived from the fibrotic and atrophic lesions compromising the AV nodule, and the His bundle and branches, and autonomic dysfunction as well. Dysautonomia may affect blood pressure and cardiac frequency, eventually leading to orthostatic hypotension, syncope, and heart failure. Myocardial alterations are associated with chronic inflammation, fibrous hypertrophy, fibrosis, myocytolysis, tissue depletion of neurons, and vascular damage contributing to sudden death or heart failure. Some clinical features may result from upstream molecular mimicry and cardioactive autoantibodies production. Patients with chagasic cardiomyopathy produce anti-ß1 and -ß2 adrenergic, and anti-M2 cholinergic autoantibodies in the heart. These autoantibodies, originally directed against the parasite, would indistinctly recognize similar antigenic determinants in the host, a phenomena known as mimicry.

6. Factors that define blood inflammatory outcome in Chagas disease

The sustained systemic inflammatory burden may result from many host-parasite interactions, whereby the interplay of the natural and adaptive immune host response with the parasite will result in a varying degree of tissue damage, host aggression, and clinical outcome.

Then, the perpetuation of the Chagas disease inflammatory phenotype [10] is critical in understanding the complexity of the clinical outcome given that immunological homeostasis in chronically infected hosts could be spoiled by both parasite and host immune molecules and cells [10].

6.1. Factors that promote parasite evasion and persistence

The presence of the parasite [11] or its products like DNA, and other constitutive molecules in blood, myocardium, and the autonomic tissues lead to sustained immunological stimulation and *T. cruzi* evasive strategies. Today, the DNA amplification by polymerase chain reaction and other sensitive assays allow detecting parasites or their components in chronic symptomatic patients.

6.1.1. Parasitemia and sustained antigen stimulation

There is scarce evidence linking Chagas seropositivity with cardiovascular events in asymptomatic patients [9]. The perpetuation of *T. cruzi* antigens in cardiac tissue and the immune-mediated dysautonomia might be implicated in the cardiovascular pathogenesis [12] being responsible for the asymptomatic-to-the *cardiac* phenotype transition in Chagas disease. Different studies have shown the relevance of effector or memory CD4/CD8 lymphocytes and their effector cytokines not only in controlling parasite multiplication over the course of acute and chronic infection, but also participating in the pathogenesis of chronic Chagas disease [12]. The Th1 lymphocytes are critical in the control of Chagas disease during the acute stage unlike the chronic phase when they could be harmful. The adoptive transfer of *T. cruzi*-specific CD8+ T cells confers mice partial protection from *T. cruzi* inoculation [13], while cardiac damage may still develop. Conversely, the human being develops myocarditis in the presence of $CD4^+$ and $CD8^+$ T cells, and parasite components as well [14].

6.1.2. Cruzi virulence and immunomodulatory factors

Cruzi parasites cause pathology depending on factors like the parasite species and strains, the route of infection [15], and the host genetic background. Different parasite strains coexist in infected patients, and in natural reservoirs in domestic and peridomestic areas. The strain-dependent immunomodulatory effects of the parasite might influence parasite-host interactions [14]. Mucin-like sialic acid-acceptor glycoproteins and other parasite cell membrane products determine *Cruzi* virulence [15]. One of the hallmark parasite-derived molecules are the glycol-inositol-phospholipids (GIPLs) covering the parasite cell surface that alter the B cell compartment, work as TLR4 agonists, and mediate proinflammatory effects [16, 17]. In sum, *T. cruzi* virulence factors actively subvert the host immune system leading to chronic infection [18–21].

6.1.3. White adipose tissue is an immune active endocrine organ

Another survival strategy of *Cruzi* parasites is targeting the adipose tissue (AT) [22], both brown (BAT) and white (WAT), the largest endocrine organ in the body shaped by adipocytes, fibroblasts, macrophages, and endothelial cells. The adipose tissue is involved in many

physiologic functions including energy homeostasis and immunity [23], and might warrant long-term parasite persistence by providing a safe reservoir to avoid the host-defense mechanisms. Then, immune system suppression would result in parasite recrudescence and multiple tissue invasion [24]. Besides, the AT might be the major site for parasite reactivation as indicated by the finding of parasite-derived DNA in AT [25] in patients with CCC [25]. Likely, the AT serves as a parasite reservoir favoring opportunistic reinfection upon immunosuppression, as observed in chagasic transplanted or HIV patients, or those under immunosuppressive therapies [26]. As infection increases the level of TLR4 and TLR9, and of the mRNA of cytokines, chemokines, and of their receptors, the adipose tissue appears to be both a target and a sensor of parasitic infection even in the early, latent stage of *T. cruzi* infection. Recently, the *T. cruzi* was detected in the adipose tissue of chronically infected individuals [25].

6.2. Host-protective and pathogenic anti-*T. cruzi* immune-mediated-response

6.2.1. The innate immune system

Innate, nonspecific, immunity involves any pathogen-eliminating mechanism triggered promptly without memory requirement. The acute nonspecific inflammatory molecules serve not only as 'gateway' signals, generating conditions unfavorable for the invading agent, but they are also implicated in chronic inflammatory diseases. Chronic sustained inflammation actually contributes to cardiac hypertrophy.

6.2.1.1. Toll-like receptors agonists expressed by T. cruzi activate inflammatory pathways

Nonspecific immune system cells modify their functional repertoire (phagocytic activity, activation, antigenic presentation, migration, and adhesion) through pathogen-associated molecular pattern recognition receptors (PAMPs) like the toll-like receptors (TLRs), many of which can recognize several types of structurally unrelated PAMPs. These receptors trigger proinflammatory pathways' activation interacting with their pathogen-derived ligands, and even with endogenous molecules, and releasing effector molecules like cytokines. This signal cascade triggers the expression of cytokine genes, where the type of TLRs bound determines the type of response.

Different *T. cruzi*-derived molecules belong to the PAMPs family and act as TLR agonists inducing the secretion of inflammatory cytokines, chemokines, and the production of nitric oxide (NO) by cells of the monocytic lineage. In this regard, the first evidence was the identification of the trypomastigotes-derived glycosylphosphatidylinositol (tGPI) that anchors mucin-like glycoproteins (tGPI-mucins), as a potent agonist of the human TLR2 [27] inducing proinflammatory responses on cells which express normal levels of TLR2 and TLR4 (**Figure 2**).

Another epimastigote-derived GPI family member, the glycoinositolphospholipid characterized by a lipid moiety, induced TLR4-mediated NF-κB activation. Many GIPLs freely anchor at the surface membrane of all the parasite life-cycle stage forms, whether infective metacyclic trypomastigotes or epimastigotes forms and have pleiotropic properties [28]. The variable lipid moiety composition of different GPI anchors determines the TLR type specificity for TLR2 (alkylacylglycerol) or TLR4 (dihydroceramide).

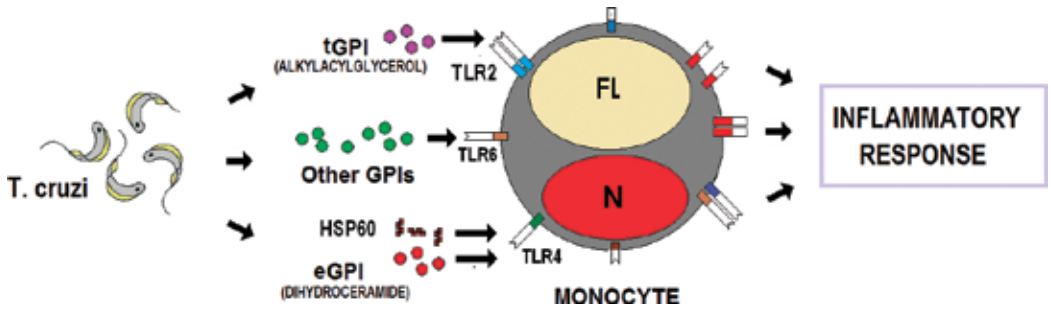

Figure 2. *T. cruzi* molecules PAMPs members are TLR agonists. The variable lipid moiety composition of different GPI determines whether their recognition is mediated by TLR2 (alkylacylglycerol) or TLR4 (dihydroceramide). TLR4 agonist triggers powerful proinflammatory molecules release. FL: Phagolysosome; GPI: Glycosylphosphatidylinositol; tGPI: Trypomastigotes-derived glycosylphosphatidylinositol; eGPI epimastigote-derived glycosylphosphatidylinositol, HSP: Heat shock protein.

6.2.1.2. Toll-like receptors and resistance to infection

Direct testing of the hypothesis that TLR triggering by PAMPs is crucial for host resistance to infection but is currently not possible due to unavailability of *T. cruzi* strains lacking the expression of any TLR agonist. Nevertheless, studying the course of infection in TLR-encoding genes-deficient mice, evaluating mortality, parasitemia, and several parameters of the innate and acquired immune responses have brought an additional understanding of the impact of impairing TLR-mediated recognition of *T. cruzi* in developing host susceptibility to the infection. In this regard, MyD88-deficient mice lacking the transducer of multiple TLR-signaling pathways first evidenced the crucial involvement of TLRs in host resistance to *T. cruzi* [29, 30].

6.2.1.3. Cardiac toll-like receptors increase in ischemia/reperfusion-induced cardiac hypertrophy

The development of cardiac hypertrophy involves TLR signaling so that MyD88 blockage attenuates cardiac hypertrophy and extracellular heat shock protein 70 (HSP70) induces cardiomyocyte inflammation [31, 32].

6.2.2. The adaptive immune system

The immune-mediated-pathology (IMP) links to parasite persistence inducing protective effector and autoimmune response and has been a subject of debate in CCC for years. Immune effector cells along with autoantibodies participate in both protective and pathogenic adaptive responses in CCC. Typically, histopathological examination in chronic myocarditis reveals inflammatory polymorphism with macrophages, eosinophils, mast cells, B and T lymphocytes, and granuloma cells, and a predominance of B cells and plasma cells in the epicardium and T cells in the myocardium, which progresses towards fibrosis. The mononuclear infiltrate and its mediators would be, at least partly, primarily responsible for myocardial damage [33].

6.2.2.1. Autoimmunity

At first post-infectious autoimmune myocarditis was proposed to reasonably explain the mismatch between myocardial areas showing parasite invasion and those with myocardial damage revealed by pathology examination that hampered reliably establishing Chagas disease pathogenesis.

- Cardiac epitopes share amino acid sequences with *T. cruzi* epitopes.
- The transfer of lymphocytes to syngeneic recipients produces inflammatory lesions in cardiac and nervous tissue.
- Chronic chagasic patients have autoantibodies in the bloodstream.
- Plasma cells obtained from murine myocardial lesions release anti-cardiac antibodies.
- T-lymphocytes obtained from human biopsies show cardiac muscle reactivity.

6.2.2.1.1. Evidence derived from the autoreactive immune response

While activation of autoreactive clones (possibly by polyclonal activation) occurs during the acute primary infection phase, autoantibodies appear to be generated during both the acute and chronic phases of the disease, likely perpetuated due to myocardial reactivity regardless of the etiologic agent. Both humoral and cellular cardiac autoimmunity might develop upon acute *T. cruzi* infection in the genetically susceptible host [34]. Another hypothesis sustained that autoimmunity develops only after sustained low-level stimulation of self-reactive cells over the chronic phase. Altogether, self-reactivity was initially proposed as a mechanism of tissue damage.

Many publications have mentioned the presence of cardiac tissue-parasite cross-reactivity. *T. cruzi* may induce antibodies and T cells also reactive to host antigens causing autoimmune reactions.

Certain antigens might induce nonspecific polyclonal activation, expanding clones that were in the anergic state as the polyclonal B cell activation associated with hypergammaglobulinemia and delayed specific humoral immunity reported in *T. cruzi* experimental infection in mice [35].

All in all, self-reactivity is accepted in Chagas disease though only subsidiarily contributing to myocardial tissue damage and deterioration as argued for the presence of autoantibodies and lymphocytes at the site of the lesion. Actually, immunosuppression not only does not cause improvement but rather aggravation of the course of the disease. Indeed, was self-reactivity relevant to injury, immunosuppression should be beneficial. Immunosuppression during acute infection reduces tissue inflammation while the parasite load increases in the infected tissues in mice. However, immunosuppression results in a generalized tissue invasion aggravating the disease. In chronically infected individuals, parasitemia is undetectable but any induced or acquired immunosuppression condition including pharmacological treatments,

AIDS, a transplant, an autoimmune disease, leukemia or pregnancy, may trigger reactivation. When immunosuppression occurs during the chronic stage, skeletal and cardiac muscle inflammation increases, allegedly explaining why a subset of patients presenting an insufficient or suboptimal immune response develop heart disease [36].

6.2.2.1.2. Evidence derived from molecular mimicry and the formation of cardioactive autoantibodies

Cardioactive substances from sera from chagasic patients. Subsequently they were characterized as antibodies with specificity towards the cardiac β-adrenergic receptors that acting as partial agonists increased mechanical tension and sinus beating frecuency in chagasic patients. The Cardiology Service of the Ramos Mejía Hospital contributed to the characterization of the anti-cardiac receptor reactions, and the results indicated that self-reactivity of this kind was caused by parasite epitopes with low affinity for cardiac receptors, later proved by the identification of anti-*T. cruzi* P ribosomal proteins in chagasic individuals. In *in vitro* or *ex vivo* experiments, IgG-enriched serum fractions obtained from chagasic patients modify baseline heart beating frequency in cultured cardiomyocytes, increase cardiac inotropism, and trigger atrioventricular blockage in isolated hearts. Rabbits immunized with the major immunogenic region of β-adrenergic receptors develop cardiomyopathies and malignant arrhythmias, and cardioactive antibodies have also been observed in other pathologies. Patients with ethiologically different chronic heart diseases like the idiopathic dilated cardiomyopathy or presenting primary electrical alterations produce chagasic IgG-like enriched fractions with antibody activity. Circulating autoantibodies to cardiac beta-adrenergic and muscarinic receptors may affect cardiac function in chagasic patients. The prevalence of anti-autonomic receptors antibodies was higher in patients with chronic chagasic heart disease and other forms of heart disease than in the healthy counterparts [36].

6.2.2.2. Immune origin of the disease and the localized inflammatory response

The 'tissue load of parasites' is associated with the 'severity of the lesion' and might predict not only the characteristics of the immune response in the acute or chronic (reactivation by immunosuppression) stages of the disease of the disease but also of the localized inflammatory response. Accordingly, tissue specificity would result from the local 'parasites persistence' in the site of inflammation. Whole, fractionated, or recombinant parasites immunization triggers inflammatory lesions and electrocardiographic alterations. The spectrum of clinical presentations might result from both the efficacy of the immune response during the acute stage and the parasite strain involved. Individuals infected with less virulent strains and immunocompetent hosts should become asymptomatic over the chronic stage (**Figure 3**). The immune response so results from the net balance of the *T. cruzi* strain immunogenic potency and the regulatory T cells and effector lymphocyte subpopulation Th1/Th17 during the infection. The IL-17 produced during experimental *T. cruzi* infection regulates Th1 cells differentiation and parasite-induced myocarditis. A low regulatory T cell activity and the frequency of IL-17-producing T cells correlate with CCC severity [37], not precluding a minor participation of the self-reactive immune reactions in producing injury.

Induced immunosuppression fails to cause relevant autoimmune-mediated damage for it aggravates rather than ameliorates the disease. Adoptive lymphocytes transfer to syngeneic

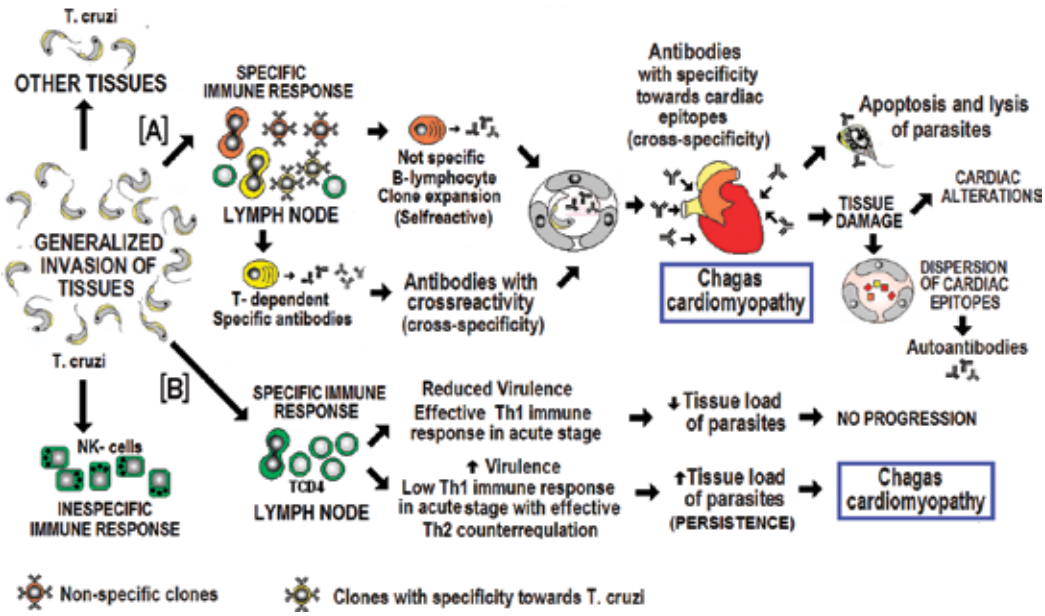

Figure 3. Possible mechanisms involved in the immunopathogenesis of Chagas disease. Although the immune system protects man from massive infection, he is unable to completely eliminate all parasites. (A) The humoral protective and immunopathogenic immune response to *T. cruzi* has been intensely studied. Although many questions remain, the antibody response is always present in infected individuals and many of the antibodies have a protective capacity. Several aspects are related to the presence of autoreactive antibodies in chagasic patients and in experimental chagasic models. The presence of similarities between the epitopes of the parasite and cardiac tissue leads to the expansion of autoreactive clones. Some authors argue that some autoantibodies should be due to the existence of polyclonal activation. Other autoantibodies may be the result of the autoantigen release of damaged tissue (epitopes dispersion epiphenomenon). (B) Th1 lymphocytes have great relevance in the control of the disease. The importance of the T-dependent response is evidenced by the observations recorded in immunosuppressed patients in whom the disease worsens. In vitro studies would indicate that the NK cell-mediated immune response (ADCC and natural cytotoxicity) could participate in the in vivo response. According to some authors the antigenic persistence would be the main mechanism inducing the inflammatory immune response in the chronic stage and IL17 seems to be involved in CCC. This interpretation postulates that the sustained activation of the immunoinflammatory response is the main cause of tissue damage.

recipients produces inflammation indicating that the T-response can effectively control the disease. The cellular immune response is critical in controlling *T. cruzi* infection, and the developed vaccine so far exacerbates damage and progression of the disease. Then, the vaccine either failed in enhancing the adequate response and/or its design requires further adjusting the epitopes. Of note, despite cellular control the infection, immune response persistence and cardiac infiltrated cells may cause injury. Finally, the contribution of autoantibodies is complex as it is likely involved in further enhancing cardiovascular damage.

7. Suppressor of cytokine signaling proteins, immune regulation, and dilated cardiomyopathy

Cell-cell signaling is an essential hallmark of multicellular organisms for communicating different cell populations [38] and is particularly crucial for the immune system function. The 'suppressor of cytokine signaling' (SOCS) plays a critical role in the regulation of all

SOCS type	Factor	Effect	References
SOCS3	G-CSF	Hematopoietic neutrophilia/inflammatory conditions	Chen et al. [35]
	IL-23 (↑IL17), (↑IFN-γ)	Enhance Th1 and Th17 polarization	Chen et al. [35]
	Leptin	Resistance to diet-induced obesity	Yang et al. [39]
	IL-6, (+IL-27)	Reduced CD8+ T cell proliferation	Yang et al. [39]
	TNF-γ, IL1β	Hypertrophy, fibrosis and inflammation	Liongue et al. [33]
	Global action	• Critical role in regulation of cytokine signaling • Control of hyperproduction of IL-10 and TGFβ • Promote Th2 response • Indirect sustained Th1 activity	Kinjyo et al. [34], Chen et al. [35]

Table 1. Main action of SOCS3 as a critical regulator.

crossroads of the cytokine-induced pathogenesis of dilated cardiomyopathy. The SOCS3 transgene induces Th2 responses, and SOCS3 gene deletion did not enhance Th1 polarization as expected but induced a negative regulator of the Treg subset with increased IL-10 and TGFβ production in mice [39, 41]. Not only SOCS3 is essential for G-CSF, IL-6, LIF, and leptin signaling, but is also an indirect regulator of IFNγ signaling and a negative regulator of IL-23 signaling, inducing IL-17-secreting T cells (Th17) polarization (**Table 1**).

The SOCS3 pathways are important, altering cardiac physiology by affecting molecular targets associated with myocardial changes implicated in structural pathologies. The SOCS3 proteins regulate specific cytokine pathways related to cardiac growth and enlargement and seem to be consistent with their roles critical regulators of hypertrophy, contractile dysfunction, and ventricular arrhythmias. They were, as their name suggests, first described as cytokine signaling inhibitors as observed for the Janus kinase/Signal Transducer and Activator of Transcription/Suppressor of Cytokine Signaling (JAK/STAT/SOCS) signaling pathway (JSS-SP). The remodeling by cytokine receptor signaling mediated by the JSS-SP provides a morphological basis explaining the pathogenesis of myocardial hypertrophy, fibrosis, and inflammation [41] in CCC. The inflammatory markers TNFα, and IL1-β represent potential targets in cardioprotection and therapy [42].

The SOCS3 protein is a key negative-feedback regulator of the gp130 receptor involved in signaling pertaining cardiac hypertrophy and survival. Activation of the gp130 without SOCS3 regulation leads to cardiac hypertrophy, in line with their roles as negative regulators of cardiac growth. Also, SOCS3 regulation on cardiac gp130 signaling participates in the pathogenesis of contractile dysfunction and ventricular arrhythmias. Consistently, human CCC is characterized by segmental left ventricular wall motion abnormalities (WMA), mainly in the early stages of the disease.

The failure of the SOCS3 protein, also a major negative regulator of both leptin, and insulin signaling, might participate in the pathogenesis of obesity, and associated metabolic abnormalities as found for diet-induced and genetic obesity, systemic inflammatory burden, and

hyperlipidemia. In sum, the SOCS3 may be critically negative regulators of inflammation, cardiac hypertrophy, contractile dysfunction, and ventricular arrhythmias. However, our understanding of the origins of the individual pathway components and their assembly into a functional pathway has remained limited.

8. Long-lasting systemic inflammatory burden and clinical Chagas progression

The immune-inflammatory response plays a key role in cardiovascular damage [40, 41], and *T. cruzi*-derived molecules may sustain the TLR-mediated innate immune response inducing inflammatory cytokines and chemokines secretion. The adaptive immune response to Chagas antigens may protect the host from secondary reinfection, though damaging the CV system due to inflammation, and the associated connective repair (fibrosis).

Crossroads between "natural, and specific immunity effector cells and molecules" and "parasite persistence strategies in blood, adipose tissue, the heart, and other infected tissues" seem to contribute to cardiovascular risk. Tissue damage induces inflammatory reactions. Immune-activated pathways are the main contributors to systemic inflammation in human CCC by active crosstalk between different CVRFs, metabolic and immune-mediated innate and adaptive host-parasite interactions. The contribution of natural and specific immunity against Chagas antigens enhance systemic inflammatory burden (SIB). Certainly, the immune system is not the only source of inflammatory molecules, but other tissues also contribute to enhancing systemic inflammatory burden as the WAT which releases inflammatory cytokines.

In a recent study, the levels of IL1β, IL6, IL10, TGFβ, IL12, IL17, TNFα, and serum IFNα were different in either chronic asymptomatic or cardiac chagasic patients compared with healthy controls. The asymptomatic patients had a higher plasma TNFα concentration (eightfold) and IL10, and lower IFNα than in normal controls, suggesting a process of immune regulation. Neither the interaction with traditional CVRFs and their contribution to CVR nor control-matching for age, sex, weight, or BMI were considered in this study. The advent of noninvasive imaging techniques allows studying the relationship between inflammatory markers in subclinical atherosclerosis development. The association of many systemic diseases with an increase in the prevalence of cardiovascular diseases involves immunoinflammatory mediators related to chronic inflammation and cannot be explained by the classic CVRFs. Unlike the classical CVRFs like the lipid profile, the approaches based on the contribution of the inflammatory milieu to cardiovascular disease development have not yet allowed identifying clinically validated biomarkers regardless the evidence suggesting their association with increased morbidity, and mortality except CRP [43].

Scientific research is encouraged to delve into the important role suggested for the inflammatory response in the metabolism and control of the atherogenic potential [44]. Hypercholesterolemia and inflammation are certainly considered contributive partners in atherosclerosis.

Only lipid-related indicators like LDL- and HDL-cholesterol fractions, and triglycerides are currently recommended in predicting cardiovascular risk. Paradoxically, plasma total

cholesterol level is accepted as a marker of relative CVR, though more than 50% of all cardiovascular events can occur in individuals with concentrations below the accepted normal total cholesterol level [56].

With a complete understanding of atherogenesis, the atherothrombotic markers, and the already mentioned inflammatory markers [45] are potentially available for CVR estimation.

Recent data suggest that the measurement of related inflammatory markers may improve cardiovascular risk assessment. Certain markers being evaluated include a group termed as "cellular" cytokines (e.g., IL1, IL2, IL12p40, IL15, IL17, TNF, IFN, and IFN), and "humoral" cytokines (e.g.IL4, IL5, IL6, IL10, and IL13), growth factors and angiogenic [e.g., EGF, VEGF, FGF, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-colony-stimulating factor (GM-CSF)], and chemokines [e.g. CCL2 (MCP1), CCL3 (MIP1), CCL11 (Eotaxin), and CXCL8 (IL8)]. The role of some of them in cardiovascular pathogenesis is described below.

8.1. Soluble ICAM1

Inflammatory stimuli trigger a dramatic increase in ICAM1 expression on the vascular surface. Later on, inflammatory cytokines induce the endothelial expression of VCAM1 which, like ICAM1, interacts with leukocyte integrins promoting the firm adhesion of leukocytes to the surface of the endothelial cells. After proteolytic cleavage, the endothelial ICAM1 molecules can be released into the circulation as soluble molecules (ICAM1s), the level of which correlates with CVRFs like smoking, hypertension, hypercholesterolemia, and hypertriglyceridemia and with acute phase reactants like the PCR, and increases in patients with coronary disease. The circulating level of the soluble forms of ICAM-1, VCAM-1, selectin, and CD44 are remarkably high during the acute *T. cruzi* infection, while the soluble forms of VCAM-1 and P-selectin increase in chronic infection [46–52]. As inflammation markers, the soluble forms of ICAM-1 might be notably higher in patients with DCM [53–55].

8.2. Chemotactic monocyte-1 protein

An important early step in atherosclerosis is the adhesion of monocytes to activated endothelial cells. The endothelium produces several molecules critical in the proatherogenic events like the endothelium-released MCP1 which contributes to increased monocyte recruitment, and activation of nuclear factor kappa B (NF-kB) involved in the transcription of many functional genes in the inflammatory process [64]. Recently, CCL2/MCP-1 has emerged as a critical factor in infectious and autoimmune myocarditis, is largely produced in *T. cruzi*-loaded mice hearts, and it promotes macrophages infiltration and parasite destruction. *T. cruzi*-infected $CCL2^{(-/-)}$ mice developed higher parasitemia, dying prematurely, and showed increased levels of TNF, IFN-gamma, and IL-10 in plasma, clinical signs of systemic inflammatory response. Cardiac density of amastigote nests was associated with leukocytes infiltrates. Other studies demonstrated that CCL2 contributes to the reduction of parasite growth by controlling the distribution, cellular composition, and the status of inflammatory infiltrates in acute *T. cruzi* infection [21]. More recent evidence shows that polymorphisms involved key molecules related to innate immunity, and that cell migration plays a critical role in genetic susceptibility to CCC [56–58].

8.3. Interleukin 10

Within the group of the so-called anti-inflammatory cytokines, the human IL10 is associated with an anti-atherogenic action reducing inflammation. Cytokine knockout of the Th1 inhibitor IL10 increase vascular lesions [59]. IL10 has strong regulatory properties on macrophages and T cells, and negatively regulates many cellular processes involved in atherosclerotic plaque development and stability.

A recent study revealed comparable serum cytokine levels in cardiac chagasic and asymptomatic patients though IL10 level was higher, and IFNγ level was lower in the former suggesting a greater regulatory activity in cardiac patients. Presumably, the IL10 level is ineffective to restore homeostasis. Likewise, circulating IL10 increases in patients with DCM.

8.4. Transforming growth factor beta (TGFβ)

The transforming growth factor beta is produced by different cell types including adipocytes, macrophages, endothelial cells, smooth muscle cells, platelets, and regulatory T cells. The TGFβ1 factor stimulates PAI-1 release and suppresses leptin release from the human adipose tissue. It inhibits atherogenesis modulating T-lymphocyte activity rather than modulating the prothrombotic and fibrinogenic activity, as confirmed in TGF II receptor type-KO models. It is also involved in regulating host tissue fibrosis. The oral administration of GW788388, a novel kinase inhibitor type associated with TGFß I and II receptors, remarkably increased cardiac cells' survival time and decreased cardiac fibrosis, offering a potential alternative to the current asymptomatic Chagas treatment. However, the cost-benefit balance is uncertain since circulating TGFβ modulates the pathogenic effector immune response avoiding immune damage but exacerbating fibrosis. Recent studies indicate that the deep alterations induced by circulating TGFβ increase in patients with DCM with or without cardiac fibrosis [60–62].

8.5. Interleukin-17A

The interleukin-17A cytokine released by Th17 cells is elevated in plasma in atherogenesis mice models. Increased serum levels of IL17 and IFNγ are found in patients with coronary atherosclerosis. The proatherogenicity of IL17A results from the monocytes/macrophages recruitment into the aortic wall [63]. The differentiation of Th17 depends on IL23 and IL6 released by myeloid dendritic cells, IL1, IL6, and IL21 derived from macrophages and T lymphocytes [64]. In humans, TGFα acts as a negative regulator of IL17. For years, Chagas-associated cardiac damage has been attributed to immunological dysregulation, including an imbalance between pro- and anti-inflammatory cytokines, Th1-Th2 immune deflection, and regulatory T cell activity. Recently, IL17 produced during experimental *T. cruzi* infection regulated Th1 differentiation, and parasite-induced myocarditis. The decrease in IL10 and IL17 cytokines' production in association with high levels of IFNγ, and TNFα correlates with the severity of human chagasic cardiomyopathy. This immunological imbalance might be causally related to a poor suppressor activity of the regulating T cells controlling myocardial inflammation. Finally, the derived IL17A-fibroblast and the derived -GM-CSF-macrophage axis are potential targets for the treatment of DCM and related inflammatory cardiac disorders [64].

8.6. C-reactive protein

The CRP marker level is used as a predictor of future cardiovascular events, being a good estimator of mortality risk in different contexts, particularly in metabolic syndrome in the general population. It has also been suggested as a direct stimulator of plaque formation decreasing endothelial nitric oxide synthase (eNOS) activity [65], and implicated in other deleterious effects. *In vitro* studies provide evidence of the proatherogenic direct effects of CRP as found in endothelial dysfunction, Also, an increase CRP serum level in chagasic patients has been associated with a greater progression towards heart failure. High hs-CRP level is associated with a higher incidence of the long-term combined endpoint of all-cause mortality and hospitalization in patients with DCM. Besides, oxidative stress molecules and hs-CRP are both associated with heart failure and damage severity in patients with DCM [66, 67].

8.7. Tumor necrosis factor alpha (TNFα)

The pleiotropic TNFα cytokine is one of the most potent mediators of inflammation. It is associated with an increased CVR. It can induce proatherogenic lipid alterations, including increased LDL-cholesterol and HDL-cholesterol [68] and promote hypercoagulability inducing tissue factor (TF) expression in endothelial tissue and suppressing anticoagulant activity through activated thrombomodulin-activated protein C [69]. It also induces endothelial dysfunction through decreasing nitric oxide and regulating adhesion molecules, an early critical step in atherogenesis. Several investigations in animal and human models provide convincing evidence on the action of TNFα as one of the major regulators of vascular homeostasis. Blocking of TNFα results in a significant decrease in Lp (a), homocysteine levels and increases in Apo AI, triglycerides, and Apo B concentration. The prolonged use of TNFα blocking agents interferes with proatherogenic action, reducing the incidence of cardiovascular events. Taken together these studies confirm a critical role of TNFα at the prothrombotic, proinflammatory and metabolic level. An association between TNFα levels with heart failure was observed in chagasic patients. Autopsies specimens confirmed the presence of cardiac inflammatory cell infiltrates showing a Th1 cytokines pattern. Chronic asymptomatic chagasic patients have TNFα plasma concentrations roughly eightfold higher than healthy controls, and TNFα may play a role in progression to heart failure, The increased level of TNFα might also be related to disease severity in chronic Chagas disease as found in patients with DCM. Interestingly TNFα blockage aggravates experimental CCC [70, 73].

8.8. Interleukin-1 (IL-1)

The IL-1 interleukin is a crucial proinflammatory mediator in acute and chronic inflammation, and also a powerful innate immune response inducer. It induces the synthesis and expression of several hundreds of secondary inflammatory mediators in different diseases [74, 75]. The inflammatory response is associated with the expression of numerous cytokines [interferon gamma, interferon alpha, tumor necrosis factor (TNF), and interleukin-3 (IL-3)], which stimulate xanthine oxidoreductase (XOR). The main circulating form of IL-1 is the IL-1β, initially synthesized as the pro IL-1β a precursor which is activated by caspase-1 cleavage in the setting of a macromolecular structure known as the inflammasome [71]. Many potential triggers

of the inflammasome have been identified, including microbial agents, ischemia, damaged cells, cholesterol crystals, and TLRs ligands such as danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). The IL-1 molecule has been associated with endothelial dysfunction, hypertension, heart failure, and diabetes [75]. Recent studies indicate that XOR and XO serum levels are considerably increased in both cardiac and asymptomatic patients following *T. cruzi* invasion. Serum levels of IL-1β could be used in predicting the long-term outcome of patients affected by idiopathic DCM [71, 76]. Cardiac fibrosis and heart failure progression in inflammatory dilated DCM might be related to the myeloid differentiation factor-88/IL-1β signaling pathway [72, 77].

8.9. Interleukin-6

IL-6 provides a link between innate and adaptive immunity through the regulation of leukocyte activation, differentiation, and proliferation. During the acute and chronic inflammatory response, macrophages release TNFα in the presence of a variety of stimuli including atherogenic factors. In the macrophages, TNFα triggers the release of TNFα, and of more IL1B, which stimulates endothelial cells to produce IL6 and IL8. To date, both the scientific outcome of experimental studies, and the abundant clinical evidence in atherosclerosis indicate that low-intensity sustained inflammation plays a key role in atherosclerotic plaque formation, progression, and destabilization leading to clinical endpoints like myocardial infarction, sudden death, or stroke. The underlying mechanisms are still unclear, despite the intense research over the past two decades. Both IL6 and its signaling events contribute to atherosclerotic plaque development and destabilization. Increased levels of IL6 [78, 79] and CRP, an accepted CVRF, can also contribute to atherosclerosis and arterial thrombosis by activating tissue factor production, increasing adhesiveness of endothelial cells, fibrinogen and factor VIII and stimulating platelet production, and aggregation [91]. In addition, smooth muscle cells (SMC) also produce abundant IL-6. Other inflammatory factors generated by adipocytes like IL6, CRP, and TNFα are also implicated in the pathophysiology of the metabolic syndrome. The polymorphism of IL6 genes correlates with the severity of coronary artery disease, and with myocardial infarction risk [80, 81], but not with carotid atherosclerosis, which seems to be independent, These findings clearly suggest a strong association between IL6 levels, atherosclerosis, and risk of cardiovascular death. Produced locally IL6 in the endothelial vasculature and by SMC, IL6 induces ROS production, proliferation, and SMC migration. IL6 is an important autocrine and/or paracrine regulator of SMC proliferation and migration, critical steps in atherosclerosis progression. Besides, circulating IL-6 levels (in parallel with an increase in circulating CRP) increase with progression to heart failure in Chagas disease. These observations agree with the polymorphisms analyzed in patients with idiopathic dilated cardiomyopathy (IDCM) that relates to TNF, IL-6, and CRP profile. A recent study shows that a rough increase in serum IL-6 is incidental with chronic IDCM [82].

9. Conclusion

Search results illustrated that immune-activated pathways are the main contributors to systemic inflammation in human CCC due an interplay and active crosstalk between different

traditional risk factors, mainly metabolic and inflammatory factors derived from immune-mediated host-parasite interactions, both innate and adaptive.

Tissue damage induces inflammatory reactions leading to dilated cardiomyopathy in genetically predisposed persons. The DCM represents an essential hallmark of CCC. Evidence suggests that the pathway of inflammation in DCM culminates in altered concentrations of various markers in peripheral blood, including oxidative stress molecules and markers of vascular and systemic inflammation. These scenarios necessarily require a means of communicating between different cell populations. Crossroads between "natural and specific immunity effector cells and molecules" and "parasite persistence strategies in blood, adipose tissue and heart and other infected tissue" would appear to contribute to cardiovascular risk. The Suppressor of Cytokine Signaling (SOCS) plays a critical role in the regulation of all crossroads of cytokine inflammatory network that induced pathogenesis of dilated cardiomyopathy and seems to play a critical role as negative regulators of inflammation, hypertrophy, contractile dysfunction and ventricular arrhythmias. However, our understanding of the origins of the individual pathway components and crossroads and their assembly into a functional pathway is limited so far. Unlike classical risk factors, approaches based on inflammatory status needs clinically validated biomarkers and its contribution to the development of cardiomyopathy in chagasic and IDCM needs additional studies, even when there is strong evidence suggesting increased morbidity and mortality associated with the systemic inflammatory burden and inflammatory cardiomyopathy.

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Author details

Rodolfo A Kölliker-Frers[1,2†], Matilde Otero-Losada[3†], Gabriela Razzitte[1], Mariela Calvo[1], Justo Carbajales[4] and Francisco Capani[2*]

*Address all correspondence to: franciscocapani@hotmail.com

1 Parasitology Unit, José María Ramos Mejía Hospital, Buenos Aires, Argentina

2 Laboratory of Cytoarchitecture and Neuronal Plasciticy, Institute of Cardiological Research, "Prof. Dr. Alberto Taquini", ININCA-UBA-CONICET, Buenos Aires, Argentina

3 Laboratory of HPLC, Institute of Cardiological Research, University of Buenos Aires, National Research Council, ININCA-UBA-CONICET, Buenos Aires, Argentina

4 Cardiology Unit, José María Ramos Mejía Hospital, Buenos Aires, Argentina

† RAKF and MOL share authorship of this paper.

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Section 3

Modern Medicine and Back to Nature

Chapter 8

Efficacy and Safety of Chagas Disease Drug Therapy and Treatment Perspectives

Wilton H. Kawaguchi, Leticia Bonancio Cerqueira,
Mariana Millan Fachi, Michel L. Campos,
Iara J. Messias Reason and Roberto Pontarolo

Additional information is available at the end of the chapter

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Abstract

Chagas disease, also known as American trypanosomiasis, is a neglected disease caused by the protozoan parasite *Trypanosoma cruzi*. The disease affects about 6–7 million people worldwide, mostly in Latin America. Although Chagas disease was discovered more than 100 years ago, and the first treatments over 40, only 2 drugs were used to treat this pathology, it is still considered one of the neglected diseases. In this chapter, the subjects related to conventional etiological therapies, benznidazole and nifurtimox, such as the drug, the mechanism of action, the therapy schedule for treatment, efficacy and safety and their adverse effects will be discussed. Additionally, it will address alternative therapies of comorbidities related to the progression of Chagas' disease in patients with chronic disease, such as heart disease and dysfunction of the digestive system. Finally, novel pharmacological strategies and their related compounds will be reviewed accounting for their progression in pharmacological studies and their success rate.

Keywords: Chagas disease, benznidazole, nifurtimox, symptomatic treatment, new strategies

1. Introduction

Chagas disease or American trypanosomiasis is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). Endemic largely in Latin American countries, it is transmitted primarily by vectors, the insect vector triatomine, also known as "kissing bug". It is estimated that 6–7 million people are infected worldwide, and that more than 10,000 people die each year as a result of the disease, with the highest number of cases in Latin American countries [1].

Although Chagas disease has been discovered more than 100 years ago, there are currently only two drugs used to control Chagas disease (CD), benznidazole and nifurtimox [2–4]. Benznidazole has a similar efficacy profile to nifurtimox. They have high cure rates in the acute phase and at the beginning of the chronic phase, being little or ineffective in the late chronic phase [5]. Benznidazole and nifurtimox belong to the nitro heterocycles class of drugs; however, each presents different mechanisms of action that are considered very aggressive and may cause several adverse effects.

In the chronic phase of CD, the treatment at this stage aims to reduce parasitemia and prevent complications that may lead to progression of visceral lesions. However, the administration of benznidazole and nifurtimox remains controversial [5, 6]. To treat the cardiac and gastrointestinal manifestations attributed to this phase of the disease, it requires specific pharmacological approaches, highlighting the combination of diuretics, angiotensin-converting-enzyme inhibitors or angiotensin receptor blockers, and adrenergic beta blockers. The choice of palliative treatment is in relation to the symptoms of the patient.

Currently, the main treatment for CD is benznidazole and nifurtimox and both compounds are effective in the acute phase to reduce parasitemia and the persistence and the clinical severity of the disease [5]. However, in most countries, benznidazole is the primary choice to begin the treatment, since it has shown less adverse events. The predominant reasons to use nifurtimox are the occurrence of benznidazole resistance or its unavailability [6, 7]. Benznidazole can achieve over 70% of cure in cases of congenital and acute phase, while nifurtimox reaches 80% efficacy. However, these compounds have limited efficacy in the chronic phase, with efficacy rates of only 6–10% [8–10]. Treatment complications include, need for increasing doses, duration varying according to the stage of infection, and high occurrence of adverse effects caused by the long-term treatment with high doses. In addition, resistance may occur depending on the *Trypanosoma. cruzi* (*T. cruzi*) strain [10].

Although the aforementioned therapy is over 40 years old, there are limitations in this first line of treatment for CD. Several studies aimed to bring new options to its arsenal, mainly for the chronic phase. Among them, are drugs used to treat other conditions, such as posaconazole, and new compounds, such as E1224. Each of these has been tested in clinical trials. Other strategies have been suggested as groundbreaking approaches to eradicate, or at least control, CD. These include drugs, such as cruzipain and trypanothione reductase which specifically targets the ergosterol biosynthesis pathway.

Considering this the classical treatment of CD, with a focus on the evaluation of efficacy and safety of the current drug therapy, will be properly addressed in this chapter. In addition, a review of new pharmacological therapies will be discussed.

2. Etiological treatment: benznidazole

2.1. Treatment history

Until the publication of the *Manual de Doenças Tropicaes e Infectuosas* in 1935 by Carlos Chagas and Evandro Chagas (Manual of Tropical and Infectious Diseases), there was no

pharmacological treatment available for trypanosomiasis. Drugs with trypanocidal activity have been investigated by a number of researchers; however, without success [11]. In 1936, quinolinic compounds were successfully used to treat an acute case of trypanosomiasis [12]. In the following years, nitrofurazone was administered to treat trypanosomiasis in mice and achieve efficacy rates between 20 and 100%, depending on the therapeutic schedule [13]. These results motivated the experimental trials in humans, in which nitrofurazone demonstrated to be effective against trypanosomes in the circulating blood, cerebrospinal fluids [14] as well as other promising clinical results [15, 16].

Novel studies were performed to identify an alternative therapeutic schedule and main side effects [17, 18]. In 1962, Rio de Janeiro was the host city for the "Meeting of Debates on Chagas Disease", where the standardization of the methodology and establishment of criteria for the evaluation of attempted pharmacological treatments was discussed. Nifurtimox and benznidazole remain as the only two drugs available for treatment of CD [19].

2.2. The drug

Benznidazole (BNZ) N-benzyl-2-(2-nitro-imidazol-1-yl)acetamide (**Figure 1**) is a nitro heterocyclic drug. It was nitroimidazolic derivative synthesized in the early 1970s by Wineholt and Liebman and produced by Hoffman-La Roche, Switzerland. In 2003, the rights and manufacturing technology of BNZ was granted to the Laboratório Farmacêutico do Estado de Pernambuco (*LAFEPE*) – Recife/PE – Brazil [5, 9, 10, 20, 21]. According to the Biopharmaceutical Classification System, BNZ is categorized as class 4, thus exhibiting limited and/or variable absorption due to the characteristics such as low solubility and low permeability. Therefore, there are interests in strategies to improve the absorption rate, hence increasing its bioavailability [22, 23].

BNZ is administered orally in a tablet form of 100 mg for adults and 12.5 mg tablets for children [24]. The drug is fully absorbed in the gastrointestinal tract and the plasma peak concentration was achieved within 2–4 hours. The half-life is 12 hours and its metabolites were eliminated in urine and feces [5, 10, 20].

2.3. Mechanism of action

The mechanism of action of BNZ is not fully understood [9, 25]. However, some reports associate its action with the formation of free radicals and/or electrophilic metabolites. In the nitroimidazole derivatives, the reduction of the nitro group (NO_2) in the amino group (NH_2)

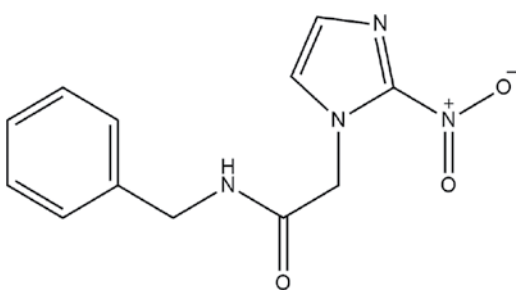

Figure 1. Chemical structure of benznidazole.

occurs by the action of nitroreductase present in protozoan cells or bacteria. This reaction leads to the formation of radical intermediates, as well as electrophilic metabolites. The process is initiated by the action of the enzyme NADPH cytochrome P450 reductase, which acts on the nitro group (R-NO_2) of the molecule nitroimidazole, inducing the production of an intermediate nitro radical (R-NO_2) and the formation of hydroxylamine (R-NHOH). These intermediates act on the covalent modification of macromolecules, such as DNA, causing fragmentation in the chain and destabilization of the helix, inhibiting DNA synthesis leading to cell death of protozoa parasitic and/or bacterial. In addition to this alteration in DNA, it involves the modification of other macromolecules, such as lipids and proteins, affecting *T. cruzi* metabolism. An additional mechanism of action of BNZ is the increase in phagocytosis, leading to lysis and inhibition of *T. cruzi* growth through an interferon-gamma (IFN-γ)-dependent mechanism and through the enzyme NADH-fumarate reductase, respectively [9, 11, 26–28].

Concomitantly, the electrophilic metabolites mentioned above due to their high reactivity and low specificity, may present human host action and cytotoxic effects observed during the treatment of patients [27–29].

2.4. Therapeutic schedule

The dosage of BNZ in the acute phase of the disease should be provided in two or three administrations daily, during the 60-day period. The dose is established according to the patient's age and weight. For adults, it is recommended 5 mg/kg/day, whereas for children weighing less than 40 kg, 5–10 mg/kg/day is recommended. Furthermore for children, the therapeutic regimen should be as small as possible to achieve therapeutic adherence. A recommendation for infants is 10 mg/kg/day. In congenital infections, the treatment is indicated for children born to mothers who were serologically positive for CD with the presence of *T. cruzi* in umbilical cord blood, serum-specific IgM soon after birth, and IgG after 6 months. In the newborn, the recommended etiologic therapy is the administration of BNZ twice a day for 30 days, it is suggested to be provided at 5 mg/kg/day [5, 11, 20, 30–32].

In the chronic phase, the dosage is 5–7 mg/kg/day divided into two administrations with an interval of 12-hour between doses, and for 30–60 consecutive days [5, 20].

2.5. Efficacy and safety

It is considered as the first choice treatment because it exhibits a better safety and efficacy profile when compared to nifurtimox. Moreover, there is evidence of greater efficacy [7, 28]. It has antiprotozoal and antibacterial performance, acting against the trypomastigote and amastigote forms [5].

In the acute phase, if the treatment is initiated immediately following the confirmation of the presence of *T. cruzi* in the direct examination of the peripheral blood, the cure percentages are greater than 70–80%, independent of the transmission route [5, 7, 9, 33]. It should be administered to patients in the early chronic phase, children less than 12 years, laboratory accidents and congenital infection. In newborns treated during the first year of life, the chances of cure are greater than 90%; otherwise, in the late chronic phase the patient has lower chances of cure (~10%) [20, 34–36].

Two placebo-controlled clinical trials were performed in the USA in children between 6 and 12 years old for the evaluation of treatment using BNZ, in which the results were satisfactory. In the first trial, 60% of the children treated had a change from positive to a negative in serology for CD, compared to 14% of them receiving placebo. In the second trial, similar results for children treated with BNZ showed a 55% change from a positive to a negative antibody test, compared to 5% of those receiving placebo [37].

For treatment in cases of organ transplantation, it is essential to know if the donor or recipient presents positive serology and can transmit or reactivate the infection. In cases of absolute requirement for transplantation, the serum-reactive donor in which the recipient is serologically negative for a chagasic infection, the donor should receive BNZ therapy following the traditional dosing regimen, within 60 days prior to transplantation. For the recipient following transplantation, it is recommended to initiate therapy by performing serological tests over time. There is a serum conversion with the treatment for the acute phase. If an acute infection is detected, the treatment need to be started [38–40].

Immunocompromised patients, carriers of hematological malignancies, users of immunosuppressive drugs, or those co-infected with the acquired immunodeficiency virus may reactivate CD. The treatment is the conventional treatment, lasting 60 days, and depending on the clinical conditions of the patient, it may be increased to 90 days [40–42].

When accidental infection occurs, the individual who accidentally had contact with contaminated materials; needle puncture, contact in lesions, wounds or mucous membranes, or any other means that indicates the possibility of having been infected by the parasite, is evaluated by serological tests and treatment begins immediately. During the 10–15 days of the treatment period, the serological tests will be repeated [5, 11].

At the beginning of the chronic phase in children, treatment follows the same reasoning for acute phase cases in children younger than or equal to 12 years with positive serology. For adults, therapy is recommended to prevent or reduce the progression of CD in more severe forms, such as cardiomyopathy, and to prevent congenital transmission in pregnant women [5, 11, 20, 43]. Its use in the chronic phase has generated vast discussions. The BENEFIT project – Benznidazole Evaluation for Interrupting Trypanosomiasis, is an international multicenter, randomized, double-blinded, placebo-controlled trial of BNZ for the treatment of patients with mild to moderate Chagas cardiomyopathy. This study has conducted and produced results that indicated that the use of BNZ in the chronic phase significantly reduced the detection of circulating parasites; however, did not attenuate the cardiac clinical progression [20, 33].

Treatment for patients older than 50 years should take into account the risk of toxicity of the drug against the benefits of the therapy individually [6, 20].

2.6. Adverse effects

Therapy with BNZ contains some challenges, such as the large doses administered, time duration varying according to the stage of infection, and high occurrence of adverse effects. These challenges are less frequent when compared with nifurtimox (**Table 1**). The adverse effects in patients receiving BNZ may be classified into three groups: manifestations of

Adverse effects	Benznidazole	Nifurtimox
Anorexia and weight loss	5–40%	50–75%
Nausea	0–5%	15–50%
Vomiting	0–5%	15–26%
Peripheral neuropathy	0–30%	2–5%
Leukopenia	Rare: <1%	Rare: <1%

Table 1. Frequencies of adverse effects associated with benznidazole and nifurtimox.

hypersensitivity (dermatitis with rashes, generalized or peritoneal edema, fever, lymphadenopathy, muscle and joint pain), bone marrow depression (agranulocytosis, neutropenia, and thrombocytopenic purpura), and peripheral polyneuropathy (paresthesias and polyneuritis) [7, 11].

Adverse effects of dermatological cause appear in approximately 30% of the patients being treated with the drug. Rashes occur due to photosensitization, dermatitis is usually mild to moderate and may be treated with topical systemic corticosteroids. However, treatment should be discontinued immediately in cases of severe or exfoliative dermatitis or associated with fever and adenopathy. Bone marrow suppression is rare, but if occurs the treatment should be discontinued immediately. Additional adverse effects include weight loss, nausea and/or vomiting, anorexia, and insomnia [8, 11, 20, 29, 43].

Treatment is contraindicated in pregnancy and in patients with severe renal or hepatic impairment [11, 20, 44].

According to the World Health Organization (WHO), the ideal requirements for treatment are: parasitological cure in both phases (acute and chronic), effective doses in single or few doses, low cost, no side effects or teratogenic effects, without hospitalization, and induction of resistance. Until now, there is no drug for the treatment of CD that meets each of these WHO requirements.

3. Etiological treatment: nifurtimox

3.1. The drug

Nifurtimox (NFX) (**Figure 2**) belongs to the class of nitrofuran compounds. Her-Linger, Mayer, Petersen and Bock from Bayer™ synthesized it in 1962 in Germany. This was the first drug designed to treat trypanosomiasis, such as sleeping sickness and Chagas diseases [34]. Its production was interrupted in 1980s due to the reduction on world demand [11]. In 2009, the WHO Expert Committee on the Selection and Use of Essential Medicines recommends the inclusion of NFX in the model list of essential medicines (EML) and Bayer resumed the production of NFX [45]. Bayer, through WHO, still provides NFX worldwide in 120 mg and 30 mg tablets under the brand name Lampit [46], in United States and it is redistributed by Center for Disease Control and Prevention (CDC) [47].

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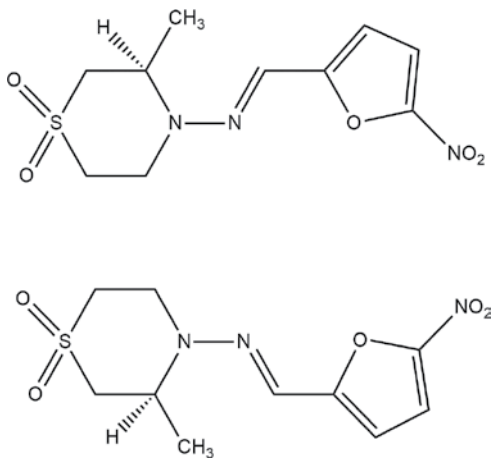

Figure 2. Chemical structure of nifurtimox.

NFX is a 5-nitrofuran (3-methyl-4-(5′-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide. NFX possesses an asymmetric center, but it is used as a racemic mixture since pure stereoisomers were not more active or less toxic.

NFX is well absorbed following oral administration. The plasma levels range from 10 to 20 mM and lower concentrations are found in urine and tissues. The therapeutic schedule must vary according to the patient's age and disease phase [9, 11, 48, 49].

3.2. Mechanism of action

The mechanism of action of NFX is based on cellular damage originating from the production of nitro anion radical through two pathways: through a redox cycle with the formation of $O^{\cdot -}$ (superoxide anion) and its reduction to the corresponding amine derivative [50–52].

This mechanism was thoroughly studied by Do Campo and colleagues [51, 53–58]. The reduction of a nitro group by nitroredutase (NTR) is fundamental for NFX mechanism of action. This enzyme catalyzes the reduction of 2-electrons of the compound, producing a nitrous intermediate, followed by a second 2-electron reduction to generate a hydroxylamine. This derivative can directly lead to cell damage, or generate other cytotoxic agents (**Figure 3**) [52, 59]. Since the *T. cruzi* detoxification mechanism is insufficient, it becomes more susceptive to reactive nitro compounds [60–62]. The presence of the nitro reductase type I in the parasite is the main responsibility for NFX selectivity. This enzyme is absent in mammals, reducing the formation of toxic agents in human.

3.3. Therapeutic schedule

Several therapeutic schedules were evaluated in the past years. The doses vary between 5 and 30 mg/kg/day, in extended therapies of 30–120 days [63–67]. Based on these experiments and considering the efficacy/tolerance ratio, the ideal therapeutic schedule of NFX is 8–10 mg/kg/day

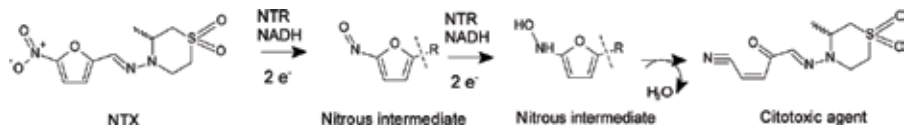

Figure 3. Mechanism of NFX based on nitroreductase type 1 action.

in adults and 15 mg/kg/day in children, for 60, 90 or 120 days, divided into three daily doses, after meals [1, 68]. It must be highlighted that even at these doses, side effects may potentially be present in adults.

3.4. Safety and efficacy

Many studies regarding the efficacy of NFX were performed between the 1960 and 1980s, at the beginning of its development and first years of commercialization [5, 11, 34]. Considering these studies, the activity of the NFX against the *Trypanosoma sp.* is expressive. NFX is capable to achieve efficacy rates of 70–100% in acute parasitemia cases. However, these rates substantially decrease when it comes to adult chronic phase, reaching values of only 7–8% in adults, although in children under 14, it remains around 86% [68, 69].

The primary reason why BNZ is often preferred compared to NFX is due to the presence of adverse effects. NFX frequently produces side effects [5, 11, 34, 70], but the majority are mild and can be managed with dose reduction or temporary suspension of medication [11, 68, 71].

3.5. Adverse effects

The treatment of CD is long and continuous and the presence of adverse effects becomes quite common between 60 and 100% of the patients [68]. The most frequent side effects described in the case of NFX are anorexia, nausea, headache, and amnesia. The possible neurological reactions are restlessness, disorientation, forgetfulness, insomnia, spasms, paresthesias, polyneuritis, and convulsive seizures. Most of the adverse effects (93%) are mild and disappear through dose reduction or after suspending the treatment [68, 70].

4. Symptomatic treatment of chronic phase

CD is classified evolutionary into two phases: an initial acute phase, followed by a chronic phase. Each phase has distinct clinical characteristics, diagnostic parameters, and treatment [72–76]. The acute phase is characterized by intense parasitism related with nonspecific symptoms, including fever, eyelid edema (denominated Chagoma), edema, and myocarditis. In general, the progression of disease can take years to reach the chronic phase, which presents four clinical forms: indeterminate (without clinical manifestations), cardiac, digestive, and mixed (association of cardiac and digestive) [77–81].

4.1. Chronic Chagas cardiomyopathy (CCC)

Chronic Chagas cardiomyopathy (CCC) is associated with high rates of morbidity and mortality, and it is categorized into stages of cardiac impairment (A, B, C, and D) according to the manifestations, electrocardiograph, radiological alterations, and changes in ventricular function [72, 74, 75, 82, 83]. Approximately 20–40% of patients with the indeterminate form will develop CCC. Several mechanisms contribute to this including the persistence of the parasite and autoimmunity. Moreover, factors such as dysautonomia (neurogenic mechanisms) and microvascular dysfunctions may potentiate and amplify this damage [74, 75, 77, 79–81, 84].

The cardiac form of Chagas can occur with or without ventricular failure [25, 82, 84]. Although the most common is the coexistence of arrhythmic manifestations with the congestive form, some patients have only arrhythmias and intraventricular and atrioventricular conductions compromising with normal ventricular function. Management of CCC treatment is based on the following clinical manifestations such as heart failure, cardiac arrhythmia, and thromboembolism [25, 79, 84].

Although, Chagas disease represents an important cause of heart failure (HF), limited studies have established the use of these drugs in Chagas patients. The treatment of CCC aims to reduce symptoms, delay the evolution of ventricular dysfunction, and prolong survival. In the asymptomatic or mild stages of HF, it is intended to delay the evolution of the disease. In the advanced stages, the objective is to improve the quality of life and the survival of patients. The efficacy and tolerability of these drugs in patients with CCC is extrapolated from the results obtained for other etiologies. Therefore, the CCC treatment is suggested to be performed in accordance with the general guidelines for HF treatment and should consist of the combination of three therapeutic classes: angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB), diuretics, and adrenergic beta blockers (BB) [40, 72, 82].

Angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) have an essential role in adverse cardiac remodeling and ventricular dysfunction progression of HF [40, 72, 82]. In experimental studies reported in the literature, captopril and enalapril demonstrated to diminish myocarditis and fibrosis of CCC [85–88]. These drugs should be administrated initially in low doses. In spite, some physicians report that CCC does not tolerate high doses of ACEI, according to the degree of tolerability; the dose can be progressively increased [25, 78, 81]. ACEI is recommended to patients with a left ventricular ejection fraction (LVEF) <45% [82, 83]. Regarding ARB, spironolactone and eplerenone have been evaluated in studies and data have shown that these drugs are effective in improving the quality of life and reduction of symptoms associated with CCC [89–91] and this class is considered as the treatment of choice for Chagas patients with LVEF <45%, or patients with LVEF<35% and New York Heart Association (NYHA) Class III/IV [82, 83]. In addition, in cases that are contraindicated to ACEI and ARB (hyperkalemia and progressive renal failure), the combination of hydralazine and isosorbide dinitrate should be prescribed [25, 73, 79, 83].

Beta-adrenergic blockers (carvedilol, bisoprolol, or metoprolol) are suggested in association with ACEI and ARB due to autonomous nervous system involvement in CCC and the production of antibodies against β-1 adrenergic and M-2 muscarinic receptors [25, 73, 79, 83]. Limited

reports address the efficacy of this therapeutic class to treat Chagas patients with ventricular dysfunction [86, 92–96]. Beta blockers have been avoided because of the presence of frequent bradycardia; therefore these drugs are not indicated for patients with bradycardia ≤50 bpm or AV conduction disorders (PR > 280 ms) [25, 73, 79, 83].

Moreover, digoxin and diuretics are considered a pharmacological option in CCC, justifying the use of digoxin for patients with symptomatic LVEF ≤45%, principally in the presence of atrial fibrillation when the ventricular frequency is increased and diuretics to improve congestive symptoms and signs. For advanced HF stages, the combination of thiazides with loop diuretics has been proven to be more effective [25, 72, 73, 79, 83].

The annual incidence of thromboembolism in Chagas patients is between 1 and 2%, affecting mainly patients with HF. Occasionally, this aggravation is the first manifestation of the CD. Cardiac emboli can reach both the pulmonary and systemic circulation, with the cerebral territory being the most clinically evident [40, 79, 82]. The treatment of thromboembolism is performed based on the established recommendations, alternating according to the extension and compromised organ. For this, a score derived from a prospective cohort study with 1043 patients was recently available to evaluate the risk and to implement preventing thromboembolisms in CCC. Through risk-benefit analysis, warfarin is indicated for patients with 4–5 point. In the case of 3 points, acetylsalicylic acid (AAS) or warfarin could be used. For 2 points, it is suggested to use AAS or no prophylaxis, and 0–1 points do not need prophylaxis [79, 81, 97].

Patients with CCC usually present ventricular arrhythmia. The most frequent ventricular arrhythmias in Chagas patients are ventricular ectopies, isolated or repetitive. The presence of these arrhythmias in asymptomatic patients with preserved ventricular function does not require antiarrhythmic treatment, whereas in symptomatic cases, the antiarrhythmic treatment can be individualized. The goal of pharmacological treatment of arrhythmias is the control of symptoms. Amiodarone is widely used, despite the high incidence of adverse events. At the usual doses of 200–400 mg/day, it can be associated with alternative agents recommended for cardiopathies of other etiologies, such as propafenone, sotalol, and beta blockers, to reduce severe arrhythmic events. However, drugs belonging to class I (sodium-channel blockers) should be avoided, principally in patients with ventricular dysfunction due to proarrhythmic effects, whereas propafenone is contraindicated in patients with left ventricular dysfunction [77, 79, 83, 98]. Bradyarrhythmia is related with sinus node dysfunction or atrioventricular blocks. For the treatment of symptomatic bradyarrhythmias in CCC, a permanent pacemaker implant is usually performed [98].

4.2. Digestive form of CD

The digestive manifestations of the CD correspond to the functional alterations observed in the esophagus and intestine, which result in the formation of mega-esophagus or megacolon, respectively. These deformations are related with the involvement of the enteric nervous system, especially the Auerbachs plexus. Degenerative phenomena in this region are caused by the presence of inflammatory processes associated with autoimmune responses. Therefore, both megacolon and mega-esophagus results in alterations in motility, and consequently, in slow transit and difficulty to empty, followed by increased organ caliber [99–102].

Mega-esophagus is classified into four groups with the objective of situating the different radiological aspects within the evolutionary spectrum of the affection. In addition, the classification of the mega-esophagus is important for the choice of treatment [99–101, 103].

The symptoms commonly reported in mega-esophagus are: dysphagia, regurgitation and esophageal pain. Treatment of mega-esophagus included clinical, pharmacological measures, dilatation and surgical procedure to aid in the transit of foods and liquids. The choice of treatment to be applied depends on the following factors: patient agreement, relevance of symptoms, degree of classification, nutritional status, clinical condition, comorbidities, age and available hospital infrastructure [40, 103, 104].

Clinical and pharmacological treatment is indicated for patients in group I, or patients at high risk of being treated with other forms of treatment, or in cases refusing invasive treatment. Patients must frequently drink water during the meals, eat slowly, and give preference to food in a pasty consistency. Hot and cold foods and drink and eating prior to bedtime are not recommended because food may be retained in the esophagus, causing pain or nocturnal regurgitation during sleep. The pharmacological treatment is based on substances that aim to relax the esophageal sphincter; however, the beneficial effect of these drugs is restricted to the period of action, being only a symptomatic treatment. The nitric derivatives (isosorbitol dinitrate) and the calcium channel blockers (nifedipine) are recommended [40, 101, 104, 105].

Alternative treatment is forced dilation of the distal segment of the esophagus and esophagic junction using a pneumatic or hydrostatic balloon or surgery. Surgery is performed for patients classified in group II (according to the intensity of the symptoms), III, and IV, and for patients without adequate response to clinical treatment. Another alternative is the injection of botulinum toxin, which acts to inhibit acetylcholine release [40, 101, 105].

The most frequent symptoms in megacolon consist of intestinal constipations, abdominal distension discomfort, occlusive phenomena associated with fecaloma, and sigmoid volvulus [106, 107]. Megacolon treatment may be clinical or surgical and varies according to patient agreement, level of complications, nutritional status, clinical condition, presence of comorbidity, and age [40]. If the clinical treatment indicates an alteration in the diet, use of laxatives, such as lubrificant laxatives (mineral oils) and emollient laxatives, and intestinal washes with water and glycerin may be used [105].

5. Therapeutic alternatives for Chagas disease treatment

5.1. Clinical trials

As previously described in this chapter, a randomized controlled clinical trial for investigation of BNZ in the treatment of CD was performed only recently: the BENEFIT clinical trial [95]. Therefore, it is not surprising that there is a lack of clinical studies with novel drug candidates for CD, since even the study to evaluate the first choice treatment is very recent.

Posaconazole (POS) (**Figure 4A**) is a triazole derivative with antifungal activity [108], that has been approved for the treatment of invasive fungal infections in humans [109]. It has shown trypanocidal activity in murine models [110, 111]. In addition, POS treatment led to the cure of the infection in one patient, resulting in parasite levels lower than after treatment with BNZ [112]. Therefore, POS efficacy and safety were compared to BNZ in phase II clinical trial CHAGASAZOL NCT01162967 [4], since it has shown trypanocidal activity in murine models. In this study, 78 patients were randomly selected to receive POS at a dose of 400 mg, POS at a dose of 100 mg, or BNZ at a dose of 150 mg. All drugs were orally administered twice daily for 60 days. During the treatment days, only two individual of POS patients treated with the dose of 100 mg had tested positive for *T. cruzi* DNA using a RT-PCR assay. The study duration was 1 year, thus the patients were retested following the treatment period. The outcomes have shown a significantly (p-value lower than 0.01) higher incidence of positive *T. cruzi* DNA results in both POS-treated groups (81–92%) compared to BNZ-treated group, since the last one led to only 38% of patients testing positive for *T. cruzi* DNA using the RT-PCR assay. While all patients from POS groups completed the study, five patients stopped the BNZ treatment, due to severe skin reactions. The adverse reactions of the current treatment with BNZ are some of the reasons why superior treatments must be pursued. However, BNZ treatment failed in fewer patients than POS [4].

CHAGASAZOL was not the only clinical trial testing the potential application of POS in Chagas Disease treatment. The clinical trial STOP-CHAGAS NCT01377480 [113] investigated if POS or POS plus BNZ were superior to BNZ monotherapy in the reduction of parasites load after 60 days of treatment and 360 days follow up. In this study, 120 subjects were randomly divided into the following groups: POS 400 mg twice a day; BNZ 200 mg and placebo, both twice a day; BNZ 200 mg and POS 400 mg, both twice a day; or placebo 10 mg. Two outcome stages were considered, the persistence of negative RT-PCR in day 180 and the maintenance of this response by the end of the study in day 360. The successful overall outcome was the RT-PCR negative result in both stages. Both groups receiving BNZ achieve 96% subjects with negative RT-PCR at day 360, while placebo group and POS alone group had 16.7% and 23.3% subjects with negative RT-PCR, respectively. Those groups receiving BNZ also had six serious adverse events reported, such as cutaneous reactions, nervous system disorders, and gastrointestinal symptoms. Therefore once again, BNZ monotherapy was superior to POS, and no advantages were observed in the combination therapy.

Ravuconazole (**Figure 4C**) is another triazole compound that has shown potent and specific anti-*T. cruzi* action *in vitro*, but with limited *in vivo* activity, probably as a consequence of its poor pharmacokinetic properties [114]. Therefore, E1224 (**Figure 4D**) was developed as a prodrug of ravuconazole. A phase II Clinical Trial was performed in 2013 to evaluate the prodrug efficacy and safety compared to BNZ, as a randomized double-blinded placebo-controlled proof-of-concept study [115]. The study was performed in 224 patients with chronic indeterminate CD. Two groups received E1224 for 60 days at doses of 200 mg (low dose) and 400 mg (high dose), one group took E1224 for 30 days at a dose of 400 mg (short dose), and one group received BNZ at dose of 5 mg/kg/day. All treatments were able to eliminate blood-circulating parasites during the treatment period. E1224 had a parasite clearance rate of 90, 89, and 76% for the low dose group, short dose group, and high dose group, respectively. However, at the 12-month evaluation clearance of all patients that received E1224 was significantly low, and only the high dose group (29%) led to results significantly better than placebo.

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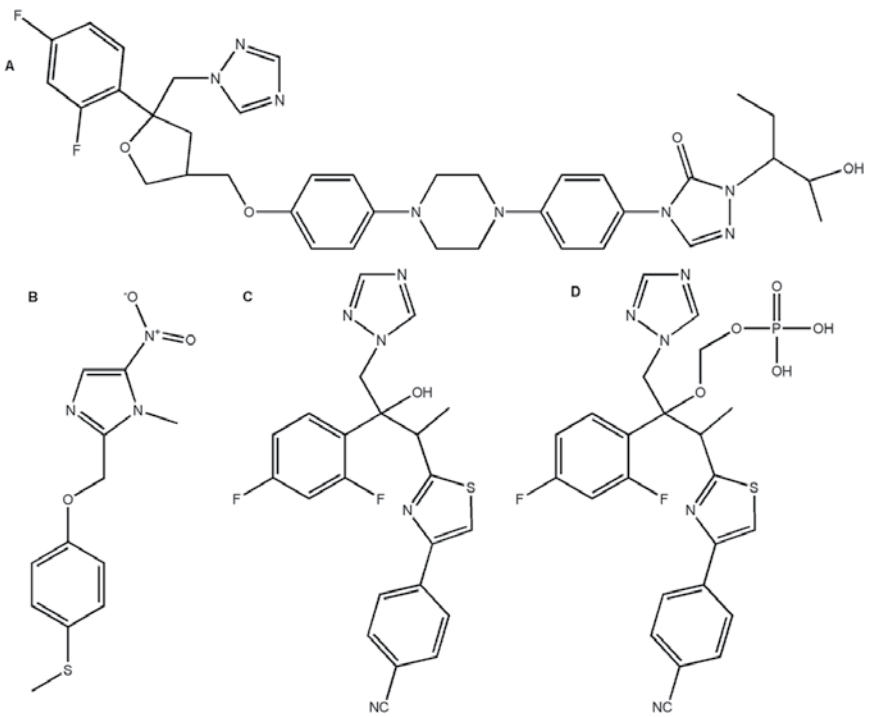

Figure 4. Structures of the compounds posaconazole (A), fexinidazole (B), ravuconazole (C), and E1224/fosravuconazole (D).

Alternatively, the BNZ clearance was 91% at 60 days after treatment and it dropped to 82% at 12 months, also being significant versus placebo. In summary, the drug candidate E1224 was as effective as BNZ at clearing the parasite following the treatment course, but the sustained efficacy 1 year following treatment was very low. Further, BNZ led to significant side effects. Therefore E1224, now called fosravuconazole, will be clinically tested in association to BNZ in order to improve treatment tolerability issues by combining them in the disease therapy.

A search for CD studies of new treatments on the clinical trials website (clinicaltrials.gov) provides one more study, with fexinidazole (**Figure 4B**). This compound is an old nitroimidazole that has shown promising results in preclinical models of CD [116]. According to Drug for Neglected Disease initiative (DNDi), this clinical study NCT02498782 was stopped due to safety and tolerability issues that occurred at the highest tested dose. High efficacy was observed at the lowest dose with acceptable safety and tolerability. Based on this, DNDi claims to be planning a new study in the near future [117].

5.2. Preclinical studies

Considering the lack of new and safer treatment options for CD, it is very encouraging to observe the efforts of many research groups investigating suitable candidates, which may improve the quality of life of many Chagas disease patients. Among the proposed targets for new alternative therapies examples are, ergosterol synthesis, cruzipain, trypanothione reductase, and type I

nitroreductase. Studies addressing these targets in several phases of development will be discussed in this text. However, only a few compounds have demonstrated *in vivo* activity and favorable disposition, while none of the published preclinical candidates seems to be in the boundary of the clinical studies.

5.2.1. CYP51

The sterol 14α-demethylase (CYP51) is an important enzyme for *T. cruzi* survival [118], which works as a target of broad-spectrum antifungal agents, such as POS and ravuconazole, or its prodrug fosravuconazole [119]. Since they were clinically tested, as previously mentioned, they are the most close that a new treatment has come to the patients, even though the clinical studies outcomes were not the best [113]. Albaconazole was considered promising after *in vitro* and *in vivo* studies, but its effect was not satisfactory against a resistant strain [120, 121].

Several others inhibitors of sterol biosynthesis have been tested in non-clinical phases. TAK-187 is another antifungal agent [122] tested for CD due to its good drug-like properties and mechanism of action aiming at CYP51. It has shown potent anti-*T. cruzi* activity *in vitro* and *in vivo* [122], while preventing *Trypanosoma cruzi*-induced cardiac damage [123]. Recently, a new formulation of TAK-187 was patented [124], indicating that a clinical study is potentially possible.

Dialkyl imidazoles have shown high potency (EC50 in the 0.4–10 nM range) against *T. cruzi in vitro*, leading two of them to be tested *in vivo* [125]. These derivatives were able to reduce the parasitemia to undetectable levels in a mouse model of acute CD. According to the authors, these compounds are less expensive to be produced than POS. Carrying systematic modifications in the proposed scaffold of the aforementioned candidates led to new candidates with an EC50 better than that of POS (1 nM); however, only *in vitro* studies have been performed [126].

Since tipifarnib has shown potent anti-*T. cruzi* activity, it is being considered as a new lead compound for CD treatment [127]. To evaluate this drug, analogs of the antitumor agent were designed and investigated *in vitro*. They were considered advantageous due to their modest potency for inhibition of human CYP3A4 [128]. One of these analogs, compound 2 was evaluated *in vivo*, but was not better than POS or BNZ [129]. Lack of adequate pharmacokinetic properties, with short half-life and low exposure, may cause the tipifarnib analog to be ineffective.

A very rational study was performed in the development of 4-aminopyridyl-based CYP51 inhibitors [130]. In this study, compounds were developed and tested for their *in vitro* activity. To assess their action *in vivo*, the pharmacokinetic study was previously performed to better understand the compounds disposition and modify the vehicle of administration. Some of the derivatives have demonstrated better *in vivo* activity when compared to the *in vitro* studies. For the authors, the disconnection between *in vitro* and *in vivo* potency raised questions about prioritizing the CYP inhibitors based only on their *in vitro* performance. They achieved lead compounds with good potency and oral bioavailability, while they admit the need for optimization of the elimination half-lives.

A similar study design was followed to evaluate analogs of fenarimol [131], where it was observed that compounds with long half-lives, that is, maintaining plasma concentration above the IC50, were those that led to undetectable parasite levels in bloodstream. The

compounds that were less effective to reduce the parasitemia were those with fast elimination and plasma concentrations equals or below the IC50. The two lead compounds were highly bioavailable with a long half-life. The *in vivo* efficacy was superior compared to BNZ and comparable to POS.

Assuming an evolution of the antifungal approach, VNI is an imidazol developed to be specifically active in the inhibition of the CYP51 of *T. cruzi*. It was able to cure the acute and chronic forms of CD in mice, with 100% survival and no observable side effects. Its pharmacokinetics were considered suitable for further development; however, the presented data were not completely explored [132]. The VNI optimization, VFV, overcame the promising results of VNI not only in the animal model of CD cure rate, but also with better pharmacokinetic properties and more information regarding distribution and biotransformation [133]. The reporting of VFV acute toxicity study have shown no observed adverse effect up to 200 mg/kg in mice, but authors did not present data to assure that the absorption was not saturated, that is, the exposure was proportional to the doses used in the acute toxicity study [134]. These imidazole-containing compounds seem to be the most advanced compounds, but the authors highlight the need for further studies.

5.2.2. Cruzipain

The cysteine proteinase cruzipain is vital for *T. cruzi* survival, since it is essential for the parasite replication and differentiation. Based on this, cruzipain has been considered as a target for drug discovery, but also for vaccines [135]. Phe-ala-PQ was a prodrug of primaquine, which had the intent to use the proteinase function of cruzipain to release the parent molecule in the parasite [136], but there are others.

Vinyl-sulfone derivatives [137], such as K11777 and WRR-483 have irreversible inhibitory activity in cruzipain, leading them to be effective against *T. cruzi in vitro* and, also *in vivo* [138, 139]. On the other hand, Cz007 and Cz008 are vinyl-sulfones containing nitrile with reversible cysteine proteinase activity that showed *in vivo* anti-*T. cruzi* action following oral administration in a dose lower than that of BNZ [140, 141]. Authors report bioavailability of both new compounds to be approximately 50%, but with short elimination half-life, what led them to administer the compounds for the efficacy study mixed to powdered chow, while BNZ was administered in water.

Additional compounds focusing on cruzipain have their anti-trypanocidal activity published only *in vitro*, such as thiazolyl hydrazones [142], thio semicarbazones [143], acylhydrazones [144], dipeptidyl nitrile derivatives [145], and triazine and purine nitriles [146].

5.2.3. Trypanothione reductase

Trypanothione reductase is responsible for the parasite antioxidant protective mechanism, thus inhibitors aiming for this target may disrupt its antioxidant defenses [147]. Some tricyclic compounds already active for other conditions were able to inhibit trypanothione reductase, and clomipramine was the most active with an inhibition constant (Ki) of 6 μM [148]. The testing for new active compounds led to the discovery of inhibitors with a Ki up to 0.33 μM

[149]. Among the phenothiazines, thioridazine is one of the most potent irreversible inhibitors of trypanothione reductase [150], and has shown better *in vivo* activity than the non-treated group [151]. When compared to BNZ, thioridazine animal survival was comparable of BNZ in mice infected with *T. cruzi* [152].

Moreover the trypanothione reductase inhibition, hydroxymethyl nitrofurazone (NFOH) is able to inhibit cruzipain by 60%, showing *in vivo* activity and experimental animal survival comparable to BNZ [153]. The NFOH pharmacokinetics were studied in rats and rabbits [154, 155], and indicated that optimization is needed despite the dual action.

Additional compounds able to inhibit trypanothione reductase *in vitro* include quaternary analogs [156] of chlorpromazine. The dibenzosuberyl-containing analogs of clomipramine were poor inhibitors of trypanothione reductase, while the polyamine derivatives containing dibenzosuberyl were potent inhibitors up to 0.26 μM, even though they were not active *in vivo*, maybe due to poor pharmacokinetic properties, which were not evaluated [157]. Studying the *in vitro* anti-*T. cruzi* activities of ethyl and methyl quinoxaline-7-carboxylate 1,4-di-N-oxide derivatives, three compounds were discovered. However, only one was non-cytotoxic on host macrophage cells [158], and no *in vivo* study was presented.

5.2.4. Type I nitroreductase

Type I nitroreductase is an enzyme responsible for the differentiation of *T. cruzi* and for the activation of nitroheterocycles [159]. Nitroreductases are among the enzymes proposed as responsible for the bioactivation of BNZ [159] and NFX [160]. Based on this hypothesis, the impairment of this enzyme would be responsible for resistance [159].

The type I nitroreductase bioactivation approach supports the *in vitro* action of aziridinyl nitrobenzamide [161] and halogenated nitrobenzylphosphoramide mustards [162] prodrugs; however, the last was assessed in *T. brucei* parasites. Several nitrotriazole-based compounds demonstrated significant *in vitro* antichagasic activity [163, 164]. Further studies identified compounds with promising *in vivo* activity that has shown good *in vitro* ADME properties [165].

Nitrotriazole compounds with dual action aiming type I nitroreductase and inhibition of CYP51 were able to clear the parasites following a 10-day treatment, better than what was observed in the previous mentioned monofunctional 3-nitrotriazole-based derivatives [166]. Prior to the positive results of the dual action compounds, some experiments led to the understanding that nitroheterocylic compounds are better than those able to inhibit ergosterol biosynthesis [167], while it seems that together, they can work better.

Many preclinical candidates have only proven to be effective *in silico* or *in vitro* becoming promising leads or proving the validity of the proposed strategy. Novel drug candidates with *in vivo* activity, in limited cases have published disposition data, such as bioavailability and elimination half-life. The crucial study before clinical studies is the achievement of the NOAEL, since it is necessary to calculate the first in human dose. However, for compounds not showing clear toxicity without the pharmacokinetic assessment, the NOAEL can be overestimated due to saturated absorption, and this can be an challenge for compounds without this data [168].

5.2.5. Combined alternatives in preclinical studies

Based on the safety issues of BNZ and NFX, strategies to reduce their administered amount would prove beneficial as it may decrease the adverse effects. To circumvent the decrease in efficacy due to dose reduction, combined therapies have been proposed.

The combination of the diamidine prodrug DB289 and BNZ orally decreased parasitemia by 99%, while alone they led to 70 and 90% for DB289 and BNZ, respectively. When the combination of BNZ and DB766 was administered, the decrease in the parasitemia was at least 99.5%. Both combined treatments provided 100% protection against mice mortality, while BNZ alone provided only 78% protection [169].

The combination of BNZ and ketoconazole were evaluated in a disease mouse model. This led to better results than single treatments with susceptible and moderately resistant (Y) strains of *T. cruzi*. [170]. The same Y strain of *T. cruzi in vivo* was used to evaluate the combination of BNZ and itraconazole and the results indicated a fourfold improvement in the disease outcome against each single treatment. Additionally, this combination led to a decrease in cardiotoxicity in the experimental CD [171].

The new compound tetrahydro-β-carboline N-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide was superior among its peers in reducing the number of infected cells and the number of internalized parasites, with low cytotoxicity [172]. After this observation, the selected compound was evaluated in combination with BNZ *in vitro* and *in vivo*, where a synergic effect was observed, leading to a reduction in parasitemia and mortality rates [173].

An additional compound tested with BNZ was the new drug candidate 2-methyl-1,5-bis(4-nitrophenyl)penta-1,4-dien-3-one, which has shown trypanocidal activity *in vitro*. Next, it was evaluated in combination with BNZ, ketoconazole, and fluconazole. Although the combination with the last one was ineffective, the combination with BNZ and ketoconazole demonstrated strong synergism [174].

These studies indicate that combination of new compounds or compounds used for alternative conditions have the potential to be a successful approach. A similar strategy has been planned for clinical trials to evaluate the combination of benznidazole and fosravuconazole, despite the absence of advantage in the combined therapy using BNZ and POS.

6. Conclusion

Once no new therapeutic alternatives have been included in its treatment portfolio after benznidazole and nifurtimox introduction there is a need for new compounds able to treat patients of CD. Many new drug candidates are evaluated to provide a better treatment for patients in the chronic phase. Benznidazole and nifurtimox are very effective, but their adverse effects cause many patients to discontinue treatment. Symptomatic treatment of the chronic phase of the disease is as important as the treatment directed in the reduction of the parasite. It

is necessary to evaluate the patient conditions correctly to improve the quality of life. It is comprehensible that the chase for new options against this disease begins with a search of new molecules, but it is important to keep in mind that this is just a small step. Significance can only be measured if the following steps are performed and the patient treatment is reached. The combined therapy and the repurposing approaches may discover the next treatment option; however, the research must not be completed until the ideal treatment is achieved.

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Author details

Wilton H. Kawaguchi[1], Leticia Bonancio Cerqueira[1], Mariana Millan Fachi[1], Michel L. Campos[1], Iara J. Messias Reason[2] and Roberto Pontarolo[1]*

*Address all correspondence to: pontarolo@ufpr.br

1 Department of Pharmacy, Federal University of Paraná, Curitiba, Paraná, Brazil

2 Clinical Hospital, Federal University of Paraná, Curitiba, Paraná, Brazil

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Chapter 9

New Approaches for Chagas' Disease Chemotherapy

Guadalupe García Liñares

Additional information is available at the end of the chapter

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Abstract

The latest advances concerning drug design and chemotherapy development to combat the Chagas' disease are discussed. This chapter is based on the metabolic differences between the pathogenic parasite and mammal hosts that led to the progress in the search for novel metabolic pathways in parasites that may be essential for parasite's survival but with no counterpart in the host. There is a considerable amount of work in the search of more promising molecular targets for drug design. However, the chemotherapy for this disease remains unsolved. It is based on old and fairly not specific drugs associated with long-term treatments, severe side effects, drug resistance, and different strains' susceptibility. Herein, a thorough analysis of selected molecular targets is described in terms of their potential usefulness for drug design. Therefore, rational approaches to the chemotherapeutic control of American trypanosomiasis describing some useful metabolic pathways are covered. Enzymes involved in ergosterol biosynthesis (squalene synthase, HMG-CoA reductase, farnesyl diphosphate synthase (FPPS), sterol 24-methyltransferase, and sterol 14α-demethylase), trypanothione system (glutathionyl-spermidine synthetase, trypanothione synthetase, and trypanothione reductase), cysteine proteases, transsialidase, and so on are discussed. The design of specific inhibitors of these metabolic activities as possible means of controlling the parasites without damaging the hosts is presented.

Keywords: *Trypanosoma cruzi*, drug development, molecular targets

1. Introduction

Chagas' disease or American trypanosomiasis is among the most prevalent parasitic diseases worldwide [1–3]. It has been estimated that around 20 million people are infected and over 40 million individuals are facing the risk of infection by the hemoflagellates protozoan *Trypanosoma cruzi*, the responsible agent of Chagas' disease. This disease is endemic in Latin

American countries, but the migration of individuals and blood transfusions have made possible the occurrence of the Chagas' disease in developed countries. Currently, no vaccine is available, so the control of the disease is limited to its detection, vector control, screening of blood banks and organ donors, and case finding of infected pregnant women. Moreover, the development of a safer and more effective chemotherapeutic intervention is crucial for the treatment of Chagas disease.

This disease is characterized by three phases: (1) an early acute phase in which trypomastigotes circulate in blood and infect cells transforming into the asexually-multiplying amastigotes; (2) a subsequent intermediate phase where the cells are broken, parasites are released to the blood and infect other cells (in this phase there are unspecific symptoms like fever, allergic reactions, acute heart failure, or meningoencephalitis); (3) a late chronic phase, a prolonged, and asymptomatic indeterminate phase, where parasites establish in their target organs (during this stage patients may have nonspecific clinical manifestations or present major complications such as cardiomyopathy and/or megaesophagus and megacolon syndromes [4]. The absence of adequate treatment in the acute phase results in the development of the above-mentioned stages of the disease.

The existing chemotherapy remains deficient; it is based on two old drugs empirically discovered, (**1**) nifurtimox, actually discontinued, and (**2**) benznidazole (**Figure 1**). Although both of these compounds are able to cure at least 50% of recent infections, they present important drawbacks such as selective drug sensitivity on different *T. cruzi* strains, serious side effects including vomiting, anorexia, peripheral neuropathy, allergic dermopathy, and long-term treatment [2, 5, 6]. Moreover, these compounds are not effective in the chronic stage of the disease. Consequently, the development of novel, safe, and affordable compounds with potent antiparasitic activity is urgently needed [7, 8]. The existence of *T. cruzi* populations naturally resistant to benznidazole and nifurtimox led to the search for compounds with a different mechanism of action [2, 8]. The development of new drugs that are more effective and safer than those currently available is urgently necessary.

There is a considerable amount of work in the search of unique aspects of the biochemistry and physiology of *T. cruzi* intending to find specific molecular targets for drug design [9]. It can be thought that the selective inhibition of a biosynthetic pathway that leads to a crucial metabolite for parasite survival would not have any significant toxic effect on the host. Based on these facts, this chapter will discuss the search for new approaches based on metabolic differences between the pathogenic parasite and mammal hosts and the development of new potential antiparasitic drugs in the last years.

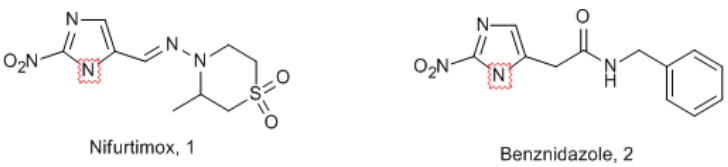

Figure 1. Drugs currently used for Chagas' disease treatment.

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2. Molecular targets

There are many metabolic pathways and enzymes unique to *Trypanosoma cruzi* that constitute excellent molecular targets for drug development. However, despite the specificity of new compounds targeting parasite molecules, the effect of these drugs on mammalian metabolism must be carefully evaluated. A few of the most studied compounds targeting specific *T. cruzi* enzymes will be reviewed here.

2.1. Sterol biosynthesis

Isoprenoid biosynthetic pathway constitutes one of the most important metabolic pathways of all organisms because isoprenoids are essential for numerous biochemical functions. In trypanosomatides diverse enzymes of this biosynthetic pathway are involved in key process. Sterol biosynthesis in parasites differs from that in mammalian hosts since the final product is ergosterol instead cholesterol, the main sterol present in the mammals. As *T. cruzi* is entirely dependent on endogenously produced sterols for survival and proliferation, the sterol biosynthetic pathway constitutes an attractive target for drug development.

Reduction of endogenous sterols induces inhibition of the multiplication of *T. cruzi*. Then, the restriction of an enzyme of this biosynthetic pathway will inhibit the growth of the parasite [10, 11]. Sterol composition in *T. cruzi* is very similar to fungi with ergosterol and 24-ethylergosterol being the primary mature sterols in the epimastigote stage. Fungisterol and 24-ethylfurgisterol are the major sterols produced by the amastigote stage. In consequence, antifungal drugs are potentially capable of decreasing pathogen growth.

There are several interesting enzymes in the pathway as potential targets for anti-trypanosomal chemotherapy. For example, sterol 14α-demethylase, sterol 24-methyltransferase, farnesyl diphosphate synthase, squalene synthase, and HMG-CoA reductase.

2.1.1. Sterol 14α-demethylase (CYP51)

Sterol 14α-demethylase is a CYP monooxygenase that catalyzes the removal of the 14α-methyl group from eburicol. Unlike other hemoproteins, the hemo cofactor in CYP51 is coordinated to cysteine residue instead of histidine. Since it is an essential enzyme in sterol biosynthesis, the activity inhibition could be lethal in organisms requiring sterols for membrane function [12]. This enzyme constitutes an interesting target as it has the advantage of being inhibited by antifungal agents currently in clinical use. Azoles are the most efficient antifungal drugs and there have been reported numerous examples of its antiparasitic effects [13–15].

The first azoles such as ketoconazole (**3**), miconazole (**4**), or fluconazole (**5**) (**Figure 2**) were found to have potent *in vitro* activity but did not cure the *T. cruzi* infection. The mechanism of action involves binding to *T. cruzi* CYP51 and a disruption of sterol biosynthesis resulting in accumulation of 14-methylated sterols [16]. New azole drugs developed to combat fungal infections have been evaluated for the activity against *T. cruzi*. The experimental azole drug D0870 (**6**) was the first to cure the chronic infection in mice [17]. Unfortunately, D0870 was discontinued as an antifungal agent due to undesired side effects.

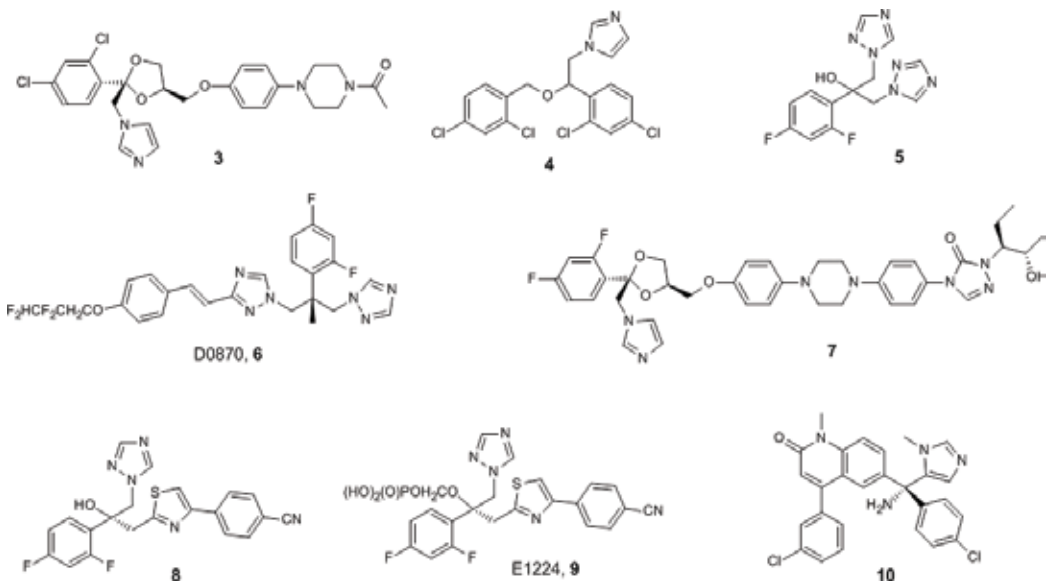

Figure 2. Structures of sterol 14α-demethylase (CYP51) inhibitors.

Among second-generation azoles, posaconazole (**7**) is the most potent drug against *T. cruzi*; because of its broad-spectrum antifungal activity, it could potentially be repurposed for use in Chagas' disease (**Figure 2**) [14]. It has been demonstrated that posaconazole is curative in the chronic murine model. In addition, it has potent activity against benznidazole and nifurtimox resistant *T. cruzi* strains [18]. Recently, a case was reported in Spain, describing the cure of chronic infection by treatment with posaconazole in an immunosuppressed patient. This compound is currently in phase II clinical trial for Chagas' disease. [19, 20] Unfortunately, posaconazole is a very expensive drug, so its application becomes impractical for patients with limited resources.

Ravuconazole (**8**) is another azole antifungal drug that has been evaluated as an antiparasitic agent and it appeared to be very efficient in restraining the parasitemia *in vitro* against *T. cruzi* in murine models [21]. However, since the half-life is much longer in humans than in mice, it is possible that this drug has curative effects. In fact, a prodrug of ravuconazole (E1224 **9**) is in a phase II trial [20]. In addition, ravuconazole has a simpler chemical structure than posaconazole, so the cost might be lower (**Figure 2**).

Tipifarnib (**10**) is an antitumor agent inhibiting human protein farnesyl transferase (**Figure 2**). It has considerable *in vitro* activity against *T. cruzi*. It was determined that the drug produces restriction of sterol biosynthesis by inhibition of CYP51 [22]. Analogs of tipifarnib have been designed and synthesized with improved CYP51 inhibitory activity and excellent pharmacokinetic properties [23].

2.1.2. Sterol 24-methyltransferase (24-SMT)

This enzyme catalyzes the methenylation of zymosterol, an important step for the biosynthesis of ergosterol and other related 24-alkylated sterols, which are the main sterols

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found in cell membranes of *T. cruzi* [24]. Azasterols are sterol compounds containing a nitrogen atom in the positions 23, 24, or 25 of the side chain. It was reported that these compounds inhibit the enzyme 24-SMT and demonstrated antiproliferative effects against trypanosomatids. 22, 26-azasterol (AZA, **11**), and 24, 25-epiminolanosterol (EIL, **12**) were the first azasterols reported to have trypanocidal activity [24, 25]. Different azasterols with modifications on their basic structure have been designed, synthesized, and biologically evaluated as antiparasitic agents [26, 27]. An important observation to take into account is that the 3β-OH group must be acylated. On the other hand, the nitrogen atom in the side chain can be located at the 23–25 position. The side chain can be attached via amine or amide bond and the presence of an ester moiety increased the activity [27]. General structures (**13**) of compounds, which have been developed as inhibitors of 24-SMT are shown in **Figure 3**.

2.1.3. 3-Hydroxy-3-methyl-glutaryl-coenzyme a reductase (HMG-CoA reductase)

The enzyme HMG-CoA reductase is involved in the first step in the pathway of isoprenoid biosynthesis and catalyzes the reduction of 3-hydroxy-3-methyl-glutaryl-coenzyme A to mevalonate. Therefore, HMG-CoA reductase is a key enzyme and constitutes a valid molecular target since its inhibition will also prevent the synthesis of compounds of the mevalonate pathway.

Current cardiovascular drugs have been tested for the treatment of Chagas' disease but new therapeutic drugs based on statins with a new anti-inflammatory approach have arisen as potential antiparasitic agents. Statins are thought to be associated with their ability to reduce cholesterol synthesis [28]. It was observed that lovastatin (**14**) and simvastatin (**15**) (**Figure 4**) have inhibited the growth of epimastigotes of *T. cruzi* and simvastatin could potentially inhibit HMG-CoA reductase both in epimastigotes and trypomastigotes [29]. Moreover, the combination of lovastatin with ketoconazole allowed the elimination of the presence of parasites into the blood flow and, in this way, prevented host death [30].

2.1.4. Farnesyl diphosphate synthase (FPPS)

The enzyme farnesyl pyrophosphate synthase, also known as farnesyl diphosphate synthase (FPPS) belongs to the E-family of the prenyltransferases and it has a key role in the isoprenoid biosynthetic pathway. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are synthesized via mevalonate pathway from acetyl-CoA [31]. DMAPP is the precursor for the biosynthesis of different and very important isoprenoids like sterols, ubiquinones, triterpenoids, and prenylated proteins.

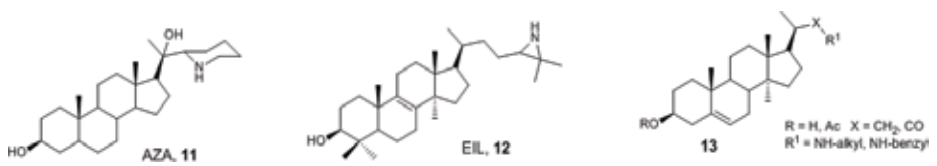

Figure 3. General structures of inhibitors of 24-SMT.

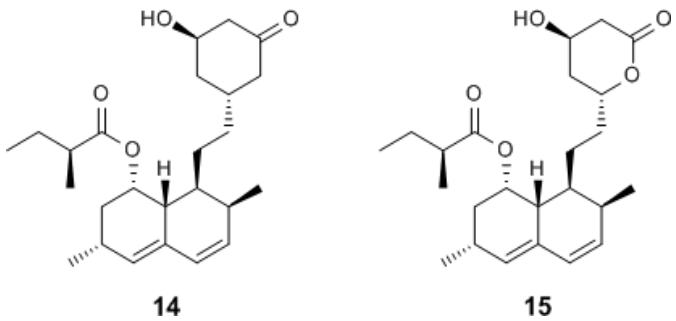

Figure 4. Chemical structures of inhibitors of the enzymatic activity of HMG-CoA reductase.

FPPS catalyzes two sequential steps: the addition of DMAPP to isopentenyl diphosphate (IPP) to form geranyl diphosphate (GPP) and the addition of DMAPP to geranyl diphosphate to produce farnesyl pyrophosphate (FPP). The inhibition or alteration of FPPS activity can regulate the isoprenoid metabolism. Therefore, FPPS has been selected as an excellent target for different disorders and anticancer, antibacterial, and antiparasitic drugs design among others [32–34].

Bisphosphonates act as inhibitors of bone resorption binding to the bone mineral. They are currently used for the treatment of several bone disorders like osteoporosis, Paget's disease, and hypercalcemia [35]. Bisphosphonates were the first FPPS inhibitors known and were reported as potent antiparasitic agents. Nitrogen-containing bisphosphonates like pamidronate (**16**), alendronate (**17**), risedronate (**18**), and ibandronate (**19**) were originally found to be effective against *T. cruzi* without toxicity to the host cells (**Figure 5**) [36]. Risedronate has shown a significantly increased survival of *T. cruzi*-infected mice *in vivo*. It was also found that diverse bisphosphonate derivatives were effective growth inhibitors of other pathogenic trypanosomatids and apicomplexan parasites [32, 37, 38].

Bisphosphonates have the disadvantage that they are highly polar and are rapidly removed from the circulatory system. Therefore, more lipophilic derivatives were developed (**20**) [38–40]. Bisphosphonates have a great potentiality as antiparasitic agents with characterized mechanisms of action involving the inhibition of FPPS, being very proper candidates to control and treat American trypanosomiasis. In addition, bisphosphonates have the advantage of being inexpensively synthesized and many compounds are FDA-approved drugs for the long-term treatment of several diseases.

2.1.5. Squalene synthase (SQS)

Squalene synthase (SQS) is also a key enzyme of ergosterol biosynthesis, which catalyzes the condensation of two farnesyl pyrophosphate molecules to form presqualene diphosphate and the subsequent loss of diphosphate, rearrangement, and reduction by NADPH to form squalene. Then, squalene epoxidase catalyzes the epoxidation of squalene affording oxidosqualene, which is cyclized by oxidosqualene cyclase to form lanosterol [41, 42]. Therefore,

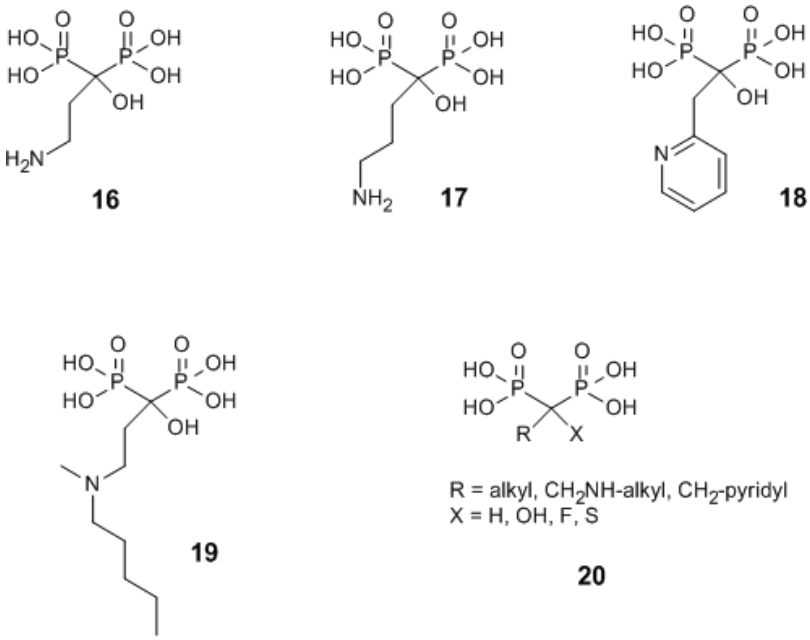

Figure 5. Chemical structures of representative bisphosphonates targeting FPPS.

any of these enzymes constitute an excellent molecular target for antitrypanosomal agent's development. SQS is also under intense study as a possible target for cholesterol-lowering drugs in humans [41].

Quinuclidines were developed as cholesterol-decreasing agents but actually, they also turned out to be potent SQS inhibitors. However, they showed poor SQS selectivity. It has also been reported that this class of compounds eliminated the parasite both *in vitro* and *in vivo* [43, 44].

SQ109 (**21**, **Figure 6**), an ethylenediamine currently in phase II clinical trials for the treatment of tuberculosis, is of great interest for the etiological treatment of Chagas' disease [45]. Studies have conveyed that SQ109 was an inhibitor of dehydrosqualene synthase from *Staphylococcus aureus*, a protein very similar to squalene synthase, suggesting that SQ109 might also inhibit *T. cruzi* SQS [46]. In fact, recently, it was determined that SQ109 is active against all life cycle stages of *T. cruzi*, detecting the most potent activity against the highly infective trypomastigote form. Furthermore SQ109 showed synergism with posaconazole [47].

Other very interesting SQS inhibitors that have been discovered include thiocyanates like WC-9 (**22**), which proved to be a potent inhibitor of this enzyme [48]. Fluorine-containing thiocyanate derivatives exhibited higher efficacy as inhibitors of *T. cruzi* proliferation (**23**,**24**) (**Figure 4**) [49]. Recently, the structures of human SQS and *T. cruzi* SQS bound to a substrate-like inhibitor were reported suggesting an interesting alternative for the development of selective drugs [50].

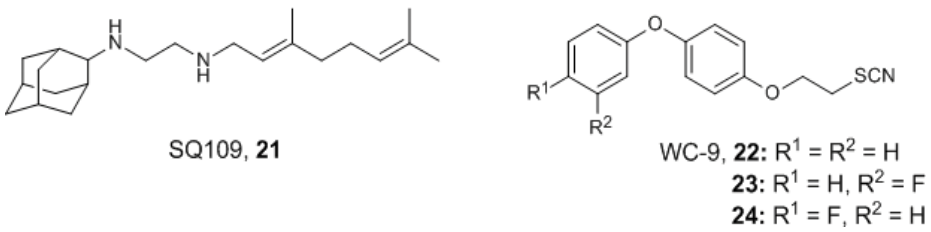

Figure 6. Chemical structures of inhibitors of the enzymatic activity of squalene synthase.

2.2. Cruzipain

Cruzipain is the main cysteine proteinase of *T. cruzi*, which is essential for the survival of the parasite. This enzyme is involved in different cellular functions such as nutrition, penetration into the host cell, defense, and differentiation processes [51]. It has been extensively studied as a valid target for new drug development [52, 53]. There are several three-dimensional structures of cruzipain with different inhibitors allowing the identification of structural regions of this enzyme that will enable the design of new agents [54, 55].

Numerous structurally varied compounds that inhibit proliferation of *T. cruzi* by inhibiting the enzymatic activity of cruzipain have been reported. Among the compounds tested, K777 (**25**), a vinyl sulfone derivative was active against a wide range of susceptible and resistant strains (**Figure 7**). Moreover, it was able to cure *T. cruzi* acute and non-acute infection in mice, showing also synergistic activity with benznidazole [56, 57].

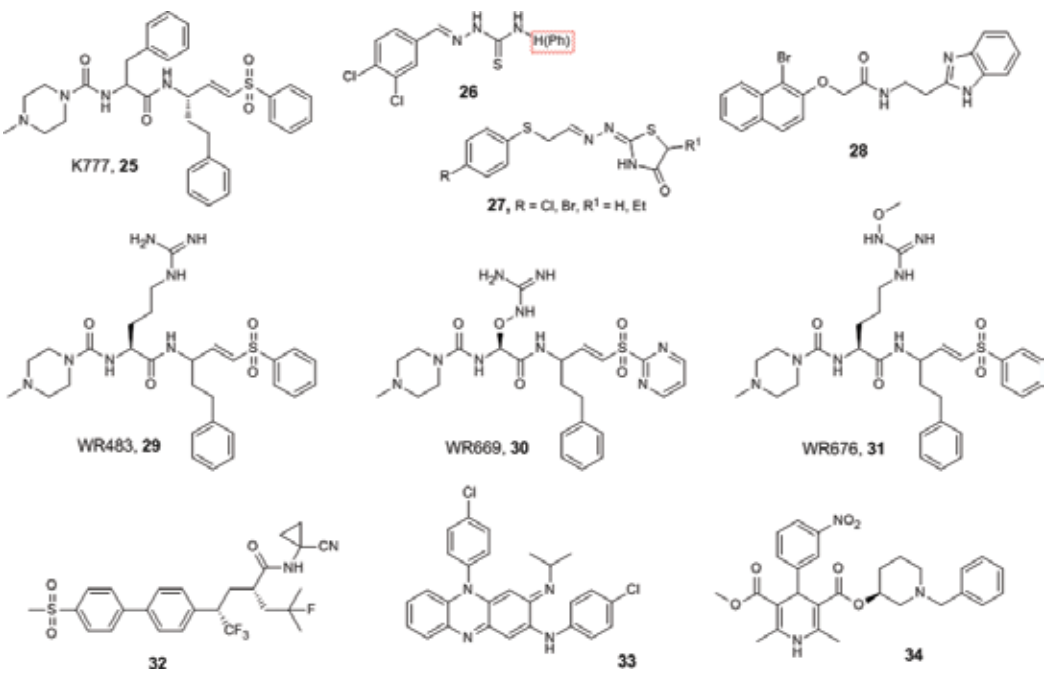

Figure 7. Structures of inhibitors of cruzipain activity.

Molecules containing thiosemicarbazones have been extensively explored [58–60]. Among these, 3, 4-dichlorophenyl thiosemicarbazone (**26**) is one of the most potent cruzipain inhibitor [61]. Recently, thiazolidinone derivatives (**27**) were identified as strong antiparasitic compounds (**Figure 7**) [62, 63].

Benzimidazoles have also been studied as cruzipain inhibitors and various derivatives have been synthesized and evaluated. *N*-(2-(1H-benzo[d]imidazol-2-yl) ethyl)-2-(1-bromonaphthalene-2-yloxy) acetamide (**28**) was the most potent enzyme inhibitor but it showed moderate trypanocidal activity [64, 65].

Other very potent cruzipain inhibitors have been developed such as oxyguanidine derivatives WRR-483, WRR-669, and WRR-676 (**29**–**31**). Some of these compounds showed suitable metabolic stability and a remarkable trypanocidal activity [66].

Very recently a series of peptidyl nitroalkenes was designed, synthesized, and evaluated as cruzipain inhibitors. Several compounds showed high activity against the enzyme observing the peptidic nature to be the determinant for their inhibitory activity [67].

Odanacatib (**32**), clofazimine (**33**), and benidipine (**34**) are examples of drug repurposing for the development of novel therapeutics for the Chagas' diseases (**Figure 5**). Odanacatib is a cathepsin K inhibitor used for the treatment of postmenopausal osteoporosis; it is a potent cysteine protease inhibitor, which is in Phase III clinical trials [68]. Clofazimine is an antibiotic applied to the treatment of leprosy [69] and benidipine is a calcium channel-blocking agent employed in the treatment of hypertension [70]. Clofazimine and benidipine were recently tested in a murine model of chronic Chagas' disease infection. Both compounds have reduced the parasitemia and the inflammatory effects have been well tolerated [71].

2.3. *Trans*-sialidase

The enzyme *trans*-sialidase (TS) belongs to the group of enzymes that are secreted by the parasite involved in the processes of cell invasion and immune evasion [72]. *T. cruzi* incorporates sialic acid (**35**) from exogenous sialoglycoconjugates by a trans-glycosylation reaction [73]. TS catalyze this transfer from host sialoglycoconjugates to glycoconjugates or mucins, which are attached to the cell membrane of *T. cruzi* via glycophosphatidylinositol anchors [74, 75]. Added to its important role, the absence of this enzyme in mammalian organisms makes it an excellent molecular target.

Within TS' inhibitors it can be mentioned fluorinated compounds like 9-benzoyl-3-fluoro-N-acetylneuraminic acid (**36**), and 3-fluorosialyl fluoride (**37**) selectively bind to the active site of the enzyme [76]. Sulfonamide-containing hydroxylated chalcones (**38**) and quinolones (**39**) are also specific inhibitors of *T. cruzi* TS, being dihydroxylated more potent than monohydroxylated derivatives (**Figure 8**) [77].

Some approved FDA drugs were evaluated on trypomastigotes deriving out of a computational screening protocol. The anti-inflammatory sulfasalazine (**40**) showed potent antiparasitic effects in *in vivo* assays, but with moderate TS inhibition. However, this drug and sulfonamide-containing compounds could be used as leading drugs in the development of new TS inhibitors [78].

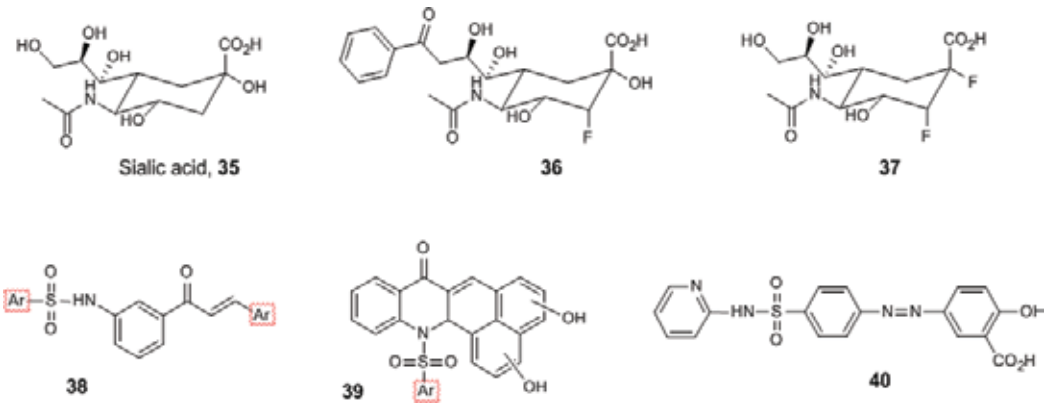

Figure 8. Representative inhibitors of *trans*-sialidase.

2.4. Trypanothione system

Trypanothione ($T[SH]_2$ or N^1, N^8-bis-(glutathionyl)spermidine, (**41**)) is a dithiol, which is responsible for the thiol metabolism in trypanosomatids by maintaining the intracellular redox balance [79]. Trypanothione is responsible for the protection against oxidative stress in *T. cruzi* trapping reactive oxygen species [80].

The protective reactions involve two key enzymes, which keep the trypanothione system operating: trypanothione reductase (TryR), homologous to mammal glutathione reductase and trypanothione synthetase (TryS) [81]. TryR catalyzes the reduction of trypanothione disulfide ($T[S]_2$, **42**) to $T[SH]_2$ (**Figure 9**); TryS catalyzes the synthesis of $T[SH]_2$ from glutathione and spermidine, keeping the amount of total trypanothione constant [82]. Taking into account the fact that trypanothione system is essential to the survival of parasites, these enzymes emerge as valid molecular targets for the search of new chemotherapeutics agents. In addition, TryS has no counterpart in mammals, making even more interesting to the design more specific and safer inhibitors [2].

Various reports have conveyed that different classes of compounds have selective enzyme inhibition activity such as tricyclic ring structures, bicyclic, and heterocyclic compounds or polyamines among others.

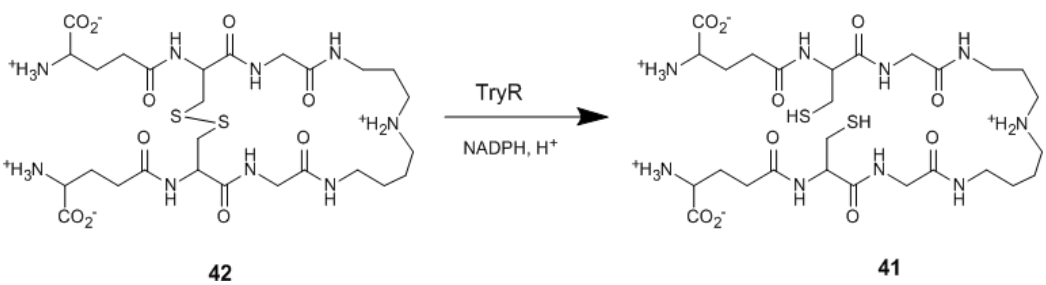

Figure 9. Structures of the oxidized and reduced forms of trypanothione.

Mepacrine (**43**) is a tricyclic antimalarial, which showed TryR inhibition without affecting human glutathione reductase and several mepacrine derivatives have been prepared. Although these derivatives presented greater potency, they turned out to be toxic to human cells [83].

Tricyclic phenothiazine-containing drugs are currently used as an antidepressant and have additionally exhibited antimicrobial activity [84]. Some phenothiazines demonstrated inhibitory activity against TryR [85]. Within them, thioridazine (**44**) is one of the most potent TryR inhibitors as investigations have suggested [86]. Clomipramine (**45**) [87] is another psychiatric drug with inhibiting action towards TryR. Both thioridazine and clomipramine seem to have *in vivo* effects against *T. cruzi* (**Figure 10**).

Library screening has allowed the identification of other classes of inhibitors that could be useful for the development of more potent TryR inhibitors. Some examples of these compounds are indatraline (**46**), a monoamine transporter inhibitor, 1-(2-(benzhydryloxy)ethyl)-4-(3-phenylpropyl)piperazine (GBR-12935, **47**) and a benzothiophene-piperidine derivative (BTCP **48**) [88]. Other or additional, studies allowed finding analogs of these compounds, which have increased the potency against TryR exhibiting higher selectivity [89]. The most interesting compounds were a piperazine-phenothiazine derivative of GBR-12935 (**49**) and a diaryl sulfide BTCP derivative [50, 88, 90].

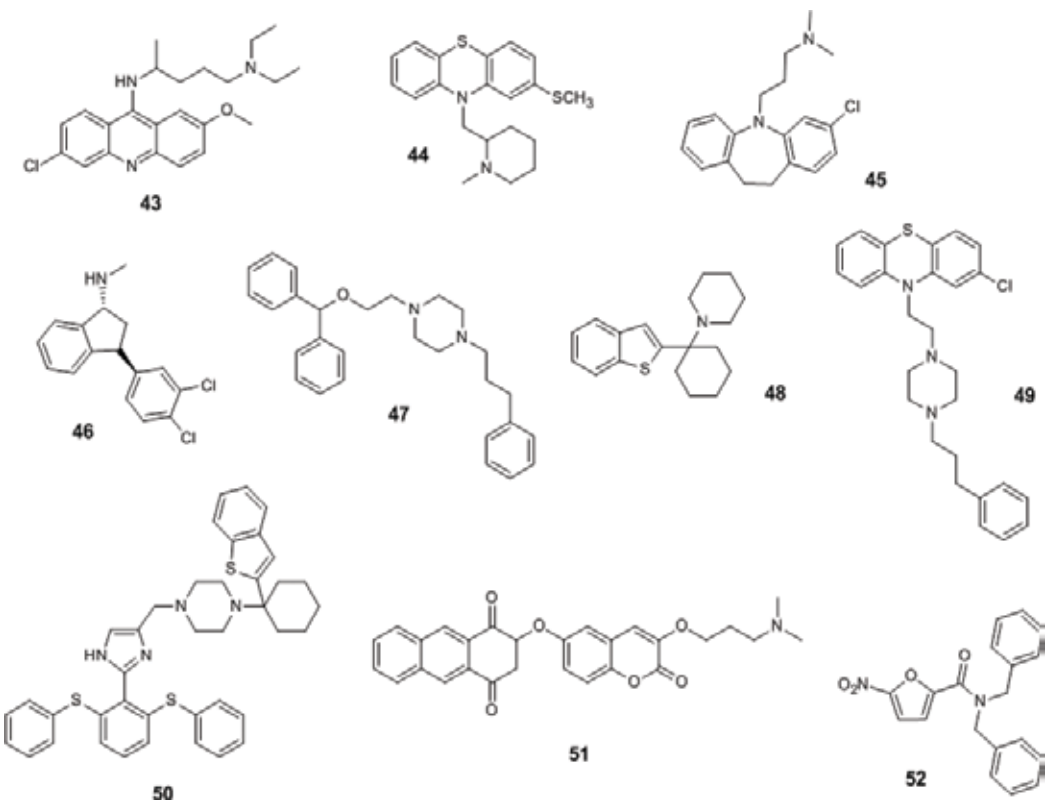

Figure 10. Chemical structures of relevant compounds targeting trypanothione reductase.

Different compounds can behave as TryR inhibitors when turning into reactive radical species through the reduction single-electron step [83, 91]. Within these structures, 1, 4 naphthoquinones and nitrofurans have been largely studied [92, 93]. The most potent derivative of 1,4 naphthoquinone was a quinone–coumarin hybrid [51] despite showing toxicity against rat skeletal myoblasts [94].

Between nitrofurans compounds, several 5-nitrofuroic acid derivatives have been synthesized and evaluated against *T. cruzi*. The best compound was 5-nitro-furan-2-carboxylic acid dibenzyl amide [52], which it significantly increased the trypanocidal nifurtimox activity being TryTR your molecular target [95].

3. Conclusions

Currently, the chemotherapy of American trypanosomiasis remains a serious problem in the field of neglected tropical diseases. There are no vaccines, and chemotherapy is limited to old drugs, which present important drawbacks. Taking into account that this disease is associated with poor populations and bad housing conditions, pharmaceutical companies have no economic motivations. Therefore, all efforts to the development of new drugs must be made by academic and/or governmental institutions and new chemotherapies are needed urgently. In order to search new, safer and efficient drugs for the Chagas' treatment, an overview of possible molecular targets based on specific features of the biochemistry of *Trypanosoma cruzi* was given. Although there are numerous potentially valid targets, only the more representative ones were discussed here. Furthermore, some of the new potential antiparasitic drugs as well as drugs applied to other human illness were described in this work. However, despite numerous efforts and progress in the searching of new or repositioned compounds for American trypanosomiasis chemotherapy, no ideal drugs are yet available for human treatment

Author details

Guadalupe García Liñares

Address all correspondence to: linares@qo.fcen.uba.ar

Departamento de Química Orgánica y UMYMFOR, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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Chapter 10

Slowed Development of Natural Products for Chagas Disease, how to Move Forward?

Javier Varela, Hugo Cerecetto and
Mercedes González

Additional information is available at the end of the chapter

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Abstract

Chagas disease, caused by *Trypanosoma cruzi*, is considered an endemic disease that affects millions of people causing generating health, economic and social problems. This study provides a review on research and development of new therapies for Chagas based in natural products of plant origin. We observed that there are more than 400 plant species that have been evaluated against different models of Chagas disease, and in some cases, there are interesting results. Challenge that hinders research work is the purification of the active compound and standardization of the chemical profile of whole extracts. The principal common factor that delays clinical testing is the lack of investment for the development of these products at the clinical phase. In the search of a natural, low cost and available drug for Chagas disease, we propose the use of new methodologies to overcome the existing challenges. The use of plant metabolomic technique is proposed as an option with high potential for the identification of biomarkers that could allow the standardization of chemical profiles. Furthermore, we describe the importance of applying good agricultural and manufacturing practices for reaching a successful development of quality phytotherapeutic products.

Keywords: natural products, metabolomics, *Trypanosoma cruzi*, Chagas disease

1. Introduction

"It does not explode like bombs or sound like shots. As hunger kills silently. As hunger kills the quiet, those who live condemned silence and die doomed to oblivion. The tragedy that does not ring, sick who do not pay, a disease that does not sell. Chagas disease is not a business that appeals to the pharmaceutical industry, nor is it a matter of interest to politicians

or journalists. Choose your victims in the pit. He bites them and slowly, little by little, and he ends up with them. Their victims have no rights, no money to buy the rights they do not have. They do not even have the right to know what they die from." *Informe clínico*, by Uruguayan writer Eduardo Galeano, in "*Chagas, una tragedia silenciosa,"* Médicos Sin Fronteras, Editorial Losada, 2005.

Chagas disease is caused by *Trypanosoma cruzi,* transmitted to humans through the bite of *Triatomine* spp. by contaminated defecation rubbed on the mucosa. Transmission can also occur by ingestion of contaminated food, through blood transfusion or vertical transmission from mother to child [1]. It is worth to highlight that this endemic disease causes health, economic, and social problems affecting 6–7 million people in 21 countries from Central and South America. Moreover, 25 million people remain under risk of infection, given the high levels of population mobility from Latin America to the rest of the world or residence in endemic regions [2, 3].

Trypanosoma cruzi (*T. cruzi*) presents a quite complex cycle with different forms throughout its evolutionary cycle. In the insect vector, different stages can be observed. The *spheromastigote*, characterized as the round form of the organism, is predominantly found in the stomach of the organism. The epimastigote stage is found in the gut of the vector, at this stage, the *T. cruzi* is intensively multiplies by binary division. And finally the metacyclic trypomastigote stage, which is the infecting form for the vertebrate host [4, 5]. When the insect feeds on human blood, it defecates on the host's skin, releasing into the feces parasites in the metacyclic trypomastigote form. Upon entry to the organ, trypanosomes are disseminated via the blood or lymphatic, affecting various organs mainly the heart, nervous system, muscle, and digestive system. Once the parasites reach the tissues they reproduce, passing through a non-flagellated stage, called amastigote, which is able to actively multiply themselves forming pseudocysts, where they are to be transformed into trypomastigotes that are later to be released [6]. It is estimated that the incubation period of the disease is between 5 and 12 days relative to each case, as well as the appearance of symptoms and the intensity of them. Often, cases are asymptomatic so timely diagnosis can be very difficult. Undiagnosed individuals may lead to conditions like serious cardiac and digestive conditions begin to arise that may lead to death [7]. It is possible to observe three well-defined stages of the disease. The initial stage is called acute or initial, which lasts approximately 2–4 months after infection. At this stage, many people do not have symptoms. Most patients with symptoms suffer from variable fever, malaise, irritability, headache, enlargement of the liver, spleen, and lymph nodes. When the inoculation is close to the ocular area, the characteristic chagoma is observed as well as a unilateral edema of both eyelids [8]. At this stage, the parasite can be found in the blood. Some acute cases can become deadly and many of these turn out to be young children and patients, who are immunocompromised (e.g. people infected with HIV), who may develop acute myocarditis or meningoencephalitis [9]. Then a second indeterminate or also called latent stage in which the infection becomes undetectable and is usually asymptomatic. It usually occurs between week 8 and 10 of the acute phase and it can last for months or even years. It is estimated that approximately 30% of individuals who reach this stage develop digestive, cardiac, and neurological problems [10]. Finally, the chronic phase which appears in the latter part of the infection, it is characterized by cardiac and intestinal problems. Sudden death can occur without the individual developing heart problems [11].

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The diagnosis of this disease is relative to infection of the patient. Direct methods (direct microscopic observation, xenodiagnosis, PCR, etc.) based on the detection of genetic material or parasites or indirect methods (ELISA, IFI, Western blot, etc.) based on the detection of specific antibodies against *T. cruzi*, mainly used in the chronic stage, either symptomatic or asymptomatic. These are usually used mainly in the acute phase, in the case of immunosuppressed persons or under 6 months of age [12].

Unfortunately, an effective chemotherapy for all the clinical forms of the disease has not been reached, although fair enough time has passed by since the discovery of the disease in 1909 by a Brazilian Doctor, Carlos Ribeiro Justiniano Chagas [2]. This intractable situation is the typical case of neglected diseases, where the lack of adequate therapies or effective vaccines provoque health crisis [13]. Despite the revealing evidence of the current situation has not received sufficient attention from the pharmaceutical industry, mainly due to economic considerations. The current pharmacological treatments are based on two nitroheterocycles compounds, which were discovered more than 30 years ago: nifurtimox (Nfx, N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitro-2 Methionine, Lampit®, suspended production, and sale by Bayer), and benznidazole (Bnz, N-benzyl-2-(2-nitroimidazol-1-yl) acetamide, Rochagan®, Roche, and currently produced By LAFEPE in Brazil). The problem with these drugs is that the significant side effects including weight loss, nausea and vomiting, rash, tissue abnormalities, leukopenia, neurotoxicity, psychosis, and peripheral neuropathy. On the other hand, Bnz can cause edema, fever, rash, peripheral neuropathy, lymphadenopathy, agranulocytosis, thrombocytopenic purpura, and joint and muscular pain. The use of these drugs during the acute phase of the disease is widely accepted, but its efficacy in the chronic phase is controversial [14].

The medicinal chemistry of Chagas disease has used different approaches in the search for new therapeutic entities. Some are oriented to the chemical development (synthesis) of new agents with interaction with key biomolecules for the parasite, or production of toxic species [15, 16]. Also, the reposition and polypharmacology of drugs have been used [17]. Other strategies have been oriented to the identification and isolation of new agents of natural origin [18]. Despite these efforts, research work in this medical area in order to find new solutions to a problem that seems to have no end is therefore, of utmost importance [14]. This review seeks answers in nature and always remembers the primary motivation: that affected people lacking the necessary resources find in their natural habitat medicinal plants that provide a possible treatment endorsed by science, effective and without side effects.

2. Natural products research

The use of plants for curative purposes dates back to the beginning of human history. The man turned to nature in search of food and health. By means of successes and errors, he learned to know the plants that healed. This knowledge was transmitted from generation to generation and was increased with experimentation. Without the resources offered by nature, humans would not have survived. Gradually humans, by dominating nature, have broken

many of the ties that bind him. Today the medicine uses synthetic or semisynthetic drugs to relieve all diseases. Many of these drugs are beneficial, but many also, by misuse or abuse have lost their efficacy and in countless cases cause harmful side effects [19, 20].

In Latin American countries, the use of medicinal plants is the usual practice of indigenous groups and is frequently used by certain sectors of society [21]. This practice is usually an economic alternative to the prices imposed by the pharmaceutical industries and in many cases the only possibility of treatment [21, 22]. Most medicinal plants have multiple physiological effects, due to the presence of more than one active principle. The latter correspond to chemical compounds of the plant, which are subject to variables, such as soil moisture, light conditions, temperature, date of planting and harvesting, drying conditions, and among others [23].

Fortunately, in recent years, there has been an increase in interest in the return to nature, and therefore, it is necessary to build a new relationship with our environment, leading a less artificial life and turning to plants not only to include them in our diet but also to alleviate our conditions [19].

Natural products contribute greatly to the history and landscape of new molecular entities (NMEs). An assessment of all FDA-approved NMEs reveals that natural products and their derivatives represent over one-third of all NMEs. By the end of 2013, the FDA had approved 547 natural products and derivatives. Since the 1970s, the relative and then an absolute number of natural-product-based NMEs began to decline and today stand at fewer than one-quarter (24%) or an average of 7.7 natural product NMEs per year [24]. Plant products represented more than one-fifth (22%) of all NMEs approved before 1950, declining by more than 50–8.7% since that time [24]. Over the past two decades, the pharmaceutical industry has shied away from research into natural products. Attention shifted toward combinatorial chemistry, which seemed to satisfy the need for compound libraries to keep up with high throughput assays based on newly discovered molecular targets. However, this approach did not result in improved productivity, nor did an increase in the number of new drugs. The number of NMEs reached a record low of 24 in 2004 with an average of 40–50 new approved drugs by year in the period of 1981–2014. In the same period of 1981–2014, 16 NMEs were approved as antiparasitic drugs. Newman and Cragg classified these NMEs as unaltered natural products, natural products derivatives, a synthetic drug, synthetic drug (natural product pharmacophore), and mimic of the natural product (**Figure 1**). Among them, 68.7% are related to natural products [25, 26].

Izumi et al. reviewed the prospect of developing new drugs for the Chagas disease, on the screening of almost 400 species belonging to more than 100 plant families for activity against *T. cruzi* [27]. The plant extracts preparation methods have been very variable, from processes involving only one part of the plant to extract with different polarity solvents as well as using all part of the plant in the same solvent. Usually, the plant part in the study is the same that is used traditionally, but this does not assure that the accumulation of the active principles is maximum in the selected part. The plant extracts screened against different intracellular

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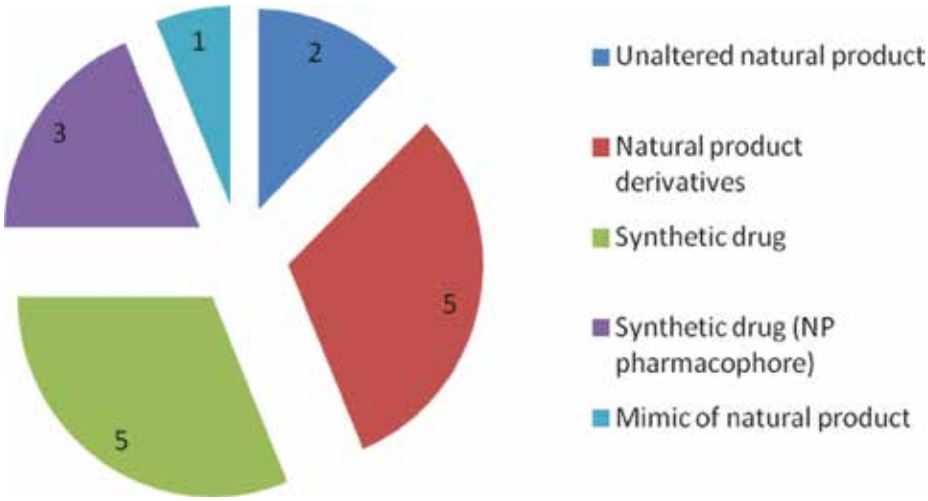

Figure 1. Classification of antiparasitic NMEs approved by FDA in the period 1981–2014.

forms of *T. cruzi* shows promising results. As a result, hexane extracts of *Polygala sabulosa* and aqueous extracts of *Polygala cyparissias* showed 50% inhibition of epimastigote growth after 72 h of treatment at concentrations of 1 and 2 μg/mL, respectively [28]. Ethanol extracts of *Physalis angulata* showed 50% inhibition of epimastigote growth after 120 h of treatment at a concentration of 2.9 μg/mL in Y strain and 7.4 μg/mL in Colombian strain [29]. Ethanol extracts of *Baccharis trimera* and *Baccharis articulata* showed 50% inhibition of epimastigote growth after 120 h of treatment at concentration 13.6 and 16.6 μg/mL in Tulahuen 2 strain, respectively [30]. For trypomastigotes, *Piptadenia africana* methanolic extract caused lysis in 50% of the parasites at 4 μg/mL after 96 h [31], and a methanolic extract from *Gardenia lutea* also promoted the same effect at approximately 22 μg/mL after 72 h [32]. The essential oil from the fruits of *Piper cubeba* showed 50% inhibition of trypomastigote form at a concentration of 45.5 μg/mL [33]. Surprisingly, by applying methanolic extracts of eight different species (*Hypoestes forsskalii, Kleinia odora* and *Psiadia punctulata, Capparis spinosa, Euphorbia schimperiana* and *Ricinus communis, Marrubium vulgare*, and *Solanum villosum*) an inhibition of 50% less than 0.25 μg/mL was found against the amastigote form with a treatment of 7 days [34]. Also for amastigotes, methylene chloride extract from leaves of *Conobea scoparioides* showed 50% inhibition after 96 h at 1.3 μg/mL [35]. Ethanol extracts of *Baccharis trimera* and *Baccharis articulata* showed 50% inhibition of amastigote growth after 72 h of treatment at concentration 9.9 and 22.3 μg/mL in Dm28c strain, respectively [30]. Given that the financial resources for research on neglected diseases are usually low, most of the laboratories cannot afford the maintenance of infected animal models or cell cultures to perform tests with the infective forms, trypomastigote, and amastigote. This leads to screenings of compounds performed only against non-infective forms, even when promising preliminary results are reached, the isolation of active compounds is not frequent. Despite the screening of hundreds

of species, the major, possibly active compound was only isolated, identified, and evaluated for antiparasitic activity in approximately 10% of the cases [27]. *In vivo* studies on the animal model of Chagas disease of plant extracts are less numerous than *in vitro* ones, but there are also reports of promising results. For example, *Serjania yucatanensis* ethanol leaves extract reduced 75% of the parasitemia in infected mice treatment at 100 mg/kg [36] and *Aristeguietia glutinosa* ethanol aerial parts extract reduced 50% of the parasitemia in infected mice at 50 mg/kg [37]. Plants extracts screening performed have been based on isolated efforts directed by research groups worldwide. Moreover, Pereira et al., after conducting an extensive bibliographic review for natural antichagasic products, conclude that there is an important geographic and ethnodirected component in the research initiatives on the relevance of plant derivatives in the treatment of Chagas disease [38]. The main obstacle that stops research work is the gap that needs to be bridged toward clinical phases, given the difficulties in scaling the purification of the active compound, standardization of the chemical profile of whole extracts, and among others. The principal common factor that delays clinical phase is the lack of investment for the development of these products at this stage of investigation [39]. **Figure 2** summarize the progress and current status of research and development of natural products for the Chagas disease.

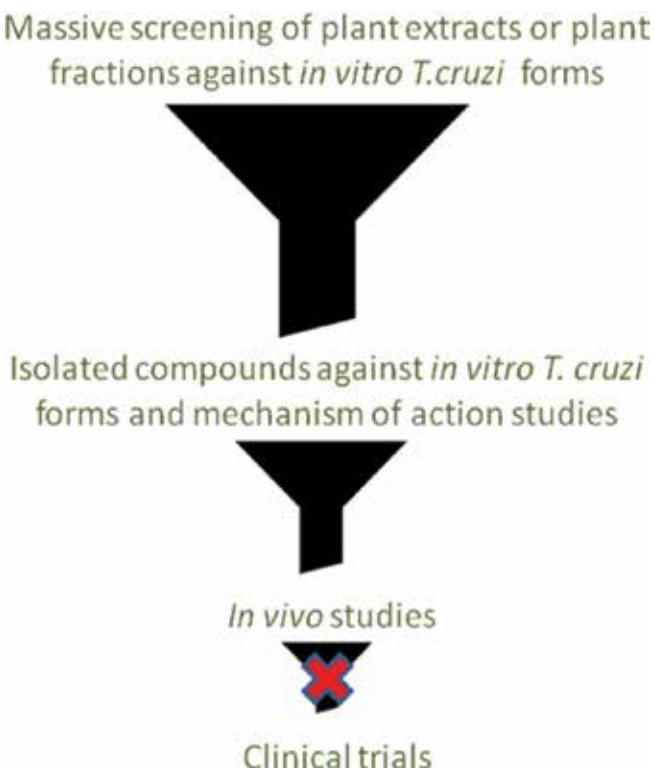

Figure 2. Current status of research and development of natural products for the Chagas disease.

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In many cases, the isolation of compounds leads to the loss of biological activity or to the achievement of a really low extraction yield. That is why work with whole extracts should be re-considered as a possible source of new treatment drugs for the Chagas disease. Given the low economic potential of the majority of the affected population, it is clear that the development of new treatment drugs should consider cost-effectiveness. In the search for new therapies for Chagas from plant origin, low cost and easy access for patients, we propose the use of new methodologies to overcome the existing challenges. The use of plant metabolomics technique is proposed as an option with high potential for the identification of biomarkers that could allow the standardization of chemical profiles of whole plant extracts that can be formulated in a simple, quick, and low-cost dosage.

3. Plant metabolomics

Metabolomics can be defined as the detection and quantification of all metabolites of low molecular weight in an organism at a given time and in certain conditions. However, adjusting to our purposes and applications can be better defined as the area of research that seeks to obtain the metabolic fingerprints to detect the differences between them and propose hypotheses that explain the differences.

The field of metabolomics in science marks its beginning when Devaux and Horning publish their research work in the metabolic profile where they apply gas chromatography coupled with mass spectrometry (GC/MS) for the analysis of extracts of human tissues and urine [40]. Immediately, the interest of different groups in using the metabolic profiles for the diagnosis and follow-up of different pathologies [41]. During the 1970s metabolomics studies expanded to a wide range of activities including: novel techniques for detection and elucidation of insect hormones [42], identification of natural products of marine origin [43], and chemotherapeutic agents derived from plant extracts (e.g.: *Hyptis tomentosa*) [44]. At the beginning of the 1980s, the first work on automated metabolic analysis emerged [45] and by the middle of the decade, the first works on metabolic profiling were published using nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC) [46–48]. In order to analyze the mechanism of action of herbicides besides performing GC/MS, a new global approach was developed in 1991 [49]. As a result of scientific cooperation, by the end of twentieth century, it was possible to apply novel technologies to metabolomics and methods of extraction, encouraging the development of metabolite databases [50], which allowed the global development of complete metabolomes [51].

In the last decade, metabolomics has developed as an important field within plant science and natural product chemistry [52–56]. Metabolic fingerprint, also known as a metabolic profile, is an objective analytical approach that seeks to quantify a group or groups of compounds found in an organism or group of organisms. The metabolic profile with gas chromatography and high-performance chromatography coupled to mass spectrometry or proton magnetic resonance (^{1}H NMR) has been successfully used the study plant biochemistry,

chemotaxonomy, ecology, pharmacology, and quality control of medicinal plants [57, 58]. The application of NMR has already been demonstrated as a suitable and sufficient method to carry out this type of analysis, since it allows simultaneous detection of various groups of secondary metabolites, in addition to abundant primary metabolites [59]. In addition, ^{1}H NMR spectroscopy has a great advantage over the other techniques, the signal intensity is dependent only on the molar concentration of the metabolites, allowing the direct comparison of these present in the sample [60, 61]. In the last years, several reports on the evaluation of the metabolic differences in *Cannabis sativa*, *Vanilla planifolia*, *Vitis* spp. and *Catharanthus roseus*, among others, and in the classification of *Ilex* species based on their metabolome are published [57, 60, 62, 63]. The major disadvantage in using NMR spectroscopy in the metabolomic analysis is overlapping signals, which however, can be solved by using different 2D-NMR techniques [52, 60, 63].

A typical approach of NMR-based metabolomic application for the identification of new natural products with biological activity that are part of whole extracts (**Figure 3**) was applied for the identification of new natural products with anti-*T. cruzi* activity in Uruguayan plants. Eighty samples of ethanolic extracts from different botanic parts, soils, and seasons, of Uruguayan specimens: *Baccharis trimera, Baccharis articulata*, *Baccharis usterii*, *Hydrocotyle bonariensis,*

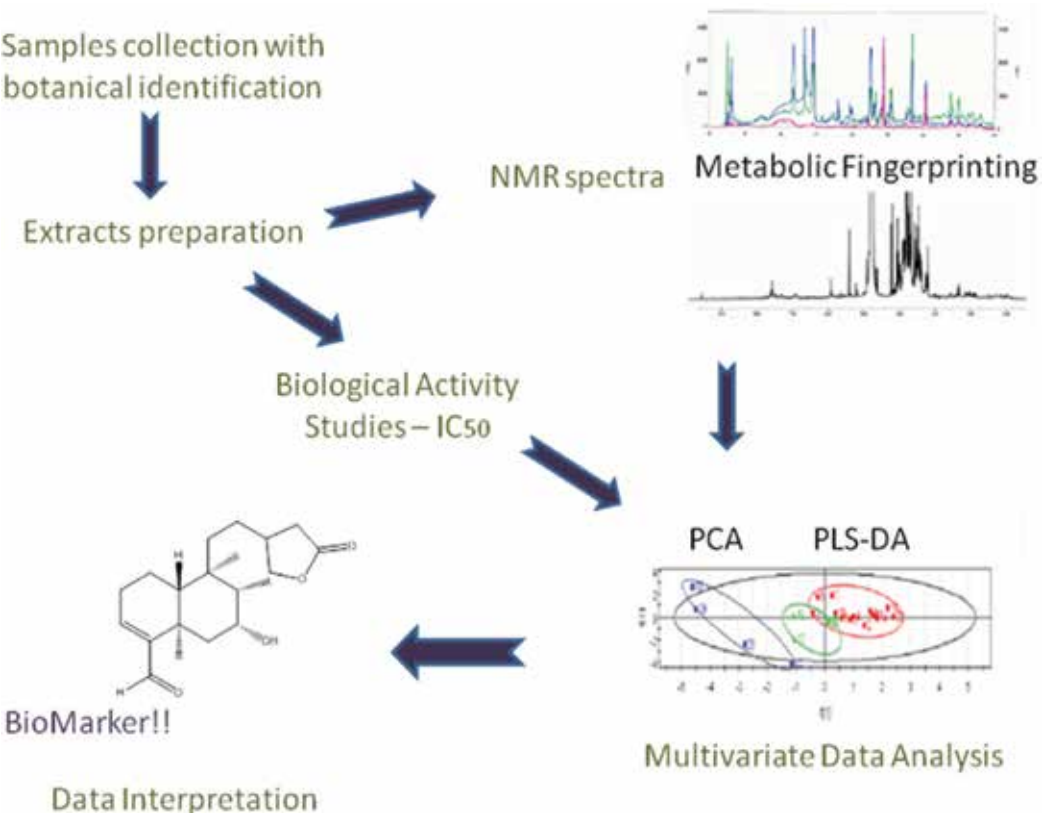

Figure 3. Typical approach of NMR-based metabolomic to identify new active compounds on plant extracts.

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Achyrocline satureioides, Taraxacum officinalis, and *Plantago major* were used. As a primary screening the anti-*T. cruzi* activity against the epimastigotes showed that three species of *Baccharis* genus and *Hydrocotyle bonariessis* displayed well to excellent antiproliferative activity. The most active fractions were additionally evaluated for their activity on amastigotes and also for cytotoxicity against mammalian cells. For the identification of the active principles was applied using nuclear magnetic resonance-based metabolomic. Through the metabolomic study of the relationship between the changes in chemical profiles and the biological activities, it was possible to identify the main active principles of the extracts and also the compounds responsible for cytotoxic activity [30]. The technique also allowed us to infer the parts of the plants with greater accumulation of the active compounds as well as the conditions for their maximum expression (soil type, harvest season).

The development of drugs from wild plants with simple growing requirements, allow us to consider the future possibility of creating standardized cultivars, in order to perform *in vivo* assays and clinical trials. We performed the standardization of cultivars of *Baccharis trimera, Baccharis articulata,* and *Baccharis usterii* with relevant results in the expression of their active molecules against *T. cruzi.* The propagation was made by cloning mother plants collected from wild nature and developing their cuttings on an environment controlled greenhouse (**Figure 4**). The plant extracts prepared from the standardized cultivars are being studied *in vivo* in a murine model of the Chagas disease.

When plant cultivars are performed for possible pharmaceutical uses it is important to consider World Health Organization (WHO) guidelines on good agricultural and collection practices (GACP) for medicinal plants [64].

Figure 4. Cultivar propagation of *Baccharis* spp. for standardization of their chemical profile for extracts preparation to be used on *in vivo* test for the Chagas disease.

4. Good agricultural and collection practices (GACP)

Medicinal plant resources are being harvested in increasing volumes, largely from wild populations. Indeed, demand for wild resources has increased by 8–15% per year in Europe, North America, and Asia in recent decades. Various sets of recommendations relating to the conservation of medicinal plants have been developed, such as providing both *in situ* and *ex situ* conservation [65].

The harvest of plants that had a wild growth is considered an efficacious source for medicinal purposes, but domestic cultivation is also a widely used and accepted practice [66–68]. Indeed, domestic cultivation provides advantages for the production of medicinal plants, allowing control for toxic components, avoiding pesticides, increasing the content of active compounds, and having precise information for the identification of botanical origin [69]. In this way, controlling the growing conditions is a relevant tool for production stability and to improve the yields of secondary metabolites, which are frequently the active compounds. Cultivation standards include providing optimal levels of water, nutrients and other environmental factors such as light, humidity, temperature, in order to improved yields of target products [70]. Moreover, controlled cultivation contributes to decrease the harvest of medicinal wild plant resources, also having a positive impact on their prices [71, 72].

This knowledge has been translated into good agricultural and collection practices for medicinal plants that were developed in order to regulate production, assess quality, and lead to the standardization of herbal drugs [73]. The application of these formal practices, summarized in GACP approaches, is important to ensure high quality, safe and pollution free herbal drugs (or crude drugs) [74]. A wide range of problems are controlled by applying GACP including the ecological environment of production, germplasm, cultivation, and collection methods, as well as quality aspects for pesticide detection, authentication in macro and microscopic terms, chemical identification of active compounds, and detection of metal elements [75]. Given the importance of GACP, many countries actively promote their implementation; however, there is still an important gap to bridge concerning knowledge and implementation. This disparity is given by the difficulties encountered for training farmers and other relevant actors for medicinal plant production, such as handlers and processors. While these kind practices are strictly applied at the level of pharmaceutical producers that are used to work in order to meet quality control requirements, it has been more difficult to successfully introduce this practices standard at the level of agricultural producers, handlers, and processors of medicinal plant material. It will be important to focus the efforts on training farmers and other relevant actors to ensure that GACP is adopted and favor the obtainment of high-quality medicinal plant materials [64].

Currently, organic farming is increasingly receiving public attention, given that these practices include a vision of sustainability and economically relevant business without forgetting the well-being of workers and creates integrated production systems for medicinal plants [76, 77]. The main characteristic of organic farming is the avoidance of synthetic fertilizers, pesticides or herbicides, and reaching the standards of organic certification. The defining characteristic of organic farming is the non-use of synthetic fertilizers, pesticides, and herbicides, which are

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not allowed according to many current organic certification standards in Europe and North America. Instead of applying synthetic fertilizers, organic fertilizers may be continuously supplied to the soil, contributing nutrients, and improving soil stability, while positively impacting the biosynthesis of essential substances. Furthermore, organic farming generates high-quality products and better yields, while taking care of the conservation of those plants. Indeed, when organic fertilizers were applied to the cultivation of *Chrysanthemum balsamita,* the biomass yield was increased and its essential oil content was higher that those free from organic fertilizers [78]. Above all, organic farming is a benign practice for our environment, based upon renewable and sustainable resources that favor the maintenance of a biological equilibrium in the medicinal plants and their ecological systems [74, 76]. For these reasons, the application of organic farming practices is highly relevant for medicinal plant production, encouraging a sustainable, and long-term systemic approach [77].

5. The novel strategy proposed to obtain effective, cheap and standardized phytoterapeutic for treat Chagas disease

Based on the use of metabolomic and the GACP, the following workflow is proposed for obtaining phytoterapeutics for Chagas disease treatment (**Figure 5**).

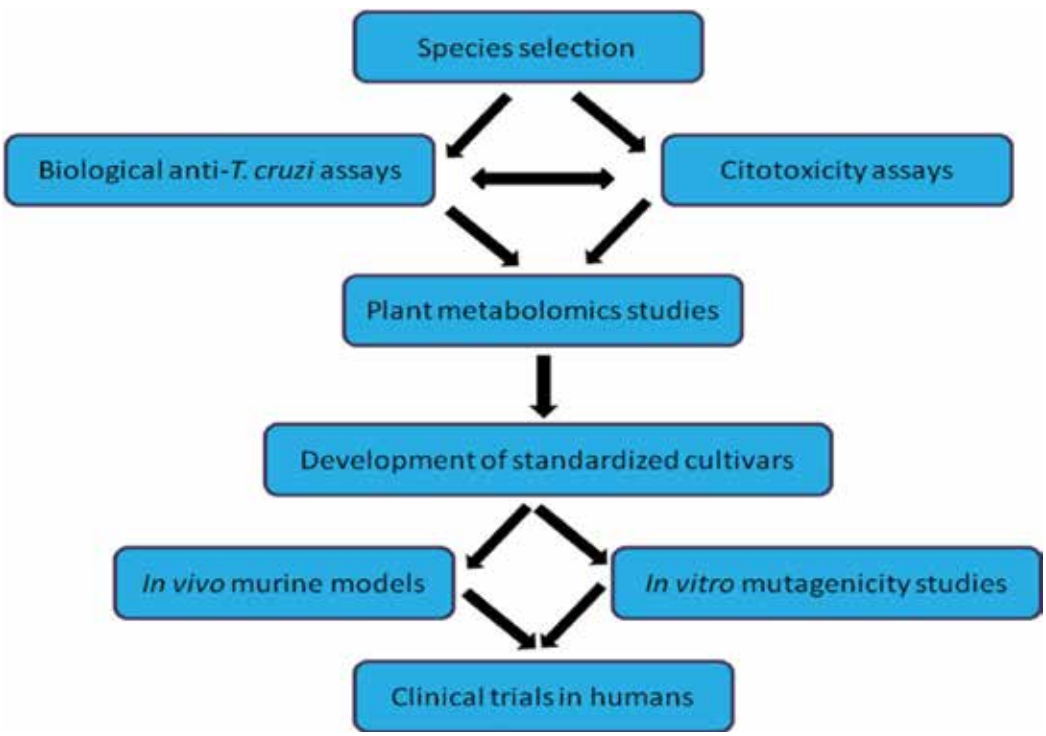

Figure 5. Workflow proposed for research and development of new natural products for Chagas disease treatment.

6. Conclusions

Traditional medicines, particularly herbal medicines have been increasingly used worldwide during the last two decades. On Chagas disease research many plant extracts were evaluated worldwide with relevant results but in most cases, the development is slowed in the scaling of the isolation of the active principle or by the lack of standardization of chemical profiles of the whole extracts. We presented a new novel strategy for the production of effective, cheap and standardized phytotherapeutic products through the use of plant metabolomics technique and the standardization of medicinal plants cultivars applying good agricultural and collection practices.

Author details

Javier Varela[1]*, Hugo Cerecetto[1,2] and Mercedes González[1]

*Address all correspondence to: jvarelaubillos@gmail.com

1 Grupo de Química Orgánica Medicinal, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay

2 Área de Radiofarmacia, Centro de Investigaciones Nucleares, Universidad de la República, Montevideo, Uruguay

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Section 4

Public Health Problem and Control Strategy

Chapter 11

Silent Information Regulator 2 from *Trypanosoma cruzi* Is a Potential Target to Infection Control

Luís Gaspar, Terry K. Smith, Nilmar Silvio Moretti, Sergio Schenkman and Anabela Cordeiro-da-Silva

Additional information is available at the end of the chapter

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Abstract

Human trypanosomiasis is a neglected tropical disease caused by protozoan parasites of the genus *Trypanosoma*. *Trypanosoma brucei* is responsible for sleeping sickness, also called African trypanosomiasis, while *Trypanosoma cruzi* causes Chagas disease, or American trypanosomiasis. Together, these diseases are responsible for significant mortality, morbidity and lost productivity in the endemic regions. There are no vaccines and treatments rely on drugs with limited efficacy, high cost, serious side effects and long administration periods. Since these diseases affect mostly the poor, there is no economic interest in the development of new drugs by pharmaceutical companies, and hopes for new treatments rely on public initiatives, public-private partnerships or philanthropic programs. The first step in the discovery of new drugs involves the identification of active molecules as starting points for further development, by either employing whole cells or by specific molecular target screenings. Research efforts undertaken by the authors' groups have focused on exploiting both strategies in the search for new molecules for trypanosomiasis drug discovery. In this chapter, we focus on Chagas disease and the recently uncovered potential of using sirtuins as targets for infection control.

Keywords: *Trypanosoma cruzi*, Chagas disease, sirtuins, drug discovery, chemotherapy

1. Introduction

Despite the efforts of many individuals and organizations over the years, human trypanosomiasis remains one of the most neglected diseases in the world. Chagas disease in particular, is a leading cause of disease and disability in Latin America, with thousands of deaths every year [1]. The negligence is particularly patented by the lack of new drugs. Indeed, the available treatment

options, benznidazole and nifurtimox (**Figure 1**), were discovered more than 40 years ago. Different strategies have been employed to control the disease, but the most impactful so far has been the control of the transmitting vector led by the World Health Organization (WHO) and Pan American Health Organization (PAHO). Vector control has caused the reduction of cases from a staggering 24 million in the 1980s to about 6 million nowadays [1, 2], with some countries considered to be free of domestic vectorial transmission. Continued and rigorous implementation of the disinfestation programs in the remaining zones should decrease even further the global numbers of *Trypanosoma cruzi* vectorial transmission. Also, the screening of donor blood and transplant organs in endemic regions and other parts of the world has greatly reduced the

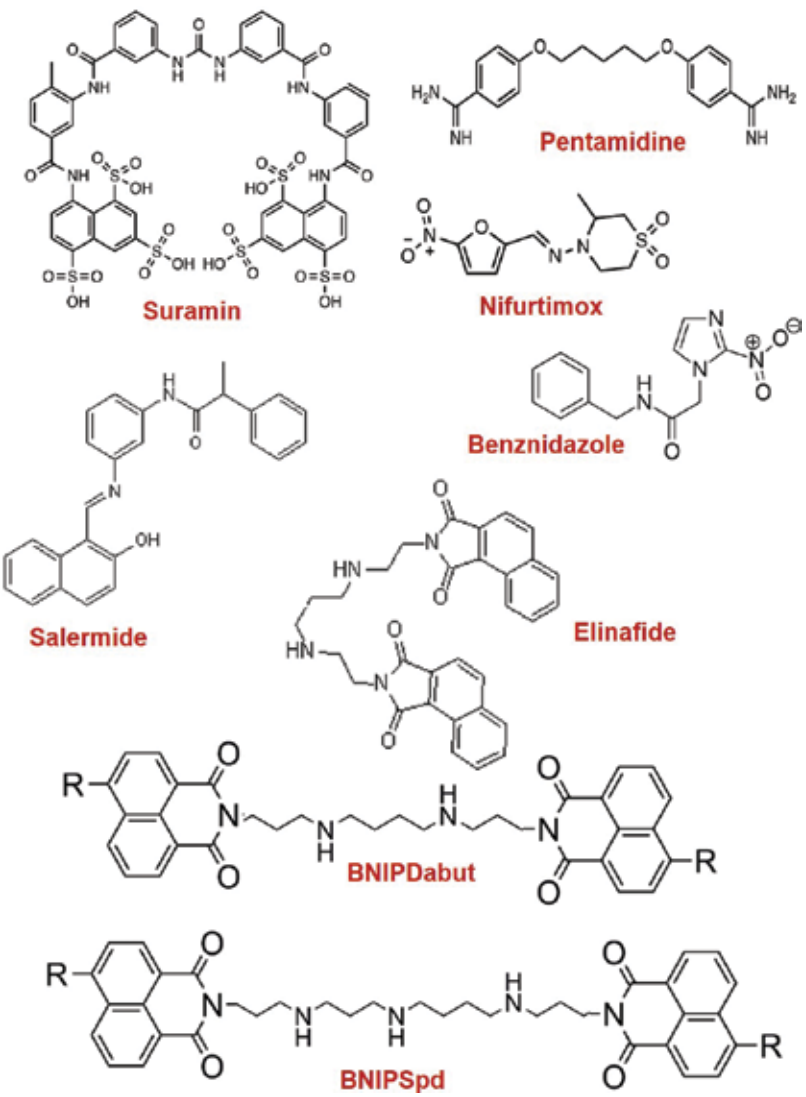

Figure 1. Various trypanocidal agents.

number of cases transmitted by this route. Whereas the global interruption of the domestic cycle will be a major breakthrough and reduce to a minimum the number of new cases of Chagas disease, complete eradication of the parasite, however, is unlikely to be achieved due to the huge natural reservoir of *T. cruzi* and the many species of triatomines capable of its transmission in the sylvatic environment [3–5].

What then, stands in the way of disease control for Chagas? First, no major technological advances are required to interrupt vectorial transmission responsible for the majority of new cases; second, decades of research in the molecular understanding of *T. cruzi* biology, the particularities of pathogenesis of the disease or the dynamics of immune response against the parasite have failed to translate into therapeutic alternatives; and finally, vaccination, either preventive or therapeutic, has remained an elusive achievement. The answer lies in new therapeutics. New, safer, cheap, easy to administer and efficacious drugs that are able to treat not only new cases, but also the millions of people already affected by the chronic stage and indeterminate form of the disease. There is growing evidence that chronic manifestations are ultimately related with inflammation resulting from parasite persistence [6, 7] and effective treatment of these cases would be highly beneficial to stop the development of symptomatology.

Active drug discovery efforts for Chagas disease have been restricted, until some years ago, to just a very limited number of groups, mostly based in academia. As a consequence, results have been sporadic, slow, ineffective and highly dependent upon intermittent funding, failing to deliver an alternative treatment. Chagas disease is as much neglected by the pharmaceutical industry as it is by research funding organizations, whose majority of funds are directed to developed world diseases.

Only recently has drug discovery for Chagas been met with concerted, focused efforts. While still not privately embraced by pharmaceutical companies, public-private partnerships have been set up with the objective of bringing together the biology expertise from academia and the technical expertise, facilities and resources from pharmaceutical industries. One organization that is leading the efforts to find new therapies for neglected diseases, including human trypanosomiasis, is the Drugs for Neglected Diseases Initiative (DND*i*), that has been involved in coordinating activities from early drug discovery to the launch and conclusion of clinical trials for some candidates like inhibitors of ergosterol biosynthesis of *T. cruzi*. The Bill and Melinda Gates Foundation, a non-governmental organization devoted to human development in underdeveloped countries, has committed to help control neglected diseases by signing The London Declaration on Neglected Diseases together with the WHO, the World Bank and 13 leading pharmaceutical industries. The Declaration states that by 2020 the signers will achieve, among other ambitious milestones, the eradication of Human African trypanosomiasis (HAT) and the control of American trypanosomiasis. Since then, other initiatives have been launched with the objective of boosting the research in new drugs for *Trypanosomal* diseases, like the European Commission Seventh Framework Program (FP7) consortia NMTrypI and KINDReD. The recent award of the Nobel Prize in Physiology or Medicine 2015 to William C. Campbell, Satoshi Omura and Youyou Tu is a recognition of the importance of drug discovery for parasitology and should further increase the awareness of neglected diseases by the international community.

Pharmaceutical research and drug discovery for infectious disease have historically began with what would be classified today as phenotypic assays, and can be traced to the pioneering work of Paul Ehrlich in the nineteenth century, while testing the effect of different dyes in trypanosomes [8]. Cultures of the microorganism of interest, bacteria or parasites, were incubated with a compound of interest, and the selective staining of the dyes was monitored by microscopy. Products of such "dye therapy" approaches were in the origin of well-known chemicals like the crystal violet dye that was proposed to be used in blood banks of endemic areas to kill *T. cruzi* parasites present in transfusion blood as a way to reduce transmission by this route [9]. Another example is trypan blue, that is still widely used as a cell biology reagent and that was the starting point for the design of the colorless analogue Suramin, a drug still in use today for the treatment of HAT and infected animals as well [10]. Such early whole cells drug screening principles were also central to the development of nifurtimox and benznidazole (**Figure 1**) in the 1960s and 1970s, by the pharmaceutical companies Bayer and Roche, respectively [11].

With the genomic era there was a dramatic shift in the way new drugs are discovered. The past 20 years have witnessed incredible advancements in genomics, proteomics, structural biology, computational chemistry and structure based drug-design, that coupled with high-throughput screening and combinatorial chemistry have helped to shape the reductionist mentality "one gene—one protein—one drug" [12]. However, the complexity of many diseases and the capacity of adaptation to adverse conditions, like the presence of a xenobiotic, evidenced by many living cells, have brought the more naïve phenotypic whole-cell screening strategies back to the spotlight. With modern phenotypic approaches, the effect of a pure molecule in a fully intact whole living organism, bacteria, parasites or human-derived cell lines, results in the identification of hit compounds that are potentially useful as scaffolds for further medicinal chemistry optimization.

While early phenotypic screenings for *T. cruzi* sometimes used the insect-specific epimastigote stage due of its extracellular nature and ease to culture, the use of reporter genes expressed during the clinically relevant stage of the disease, the intracellular amastigotes, has met a widespread application. The first of such assays was based upon the β-galactosidase-expressing parasites that made possible the detection of anti-*T. cruzi* activity by a colorimetric reaction [13]. Later, tdt-tomato and luciferase genes were also constitutively expressed in parasites, allowing more sensitive measurement of a fluorescent or luminescent signals, respectively [14].

However, the use of genetically unmodified parasites has always been an attractive pursuit, made available only recently due to technologic advancements. Such cell-based assays were developed by researchers at Institut Pasteur Korea and have met widespread use [15–18]. This assay employed the use of wild type parasites of *T. cruzi* infecting a non-modified cell line and the imaging of the resulting infection (in the amastigote stage) by high-content analysis (HCA) microscopy. Furthermore, the assay was developed in the 384-well format, allowing a high throughput testing of compounds. Preliminary cell toxicity is concomitantly determined by quantifying the ratio of host cell nuclei, a clear advantage since it reduces the need of an independent assay to assess this parameter. Using this screening assay, the authors of this chapter have also screened a library of 4000 kinase/phosphatase-like inhibitors that allowed

the identification of 11 compounds with strong anti-parasitic activity and selectivity, suitable for follow up hit-to-lead optimization (unpublished results). In addition, a complementary assay developed for phenotypic profiling also allowed the identification of several compounds that interfered with the development and intracellular differentiation of *T. cruzi*. Compounds that hindered the differentiation from trypomastigotes to amastigote and the replication of amastigotes inside host cells are among the examples of "phenotypic" hits discovered (unpublished results). Due to the complex genetics and still many unknown aspects of *T. cruzi* biology, these types of compounds have the potential to constitute important chemical genomic tools that may help answering fundamental questions like: what triggers stage differentiation and what are the pathways involved? What factors are responsible for parasite persistence? How are amastigotes kept dormant for years to decades in host cells, hidden from the immune system? Due to the nature of the chemical library, it is likely that the compounds target kinases, of which the *T. cruzi* genome has 190 annotated potential members [19]. *T. cruzi* and other Trypanosomatids have a relatively big kinome when compared with other parasites that undergo several stage differentiations and contact with distinct environments, like *Plasmodium* spp. [20, 21]. One hypothesis is that while in metazoa and yeast the ultimate targets of many signaling cascades are transcription factors, which then trigger the expression of new sets of genes, Trypanosomatids have constitutive transcription of a majority of genes in large polycistronic units, hinting at a greater role of post-translational modifications (PTMs) like phosphorylation and acetylation.

2. Sirtuins: family and functions

Post-Translational Modifications (PTMs) represent one of the major mechanisms in regulating protein function in all life forms. Through phosphorylation, acetylation, methylation, glycosylation and ubiquitination, cells greatly extend the possibilities beyond the coding genome [22]. PTMs can change the enzymatic activity of a protein, change its subcellular localization, interfere with protein complexes assembly, increase or decrease its stability and induce interactions with DNA and RNA [22, 23].

Discovered half a century ago and largely ignored for the following years, lysine acetylation has re-emerged in the last two decades as a highly important PTM [24, 25]. Initial studies had focused in the role of lysine acetylation in the regulation of chromatin structure and gene expression, but with the advances in proteomic approaches, it was possible to begin to explore the function of lysine acetylation in non-histone proteins [24, 25].

Studies based on proteomic analysis to describe the lysine-acetylated proteins repertoire of an organism, called acetylome, have shown the presence of lysine acetylation in proteins from different cellular compartments and involved in different biological processes in several organisms [26]. Because of that, lysine acetylation has been placed by some authors in the same level of biological relevance as phosphorylation [24, 25]. In fact, studies of the acetylome of mammalian cells revealed acetylation sites in 1750 different proteins [27], a number close to the about 2000 proteins found to be phosphorylated [28].

The "acetyl code" is maintained by three different protein types: the "writers", lysine acetyltransferases (KATs) that add acetyl groups to proteins, the "erasers", lysine deacetylase (KDACs) that remove acetyl groups, and "readers", proteins that specifically recognize and bind acetyl-lysine groups [26].

KDACs in particular have been the focus of great interest by the scientific community due to their many roles in cell function and disease. KDACs are interchangeably called histone deacetylases (HDACs), because the first discovered reactions catalyzed by these proteins were the removal of acetyl groups in histone tails [24, 29].

HDACs are separated into four different classes based upon sequence homology (class I, II, III and IV) and two different families: the histone deacetylase family and the sirtuin family, the latter being all class III HDACs. While the first family has a limited set of molecular targets, mainly composed of histones, sirtuins have a variety of substrates ranging from metabolic enzymes to structural proteins, as well as histones [30–32]. The sirtuin family seems to be ubiquitous throughout all kingdoms of life. The number of genes coding for sirtuins within an organism ranges from as little as one in bacteria, to seven in vertebrates [33]. The sirtuin family is further classified in 5 subclasses (I, II, III, IV and V) [34].

The most common reaction catalyzed by sirtuins is deacetylation. This reaction is of upmost biological importance as there is a clear relation between the acetylation status of several proteins and their cellular functions [35–38]. The deacetylase reaction requires (nicotinamide adenine dinucleotide) NAD^+, an acetylated lysine residue and produces deacetylated lysine, nicotinamide and 2′-O-acetyl-ADP-ribose (OAADPR) [39]. Studies on the kinetics and biochemical properties of the enzymes revealed binding to the acetyl-lysine substrate prior to NAD^+. This is followed by nicotinamide cleavage from NAD^+, that is the first product released, followed by deacetylated lysine and OAADPR [40] (**Figure 2A**). All sirtuins are strictly NAD^+ dependent, a distinct characteristic that distinguishes them from other deacetylases. In fact, SIRT6 is a sirtuin capable of tightly binding to NAD^+ without the requirement of an acetylated substrate, indicating that it may function as a NAD^+ sensor [41].

Besides being an endogenous product of the deacetylation reaction, nicotinamide is also a well-known inhibitor of sirtuins. Nicotinamide is an amide of nicotinic acid (vitamin B3) and is part of common enzyme co-factors like NAD^+ and NADP (nicotinamide adenine dinucleotide phosphate) [42]. Intracellular physiological levels of nicotinamide in some mammalian cells seem to be in the range similar to the IC_{50}'s of some sirtuins reinforcing the hypothesis that some sirtuins may act as NAD^+ and nicotinamide sensors [43, 44].

OAADPR is another product of the deacetylation reaction [45] (**Figure 2A**). Early studies characterizing this molecule found that quantitative microinjection into starfish oocytes led to a blockage of oocyte maturation, indicating for the first time that OAADPR can evoke a biological activity [46]. There is now mounting evidence that OAADPR can elicit downstream responses that might synergize or antagonize the biological functions of sirtuin genes. So far, OAADPR has demonstrated to be related with functions in gene silencing, ion channel modulation and cell redox state maintenance [45].

Another reaction catalyzed by sirtuins is ADP-ribosylation. Although sirtuins were firstly described as ADP-ribosyltransferases (**Figure 2B**), their deacetylase activity has quickly

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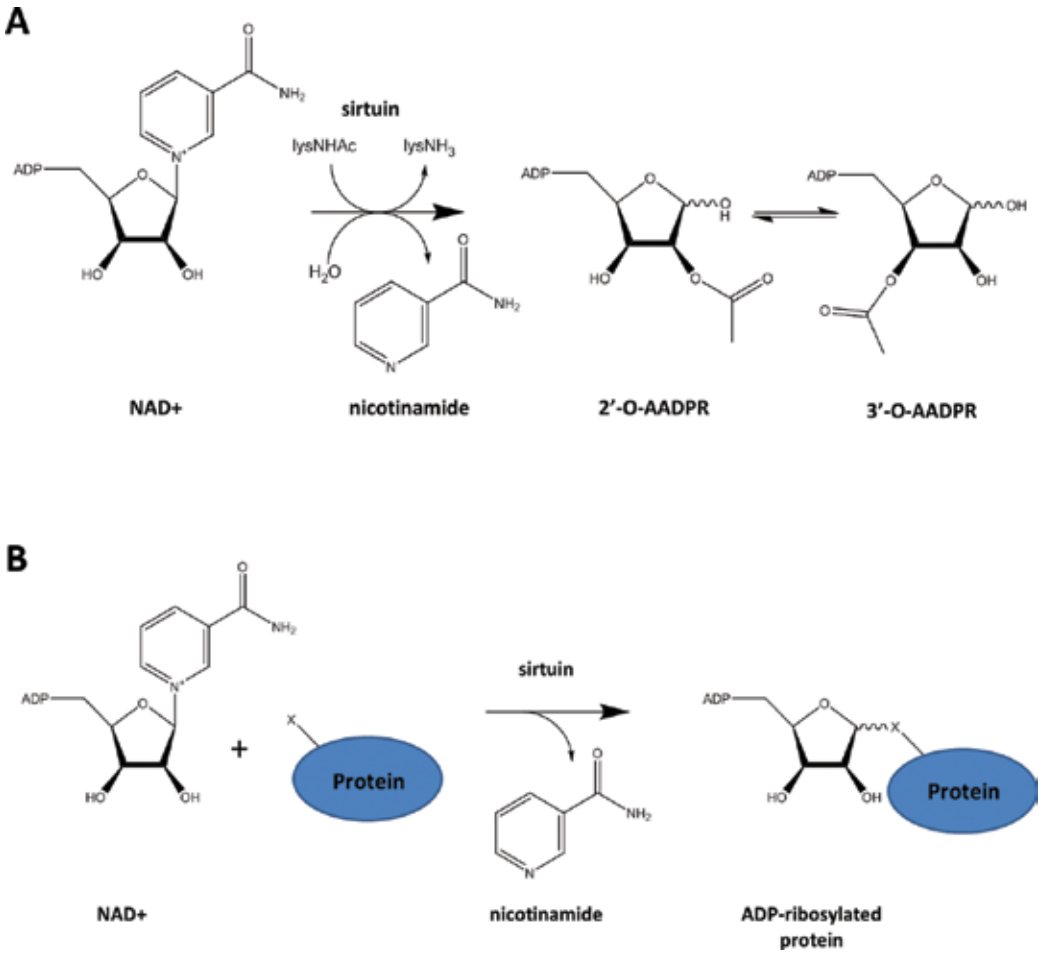

Figure 2. Sirtuin deacetylation and ADP-ribosylation mechanisms. (A) Sirtuins carry out protein deacetylation by removing acyl chains from protein lysine residues. This activity requires the cofactor nicotinamide adenine dinucleotide (NAD^+), such that nicotinamide is released with concomitant production of O-acetyl-ADP-ribose (O-AADPR). There is a sirtuin-independent equilibrium between 2′ and 3′-O-AADPR isomers. The unspecified stereochemistry at the Cl′ position of O-AADPR reflects the fast epimerization observed in solution between α and β-anomers. (B) A number of sirtuins also exhibit ADP-ribosylating functionality. This reaction is also NAD^+-dependent, as the cofactor acts as the source of ADP-ribose. 'X' represents the nucleophilic side-chain of a protein residue.

overshadowed this activity, and as a consequence the biological processes associated to this reaction remains poorly understood [47]. In truth, sirtuins may have just deacetylase activity, both, or be mostly ADP-ribosyltransferases. An example is SIRT4 that is an efficient *in vitro* ADP-ribosyltransferase of histones that only recently was discovered to possess deacetylase activity [48, 49]. There is an active debate on whether ADP-ribosylation is in fact a biologically relevant function of sirtuins, or just an irrelevant side reaction/non-enzymatic artifact [50]. Nevertheless, some of the players in the dynamics of intracellular ADP-ribosylation have only been recently identified and it is becoming apparent that this PTM might be relevant for the modulation of important cell processes and signaling pathways like signal transduction mechanisms, transcription and DNA repair [51]. The ADP-ribose hydrolysis in Trypanosomatids

has recently been studied in both *Trypanosoma brucei* and *Trypanosoma cruzi* and found to be mediated by a macrodomain with a conserved catalytic site [52].

Although (de)acetylation is the most common PTM, (de)acylation of other groups can be targeted, like succinyl and malonyl groups. SIRT5 and SIRT6 are some examples of proteins that perform deacylations of lysines other than acetyl groups, and their activities are important regulators of cell functions [53–55].

There is a wealth of information regarding the structural features of sirtuins. To date, some 83 structures of sirtuins are available in the protein databank, many of them co-crystalized with natural ligands or inhibitors. Though the available structures range from bacterial to mammalian sirtuins, the majority of the structures originate from the human genome.

Although the sequence homology can vary significantly between sirtuins, especially between prokaryotic and eukaryotic proteins, there is a conserved catalytic core of about 250 amino acids common to all members in the family [56]. The structure similarity of the *Plasmodium falciparum* PfSir2A with the mammalian SIRT5, despite a sequence homology of just 33% is a clear illustration [56]. This core contains a Rossman fold domain that is a NAD^+ binding site, and a Zn^{2+} binding domain containing four highly conserved cysteine residues arranged in the motif ($CX_2CX_{20}CX_2$). The catalytic site is located inside a hydrophobic channel formed at the interface of these two domains [56].

Whereas in HDACs from class I, II and IV, Zn^{2+} is an active participant in catalysis by producing free acetate and deacetylated lysine, in sirtuins it does not participate in reaction. However, the metal is essential for structural integrity, as was elucidated by the reversible loss of activity in a *P. falciparum* sirtuin where the zinc ion was removed [57]. Interestingly, an exception to the conservation of the $CX_2CX_{20}CX_2$ motif is found on some sirtuins of Trypanosomatids, where one of the cysteines is not present [58]. However, deacetylase activity does not seem to be affected [59]. The molecular mechanism of deacetylation is still not completely elucidated, but it is generally accepted that the first step in the reaction involves the nucleophilic addition of the acetyl oxygen to nicotinamide ribose by a mechanism of S_N2 substitution to produce O-alkylamine intermediate and nicotinamide. Then the acetyl group is transferred to ADP-ribose to form O-acetyl-ADP-ribose and deacetylated lysine [60].

The founding member of the sirtuin family is Sir2 from the budding yeast and was initially identified as part of a protein complex necessary to silence the expression of the mating-type-loci [61, 62]. Subsequently, it was also implicated in transcriptional silencing at telomere proximal sites [63] and ribosomal repeats at the ribosomal DNA (rDNA) locus [64–66]. Sir2 can be associated in distinct protein complexes that vary according to target site. For instance, at telomeres and the mating-type-loci, Sir2 forms a complex with two other homologs, Sir3 and Sir4 [63], while at rDNA locus Sir2 associates with Net1 and Cdc14 to form the regulator of nucleolar silencing and telophase exit—RENT complex [67, 68]. Yeast cells lacking Sir2 present a reduced lifespan that has been correlated with the accumulation of extrachromosomal ribosomal DNA circles originating from illegitimate recombination that are toxic to the cell and have been associated with aging [66, 69, 70].

In mammals, the nuclear SIRT1 is the most extensively studied member among the sirtuin family. The TATA box binding protein-associated factor RNA polymerase I subunit B (TAF_I68) was the first substrate to be identified for SIRT1 in mouse cells. It is a transcription factor necessary for regulating the RNA polymerase I transcriptional complex, where it was shown that deacetylation inhibits transcriptional initiation *in vitro* [71]. Studies on p53, a non-histone substrate, demonstrated that acetylation activates the DNA-binding activity and target gene expression, also increasing its stability [72]. Consistent with this proposed SIRT1 inhibition of p53 function, SIRT1 knockout mice exhibit p53 hyper-acetylation and increased radiation-induced apoptosis, suggesting that SIRT1 can facilitate tumor growth by antagonism of p53 [73]. Still, the fact that SIRT1 can be found either overexpressed or underexpressed in different tumor types, and the finding that it can also function as a tumor suppressor [74] has hindered the clarification of its role in tumorigenesis. SIRT1 also plays an important role in metabolism, and its relation with caloric restriction and life-span extension has received much attention (reviewed in [75–77]). The beneficial effects of caloric restriction have been focused on the insulin-like growth factor-1 (IGF-1) and the target of rapamycin (TOR) pathways [78, 79], but increasing evidence suggest a role of SIRT1 in caloric restriction in mammals as well. For instance, SIRT1 expression was found to be elevated in models of caloric restriction, like fasting mice, low calorie diet in rat, or humans on a 25% caloric restriction diet [80–82]. On the other hand, mice lacking SIRT1 lost the life-span extension benefits of a 40% reduced calorie diet [83]. Despite many studies, the exact molecular mechanisms of SIRT1 in caloric restriction are still to be unraveled.

In vitro studies attribute a role to human SIRT2 in cell cycle regulation through the deacetylation of both tubulin and histone H4 [32, 84, 85]. In particular, it has been found that SIRT2 overexpressing cells were significantly delayed in cell cycle progression through mitosis [86]. Some links with age-related diseases have been described for SIRT2, such has neurodegenerative diseases [87–89], and different kinds of cancer. Mice lacking SIRT2 are prone to the appearance of tumors, an effect believed to be mediated by SIRT2 negative regulation of the anaphase-promoting complex [90]. It was demonstrated that SIRT2 expression is reduced in human gliomas, some of the most frequent malignant tumors in the brain [32, 91].

SIRT3 is the major mitochondrial deacetylase and studies with double knockout mice have revealed high levels of acetylation in protein targets [92, 93]. In addition, it was observed that these mice have impaired production of ATP [92]. When fasting or fed with a high-fat diet, the mice display atypical phenotypes that include cold intolerance and decreased ketone body formation [94, 95]. This strengthened the link with thermogenesis that had been previously demonstrated [96, 97]. In fact, SIRT3 expression is induced in mice in both white and brown adipose tissue upon caloric restriction and exposure of brown adipose tissue to cold temperatures [98]. In addition, SIRT3 also has a role in the deacetylation and activation of fatty acid β-oxidation, amino acid metabolism, electron transport chain and antioxidant defenses [99, 100].

SIRT4 was originally thought to be an unusual sirtuin due to the lack of deacetylase activity [101]. However, it was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase production of ATP and has been implicated in insulin regulation of β-cells [48, 101]. Moreover, SIRT4 has recently been attributed a tumor suppressive function due to its involvement in

DNA damage protection mediated by inhibition of mitochondrial glutamine metabolism, suggesting it might have therapeutic potential for treating glutamine-dependent cancers [102]. This mechanism is inhibited by mammalian target of rapamycin complex 1 (mTORC1) pathway [103]. SIRT4 also coordinates the balance between lipid synthesis and their catabolism by repressing malonyl-CoA decarboxylase, proving that it has, in fact, deacetylase activity [49].

SIRT5 is a NAD^+-dependent protein lysine demalonylase and desuccinylase [104] and also has a deglutarylase activity [105]. It has a deacetylase activity [30], but has preference for acyl-carboxyl negatively charged groups [104, 105]. Some of its functions are related to glycolysis modulation [55]. The succinylome of mammalian cells has revealed many points of succinylation that are possible targets of SIRT5, mostly concentrated on mitochondrial metabolism [54]. SIRT5 also promotes urea cycle function via the regulation of carbamoyl-phosphate synthase [30, 35], and purine metabolism via urate oxidase [106]. Although a global protein hypersuccinylation and elevated serum ammonia during fasting were observed in SIRT5 knockout mouse model, the enzyme deficiency did not lead to any major metabolic abnormalities under either low or high fat diet conditions. These observations suggest that SIRT5 is likely dispensable for metabolic homeostasis under the basal conditions. It remains to be evaluated the role of SIRT5 in extreme conditions [107].

While most mammalian sirtuins have been implicated with metabolism, SIRT6 seems to be the only one with a direct link supporting a defined role in mammalian aging [108]. In fact, mice lacking SIRT6 gene develop a progeroid-like symptom with loss of subcutaneous fat, curved spine and lymphopenia. They develop normally for 2 weeks after birth, but then suffer from acute degeneration processes, ending up dying at 1 month of age [109]. At first considered to not possess deacetylase activity, but solely an ADP-ribosyltransferase activity [110], it was later found that SIRT6 removes both acetyl and long chain acyl groups from target molecules [53, 111]. It is localized in the nucleus, associated with chromatin, where it promotes the specific NAD^+-dependent deacetylation of H3K9 and H3K56 [111–113]. SIRT6 is involved in genome protection by assuring correct telomere maintenance [111, 112], as well as DNA repair by double-strand break repair machinery [114, 115]. Like other sirtuins it also has a role in metabolism by influencing both glycolysis and gluconeogenesis [116–118] and lipid metabolism, by regulating triglyceride synthesis [108, 119]. Conditions like inflammation, heart disease and cancer all seem to be linked with SIRT6 function [120].

SIRT7 is the least studied sirtuin of all the mammalian sirtuins, but recent findings have established new functions and roles for this protein. It is a nucleolar sirtuin [121] and its localization is associated with the main process happening at this sub-nuclear structure, namely rDNA transcription [122]. SIRT7 does not possess a very strong deacetylate activity toward common synthetic and natural peptides [121], which is in agreement with the fact that SIRT7 depletion in mice did not change the global acetylation levels of either nucleus or nucleolus proteome [123], indicating that SIRT7 deacetylase activity is specific to a limited set of proteins. One example is specific deacetylation of H3K18 [31, 124] that underlies its role in chromatin remodeling. Also, SIRT7 has been found to be closely associated with B-WICH complex, a chromatin-remodeling complex [125]. It also has a role in protein synthesis [123, 126]

and contributes to cell survival, namely by protecting against genomic insult [127, 128], hypoxia [129] and low glucose induced stress [130]. All the functions described characterize SIRT7 as a pro-survival protein. Indeed, it is currently considered to be an oncogene in all the cancer types studied so far [126, 131, 132].

3. Parasitic sirtuins

Various genome-sequencing projects demonstrated the presence of genes coding for sirtuins in most protozoan parasites of medical importance. An interesting finding was that depending on the protozoan parasite species the number of sirtuins varied (**Table 1**).

Organism	Number of sirtuins	GeneID	Subcellular Localization	Data Source
Trypanosomatids				
Trypanosoma cruzi	2	TcCLB.508207.150 (TcSir2rp1); TcCLB.447255.20 (TcSir2rp3)	cytoplasm (TcSir2rp1); mitochondria (TcSir2rp3)	TriTrypDB (v.34)
Trypanosoma brucei	3	Tb927.7.1690 (TbSir2rp1), Tb927.8.3140 (TbSir2rp2); Tb927.4.2520 (TbSir2rp3)	nucleus (TbSir2rp1); mitochondria (TbSir2rp2, TbSir2rp3)	TriTrypDB (v.34)
Leishmania spp	3	LinJ.26.0200 (LiSir2rp1); LinJ.23.1450 (LiSir2rp2); LinJ.34.1900 (LiSir2rp3)	cytoplasm (LiSir2rp1); mitochondria (LiSir2rp2, LiSir2rp3)	TriTrypDB (v.34)
Apicomplexa				
Plasmodium spp.	2	PF13_0152 (PfSir2A); PF11_0489 (Sir2B)	nucleus; cytoplasm	PlasmoDB (v34)
Toxoplasma gondii	2	TGVEG_065040, TGVEG_105780	n.d	ToxoDB (v34)
Babesia bovis	1	BBOV_I00370	n.d	PiroplasmaDB (v34)
Others				
Giardia lamblia	5	GL50803_10708; GL50803_10707; GL50803_16569, GL50803_11676; GL50803_6942	n.d	GiardiaDB (v34)
Trichomonas vaginalis	10	TVAG_549940; TVAG_409810; TVAG_026260; TVAG_319320; TVAG_180900; TVAG_190210; TVAG_362260; TVAG_413390; TVAG_146810; TVAG_016210; TVAG_146820; TVAG_256040	n.d	TrichDB (v34)

n.d=not determined

Table 1. Sirtuin genes identified in genome-sequencing programs for parasitic protozoa.

Plasmodium spp. have been shown to contain two sirtuin orthologues, called Sir2A and Sir2B. Sir2A is the most extensively studied homolog, mainly located at the nucleus [133] although it can also shuttle to cytoplasm after posttranslational SUMOylation [134]. Sir2A has been characterized as a mediator of transcriptional silencing at the telomeric regions of chromosomes [133, 135]. The telomeres of *P. falciparum* are rich in gene families involved in antigenic variation such as the *var.* family of genes. These genes are responsible for the expression of parasite-derived *P. falciparum* erythrocyte membrane protein, PfEMP1, responsible for immune evasion in humans [136]. The family of *var.* genes is tightly regulated by sirtuins, with the expression of its members being mutually exclusive [137, 138]. The switch of active *var.* is controlled exclusively at the epigenetic level [137, 139].

PfSir2B is a larger homolog with a molecular weight more than four times the size of Sir2A and is involved in the transcriptional silencing of a complementary subset of *var.* genes with distinct promoter types [140].

Sirtuins from *Leishmania* spp. parasites were among the first to be identified in Trypanosomatids, when a complementary DNA (cDNA) isolated and sequenced from *Leishmania major* showed a high homology with yeast Sir2 [141]. Antibodies raised against this LmSir2 later showed to be reactive against different life cycle stages of *L. major*, but also to *Leishmania amazonensis* and even to the serum of a patient infected with *Leishmania infantum* [142]. Furthermore, the protein was found to be among the secreted material of *L. major* [142].

Overexpression of the Sir2 protein in *L. infantum*, sharing 93% homology to the *L. major* protein, led to an increased survival of amastigotes under axenic conditions [143]. Also, when the overexpression was performed in mammalian fibroblasts, host cells became more permissive to infection by *Leishmania* infection in comparison with wild type cells, hinting at a modulation of host cell by the parasite [144]. Genetic knockouts in *L. infantum* of the Sir2 related protein 1 (Sir2rp1) gene also highlighted the importance of this protein in the parasite. While single knockouts were readily obtainable, double deletion of the alleles was only possible after the rescue by an ectopical copy of the gene, suggesting an essential role for parasite survival [145]. When single-knockouts of *L. infantum* Sir2rp1 were used to infect a macrophage cell line, *in vitro*, it was noted that although they had the same invasive capacity than wild-type parasites, they showed a hindered replication rate leading to diminished infections over-time. Furthermore, the mutant parasite also failed to establish an infection in an *in vivo* mouse model of Leishmaniasis [145]. Cellular and biochemical studies later established LiSir2rp1 has NAD^+-dependent deacetylase with ADP-ribosylation activity that co-localized to the cytoskeleton and potentially interacted with tubulin as well as with HSP83, an orthologue of mammalian HSP90 [146, 147]. The association with cytoskeleton is a characteristic feature of both SIRT2 and HDAC6 in mammalian cells [32, 148]. In addition, an orthologous from *L. amazonensis*, LaSir2rp1 was found to be a glycosylated protein, but whether this is the case for other species remains to be seen [149]. Although the Sir2 related protein 1 has received much attention, no studies have been made for the other two proteins codified by the *Leishmania* species; Sir2 related protein 2 and Sir2 related protein 3.

Sir2rp1 from *L. donovani* has also been implicated in the resistance of amphotericin B, a reference drug in the treatment of visceral Leishmaniasis. When clinical isolates were targeted for gene knockout of the protein, parasites showed a lower level of multi-drug resistant

transporter MDR1, lower drug efflux, increased ROS production and increased sensitivity to amphotericin B [150]. On the contrary, overexpression led to a resistant phenotype, thereby suggesting Sir2 as a new resistant marker for visceral Leishmaniasis [150]. Comparative transcriptomic analysis also implicates Sir2 in resistance to miltefosine, another drug used to treat the disease [151]. In addition to its potential as a novel drug target, Sir2rp1 from *Leishmania* spp. has also been suggested as a vaccine [152, 153].

Recently, LiSir2rp2 and LiSir2rp3, the others *Leishmania* sirtuins, were characterized as mitochondrial proteins, and while LiSir2rp3 was demonstrated to not be essential (**Table 1**), attempts to generate LiSir2rp2 knockout cells failed. LiSir2rp2 was implicated in parasite proliferation depending on NAD^+ availability [154].

Trypanosoma brucei, like *Leishmania* spp., has 3 sirtuins annotated in its genome (**Table 1**). The first enzyme to be characterized in the parasite was TbSir2rp1 [155]. The enzyme is localized mainly in the nucleus in association with chromosomes. The protein was shown to possess both deacetylase activity toward endogenous histones while being also able to ADP-ribosylate calf thymus histones and, to a lesser extent, bovine serum albumin (BSA). Up to that time, no ADP-ribosylation had been detected in common members of the sirtuin family like yeast Sir2 or HST2, hence TbSir2rp1 was one of the first enzymes to exhibit this dual activity [156–159]. Furthermore, mutation of a catalytic histidine essential for deacetylase activity also affected ADP-ribosylation activity, suggesting that the two activities were occurring simultaneously. Because of the increased or decreased resistance to DNA damage caused by the alkylating drug methyl methanesulfonate (MMS) in overexpressing or RNAi-induced knockdown *T. brucei* cell lines, respectively, TbSir2rp1 was also considered to have a role in DNA repair in this organism [155].

A later study performed with bloodstream forms (as opposed to insect stage forms in the previous works) characterized TbSir2rp1 and also the other two sirtuins, TbSir2rp2 and TbSir2rp3 that both with mitochondrial localization [160]. TbSir2rp1 was found in the nucleus, but when overexpressed to high levels in *T. brucei* cells, it localizes to the cytoplasm, with toxic effects to the parasite [160]. Besides, gene knockouts for the three proteins caused no growth in parasites maintained in standard conditions [160]. TbSir2rp1 mutants, however, did show an increased sensitivity to MMS damage, confirming the previous results performed with RNA interference (RNAi). The particular localization of TbSir2rp1 led to the investigation of Sir2 mediated telomere gene silencing like the one that occurs in yeast and *Plasmodium* spp., as discussed earlier [161, 162]. Although TbSir2rp1 was found to have a role in telomere DNA repair and telomere silence, it was not required for antigenic variation [160] as described for *Plasmodium* Sir2 [133, 135].

TbSir2rp1 has also been studied as a model sirtuin, with both deacetylase and ADP-ribosylation activity. Biochemical experiments revealed that ADP-ribosylation is 5-fold less active than the deacetylation reaction, and occurs only in the presence of an acetylated substrate by two distinct biochemical mechanisms [163]. Another research group demonstrated that ADP-ribosylation can also occur in arginine, independent of the presence of an acetylated substrate, as supported by mass spectrometry and molecular dynamics simulations [164].

Additional information about parasitic sirtuins can be found in a recent review by Hailu et al. [165].

4. *Trypanosoma cruzi sirtuins*

Although the draft of the *T. cruzi* genome has been published a decade ago [19], it was not until recently that the first experimental studies involving the sirtuins of this parasite have been published. Unlike *Leishmania* spp. and *T. brucei* that possess three Sir2-like proteins, *T. cruzi* only has two coding sequences annotated in its genome, TcSir2rp1 and TcSir2rp3 (**Table 1**).

One study employing parasites overexpressing TcSir2rp1 and TcSir2rp3 by a tetracycline inducible vector characterized some of the features of both proteins [58]. Localization studies employing both wild type parasites and polyclonal sera raised against both proteins, as well as localization of tagged copies in the overexpression mutants with monoclonal antibodies, attributed a cytoplasmic localization to TcSir2rp1 and a mitochondrial to TcSir2rp3 [58]. Both of the proteins' levels are regulated throughout the life cycle of the parasite, with a significant decrease in both at the trypomastigote stage [58]. Overexpression of TcSir2rp1 was responsible for higher metacyclogenesis and higher infectivity of Vero cells. Since metacyclogenesis occurs under nutrient deprivation, it is hypothesized that TcSir2rp1 may function like sirtuins from other organisms that respond to starvation [101, 166, 167]. On the other hand, overexpression of TcSir2rp3 led to a decrease in epimastigote replication time, lower infectivity in Vero cells, increased amastigote replication and normal metacyclogenesis [58]. Due to the oxidizing environment in which amastigotes replicate, it has been suggested that TcSir2rp3 performs protecting functions against oxidative stress like SIRT3 [36]. Both of the overexpressing cell lines reduced the levels of acetylation for particular proteins, as well as protected against the effect of specific sirtuin inhibitors [58].

Moretti and colleagues also independently characterized both of the sirtuins in a simultaneous study [59]. In their study, they show that salermide, a sirtuin inhibitor analogue of sirtinol found to be a strong anticancer molecule, is active against both *in vitro* cultures of epimastigotes, and against an *in vivo* model of infection by *T. cruzi*, albeit at moderate levels [59, 168]. Salermide was also found to be a strong inhibitor of TcSir2rp3 recombinant protein [59]. The authors report the same localization for both proteins, as well as the interference in epimastigote growth, metacyclogenesis, infectivity of host cells and amastigote replication in lines overexpressing the sirtuins. Differently from Ritagliati work [58], in their studies, the overexpression of the cytosolic TcSir2rp1 caused a decreased in the epimastigotes proliferation while TcSir2pr3 increased the growth rate. These differences might be due to the amount of overexpression achieved and parasite strains used [59].

5. Potential of *Trypanosoma cruzi* sirtuins as targets for infection control

A strategy that has been traditionally employed in Chagas disease drug discovery is the target-based approach. One such molecular target that has gained increasing interest as a potential drug targets against parasitic diseases is that of sirtuins [169, 170]. The hypothesis arose by the time that it was demonstrated that sirtuins are life-span regulators in organisms like yeast, flies and worms [70, 171, 172]. Therefore, many groups promptly investigated whether sirtuin

orthologues present in parasites could have important functions that could be exploited for novel therapeutic applications.

One important aspect for the viability of targeting sirtuins in parasites is the homology between the protein of interest and other proteins present in the host organisms. Although sirtuins are conserved through evolution [33], significant difference at the sequence level can be found between Trypanosomatid and human homologs. For instance, *T. cruzi* Sir2rp1 shares only 33% identity with mammalian SIRT2, its closest homolog (Multi-way protein alignment, BLOSUM 62) [173].

Another argument that has led to the consideration of *T. cruzi* sirtuins as a drug target is that this family of proteins is considered to possess structural properties adequate to inhibition by small-molecule compounds. In particular, the catalytic site is located inside a hydrophobic channel formed at the interface between the two constituting domains, the Rossman fold containing the NAD^+ binding domain and the Zn^{2+} ion binding domain [56]. Catalytic pockets buried inside the protein are considered an essential feature for target druggability [174].

One last fact that prompted the evaluation of TcSir2rp1 as a drug target was the previous evidence that a class of experimental compounds preferentially inhibited LiSir2rp1 over the human homolog SIRT1 [175]. The possibility to synthetize selective sirtuin inhibitors has been successfully achieved for human homologs, based upon structural knowledge of the catalytic site as has been demonstrated for human SIRT2 [176, 177].

Enzymatic inhibition by small molecule compounds is an essential step in the druggability assessment of novel therapeutic targets [174]. Biochemical studies performed by our research groups evaluated the effect of nicotinamide, a classic non-competitive inhibitor of sirtuins in TcSir2rp1 and TcSir2rp3 (to be published). TcSir2rp1 was shown to be inhibited by nicotinamide, albeit at a relatively high IC_{50} when compared with other sirtuins (4-fold higher for hSIRT1 and 11-fold higher for LiSir2rp1) [175]. Different nicotinamide sensitivities are found among distinct sirtuins, and may explain the differences described [177]. Nicotinamide inhibits deacetylation by binding to a conserved C pocket present in sirtuins that participates in NAD^+ binding and catalysis, where it promotes a base-exchange reaction at the expense of deacetylation [178]. A hypothesis for the high IC_{50} for nicotinamide in TcSir2rp1 could be related with structural characteristics of this conserved C pocket. Structural determination of TcSir2rp1 by X-ray crystallography currently ongoing in our group will certainly highlight these differences. Contrary to previous studies [179], we could not observe any antiparasitic activity of nicotinamide against *T. cruzi* amastigotes for up to a concentration of 2 mM. Several studies report the activity of nicotinamide against parasitic protozoa [180–182], but to our knowledge, none clearly establishes a relation between antiparasitic activity and sirtuin inhibition.

Other biochemical functions and protein interactions have been attributed to Sir2rp1 in related Trypanosomatids, and future experiments should shed light whether it applies to TcSir2rp1. One of the biochemical functions that has been characterized for both TbSir2rp1 and LiSir2rp1 is ADP-ribosylation. Both orthologous showed to ADP-ribosylate calf thymus histones and BSA [155, 183]. Later studies involving TbSir2rp1 demonstrated that this biochemical function is dependent upon acetylated histones, is coupled to the deacetylase activity of the sirtuin, but occurs at a much lower rate than the latter [163]. In fact, even though ADP-ribosylation has clear functions in both physiological and pathogenic situations when catalyzed by other

ADP-ribosyltransferases [183, 184], the reaction catalyzed by sirtuins is currently challenged to be an unspecific side-reaction [159].

Like the human SIRT2, *L. infantum* Sir2rp1 was also found to be associated with tubulin [146], the major component of Trypanosomatids cytoskeleton formed by an array of subpellicular microtubules that span the whole cell of the parasite [185]. SIRT2 is a tubulin deacetylase that displays a higher affinity for tubulin than for histones [32], and has been found to be linked to regulation of mitotic progression [86], chromatin condensation [186] and cell migration [187]. TcSir2rp1 overexpression in *T. cruzi* was found to increase the deacetylation level of endogenous tubulin [58]. It is interesting to note that Sir2rp1 from *T. cruzi* is a cytoplasmic protein like LiSir2rp1 and not nuclear like TbSir2rp1. Since *T. cruzi* shares some characteristics with *L. infantum* like the amastigote intracellular stage, it should not be ruled out that Sir2rp1 may have functions in the cytoskeleton remodeling necessary for stage differentiation. Several proteins, sirtuins included, have demonstrated the ability to shuttle from the nucleus to the cytoplasm and vice-versa [188, 189]. SIRT2, the closest sirtuin homolog of mammalian cells, is actively exported to the cytoplasm during interphase, but is accumulated in the nucleus from prophase until cytokinesis where it co-localizes with important mitotic structures like centrosomes and the mitotic spindle [190]. Curiously, analysis of TcSir2rp1 by Wregex and cNLS Mapper, bio-computational tools that identify nuclear export signals (NES) and nuclear localization signals (NLS), respectively, indicate the presence of non-canonical NES/NLS in the sequence of this sirtuin [191, 192]. Whether TcSir2rp1 does shuttle to the nucleus during specific phases of *T. cruzi* life cycle, for instance to repair DNA damage like the *T. brucei* orthologue, remains to be reported.

The mitochondrial TcSir2rp3 was found less expressed in *T. cruzi* forms proliferating in mammalian cells. Its expression increased when the parasite transformed in trypomastigotes [59]. The fact that cells overexpressing only the active form, but not the inactive form of TcSir2rp3 showed an increased intracellular growth and failed to transform in to extracellular trypomastigotes [59], suggested that it could also be a drug target to control the infection, although further experiments to generate knockout cell lines need to be performed. We recently identified several compounds that prevented intracellular growth of *T. cruzi* some of them inhibiting specifically TcSir2rp1 or TcSir2rp3 which might indicate the requirement of both enzymes for the parasite (to be published).

6. Naphtalimide derivatives as potential drugs for Chagas disease control

Naphthalimides are a class of compounds that have generated intense interest as active molecules with potential to treat a range of conditions [193]. A naphthalene ring linked to an imide group that forms a third heterocycle composes the basic chemical scaffold of the naphthalimide derivatives. This moiety has a planar nature and is considered to be responsible for the pharmacological activities attributed to compounds derived from this structure, that can be as distinct as anticancer, antibacterial, antiprotozoal, antiviral, analgesic, and anesthetic [193]. Their potential as anticancer compounds has received particular attention, mostly because of their DNA intercalating properties and also to their reported activity as topoisomerase inhibitors [194–196]. Compounds like amonafide and bisnafide have been proposed as anticancer agents and have inclusively reached clinical

trials in the past [197, 198]. Elinafide is another derivative with two naphthalimide moieties that has been evaluated in preclinical studies and demonstrated potential against various mouse xenograft models [199]. This last compound was in the origin of the synthesis of the first BNIPs that differed in the alkyl chain linking the naphthalimide and amine group, i.e. a propyl instead of an ethyl chain [200]. These derivatives showed potential activity against breast cancer MCF-7 cell line and actively bound DNA as demonstrated by thermal denaturation measurements, ethidium bromide displacement and DNA gel mobility [200]. Later derivatives that varied in the length of the chain linking the two amines of bisnaphthalimidopropyl groups were also evaluated against cancer cell lines and promastigotes of the parasite *L. infantum* [201]. While screening for enzymatic inhibitors of the recently characterized LiSir2rp1, BNIPs were identified as inhibitors of its deacetylase activity [175]. Furthermore, they were active against intracellular amastigotes, the clinically relevant stage of the parasite present in humans, at concentrations in the single micromolar range [175].

Our groups' previous results demonstrating activity toward *L. infantum* led to the testing of BNIPs as inhibitors of the related Trypanosomatid *T. brucei* and its Sir2rp1 orthologue, TbSir2rp1 [202]. BNIPs revealed to be very potent inhibitors of *in vitro* parasite growth, with one of the compounds, BNIPDabut (**Figure 1**) with an EC_{50} in the range of the reference drug pentamidine. However, when tested against the TbSir2rp1 recombinant enzyme, BNIPDabut had an IC_{50} more than 10^4 times superior to the EC_{50} against the whole cell parasite, indicating that Sir2rp1 inhibition is probably not a major mechanism of action for the compound. Whether BNIPDabut inhibits other *T. brucei* sirtuin enzymes remains to be elucidated. It should be noted that RNAi and gene knockout experiments of the three sirtuins did not led to a deleterious effect, and unlike LiSir2rp1, there is no indication of that these proteins may be essential [155, 160]. The optimal *in vitro* properties of BNIPDabut led to the testing with an *in vivo* model of trypanosomiasis by bioluminescence imaging. Although BNIPDabut maintained a strong trypanocidal activity *in vivo*, as assessed by the decrease in bioluminescent signals to levels similar to those of the reference drug control pentamidine, it was not sufficient for infection clearance, as animals' parasitaemia relapsed shortly after treatment interruption. Nevertheless, BNIPDabut should constitute a scaffold for further consideration in HAT drug discovery [202].

In a different study, BNIP derivatives were also tested against TcSir2rp1 and *in vitro* cultures of *T. cruzi* amastigotes (unpublished results). BNIPs demonstrated to inhibit the deacetylase activity of this enzyme, with BNIPSpd (**Figure 1**) as the most potent compound, showing a dose-dependent effect on inhibition. BNIPSpd also proved to be active and selective against amastigotes of *T. cruzi* in a high content screening (HCS) assay – an assay that takes advantage of computer-assisted image processing of hundreds of microscopic images originating in automated microscopes able to simultaneously capture multiple conditions or drug compounds. In this work, a new set of derivatives was also synthetized in order to improve both solubility and binding to cellular targets, mostly by including cyclic structures and heteroatoms in the carbon chain linking the two naphthalimide groups. The most active compounds were BCNIPP, also a TcSir2rp1 inhibitor, and trans BNIP-1,4-Dacyhex, a derivative of BNIPDabut that weakly inhibited the enzyme. In turn, BNIPDabut had some inhibitory activity on *T. cruzi* amastigotes, with low selectivity, but also did not inhibit TcSir2rp1 at 10 μM. The activity of BNIPSpd in a mice model of Chagas disease using bioluminescent parasites was also determined and found to be absent at the doses tested. An explanation might be the poor pharmacokinetic profile of the compounds, which fails to ever achieve at least the *in vitro* IC_{50} against *T. cruzi* amastigotes.

Altogether, our data indicate that BNIP derivatives may not be acting only by a mechanism of Sir2rp1 inhibition, with other targets contributing to the activity detected. BNIPs were originally designed and developed as anti-cancer agents [203, 204] through DNA intercalation. This property might explain some of the cytotoxic effects verified against host cells, but may also be an important mechanism of activity toward the parasite, especially since trypanosomes are highly susceptible to intercalating agents [205].

Confirmation of the mechanism of action can be undertaken by appropriate target deconvolution experiments [206]. The most common type of such experiments are biochemical methods that employ some variation of biochemical affinity purification, where the compounds are immobilized in a column, and allowed to interact with protein extracts, preferably previously fractionated. After stringent washing steps, the bound proteins are eluted and identified. Such strategy has been employed in the identification of small molecule activators of cryptochrome of mammalian cells [207]. A disadvantage is that there is a bias toward high affinity ligands, and when the target is relatively less abundant or has less affinity, important targets may not be detected. Furthermore, the washing steps may eliminate protein complexes that may be important for appropriate drug activity.

Genetic methods can also be valuable for target deconvolution. Gene knockouts and RNAi screens can be used to try to phenotype a compound's effect [208]. Furthermore, if the mutant is hypersensitive to the compound in question, the evidence that the protein could be the target for the compounds would be strengthened. The validation of trypanothione synthetase and N-myristoyltransferase as drug targets against trypanosomes are examples where the differential sensitivity of an inhibitor in wild type, overexpression, and knockout mutants is clearly illustrated [209, 210]. An additional genetic strategy is based on the generation of resistant cell lines by culturing the parasites in increasing sub-lethal drug concentrations that are posteriorly sequenced to find mutated genes related to the mechanism of action for the compound [208]. Genetic methods have recently been employed in the search of the mechanism of action of oxaboroles [211], a class of compounds in development for HAT, but also active against *T. cruzi* [212, 213].

Chemical genomics can also be applied to the discovery of novel drug targets, as exemplified by the recent characterization of cytochrome b from *T. cruzi*. This enzyme was demonstrated to be selectively targeted in relation to the human homolog by a hit compound coming from a phenotypic screening [214].

7. Perspective

Here we highlighted the potential of sirtuins, particularly in the *T. cruzi* as possible targets of drug development for Chagas disease for the following reasons:

1. The parasite contains only two sirtuins, instead of three present in *Leishmania* spp. and *T. brucei* species, and seven in the human host, which facilitates a more precise design and avoid redundant effects.

2. Several experiments demonstrate the requirement of these enzymes for growth and survival, particularly in intracellular forms of the parasite, which are clinically relevant.
3. There are several compounds already designed to be selective sirtuin inhibitors that could be modified to provide increased specificity and selectivity to the parasite enzyme. Some of these compounds have already defined druggability and specific derivatives can be repurposed more easily.
4. Both sirtuins were produced to perform enzymatic screenings and crystals of TcSir2rp1 have been obtained to be the basis of further medicinal chemistry.

Finally, several lead compounds were identified for *T. cruzi* sirtuin, which can provide the basis for future development. It is also important to note that these possible inhibitors could act synergistically with drugs already in use for treatment, as a novel combinational therapy, opening new avenues to eliminate Chagas disease.

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Author details

Luís Gaspar [1,2], Terry K. Smith [3], Nilmar Silvio Moretti [4], Sergio Schenkman [4] and Anabela Cordeiro-da-Silva [1,2,5]*

*Address all correspondence to: cordeiro@ibmc.up.pt

1 Instituto de Investigação e Inovação em Saúde, University of Porto, Portugal

2 Institute for Molecular and Cell Biology, University of Porto, Porto, Portugal

3 Biomedical Sciences Research Complex, The University, St. Andrews, Fife, Scotland, UK

4 Departament of Microbiology, Imunology and Parasitology, Escola Paulista de Medicina, Federal University of São Paulo, São Paulo, Brazil,

5 Departament of Biological Sciences, Faculty of Pharmacy of University of Porto, Porto, Portugal

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This book contains 11 chapters of significant and updated materials on what we know and what we lack and need in better understanding of Trypanosoma cruzi—a parasite that never dies—and the consequences of Chagas disease as one of the most important neglected parasitic diseases threatening the global health and wellbeing. This book is intended to increase the readers' enthusiasm to explore the four sections of the contents: Section 1 begins with biochemistry, pathophysiology, histo-immunological study, and findings to assist in the diagnosis; Section 2 further investigates the role of vector in propagation of the parasite, the intensity on epidemiology, and the severity on clinical aspects, which help us to be well perceived on the course of disease; Section 3 is seeking beyond modern medicine and what lays in the nature that helps fight against this parasite; and the last section, Section 4, deals with the impacts of public health problem and the control strategies on Chagas disease.

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