Chagas Disease 3

Challenges and advancements in the development of vaccines and therapies against Chagas disease

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Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, presents a substantial global health burden, affecting millions of individuals worldwide and posing a continual risk of infection. Despite the high mortality and morbidity rates, effective vaccines to prevent infection by the parasite remain elusive, and the drugs currently available are suboptimal. Understanding the intricate dynamics of parasite–host interactions and the resulting immune responses, which contribute to both protection and pathology, is crucial for the development of effective vaccines and therapies against Chagas disease. In this Series paper, we discuss the challenges associated with discovering and translating prophylactic and therapeutic strategies from the laboratory bench to clinical application. We highlight ongoing efforts in vaccine and new drug development, with a focus on more advanced candidates for vaccines and drugs. We also discuss potential solutions, emphasising the importance of collaborative research efforts, sustained funding, and a comprehensive understanding of host–parasite interactions and immunopathology to advance the development of new vaccines and therapies against Chagas disease.

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Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, poses a notable health burden in Latin America and has been affecting other non-endemic areas such as the US and Europe.[1] With an estimated 6–7 million individuals infected with *T cruzi* worldwide,[1] the disease often leads to severe cardiac and gastrointestinal complications. Despite the substantial burden of Chagas disease on global health, the development of effective vaccines and drugs against the disease has been hindered by the various life stages, routes of infection, and immune evasion mechanisms of *T cruzi* and the often long-lasting asymptomatic phase (figure).

The complex lifecycle of *T cruzi*, which involves different life stages, as well as invertebrate and mammalian hosts, and the diverse structure of its population has made development of vaccines difficult. The antigenic variation and geographical differences within the *T cruzi* population complicate efforts to effectively target the parasite,[2] and their direct effects on immune control, drug effectiveness, and vaccine development are yet to be fully elucidated. Additionally, *T cruzi* can circumvent the host's immune defences, leading to chronic infections.[4] The dynamics of transmission, which include vectorial, oral, and congenital pathways, further complicate vaccine development and effectiveness because the parasite load and immune response elicited can vary depending on the route of infection.[5,6] Therefore, an ideal vaccine would elicit durable protective immunity against all parasite populations (irrespective of the infection route), in addition to countering the parasite's mechanisms of immune evasion.

Challenges also persist in the development of therapeutic interventions for Chagas disease. The current drugs, benznidazole and nifurtimox, have some disadvantages, including lengthy treatment courses, adverse side-effects, and variable effectiveness, particularly in the chronic phase. The often oligosymptomatic nature of the acute phase, absence of systematic routine screening in endemic areas, and extended asymptomatic chronic phase pose substantial hurdles to early detection and treatment of infections. These hurdles shorten the window for effective intervention before the onset of severe disease manifestations. Unavailability of a reliable method to assess treatment effectiveness is another key issue that needs to be resolved.

In the following section, we discuss the importance of a better understanding of the complex host–pathogen interactions in the context of human Chagas disease as a crucial tool to identify preventive and therapeutic interventions, as well as biomarkers for vaccine development and therapeutic effectiveness.

Host–pathogen interactions and immune responses

Most insights into immune responses during the acute phase of *T cruzi* infection have come from experimental models.[7,8] Pathogen-associated molecular patterns, which are prevalent on the surface of *T cruzi*, stimulate innate cells, promoting parasite clearance and cytokine production and fostering a potent cellular response. Natural killer and $CD8^+$ T cells contribute to the control of infection through various mechanisms, including cytokine production and parasite elimination. Simultaneously, antibody production is initiated, with lytic antibodies targeting parasitic forms in circulation. Many vaccine targets were identified on the basis of their immunogenicity during the acute phase. Of note, the high antigenic variability of *T cruzi* populations leads to distinct immune responses and adds complexity to developing prophylactic and protective efforts.[4] Proteomics-based or glycoproteomics-based analysis to identify common immunogenic targets and the use of

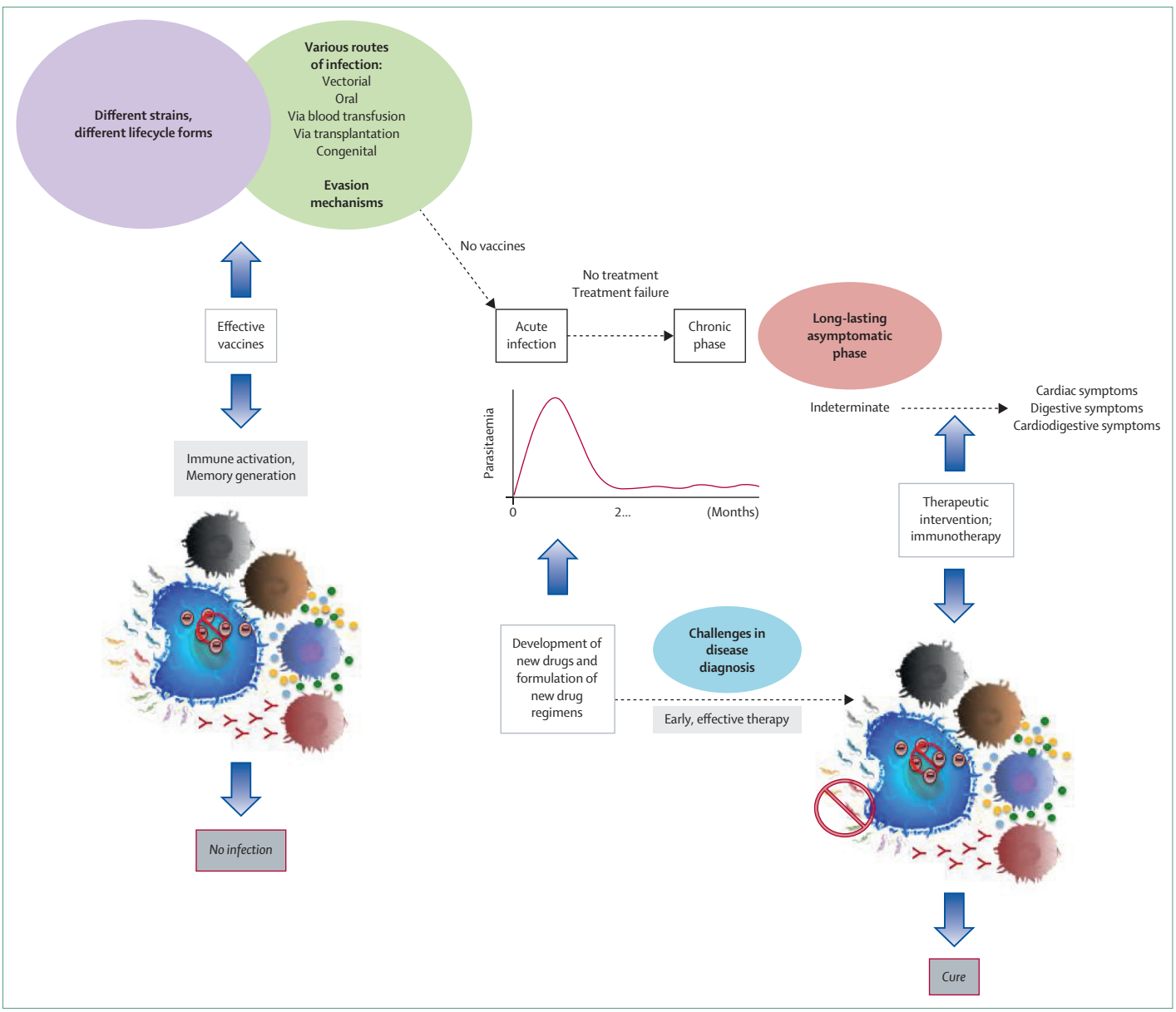

Figure: **Challenges influencing the development of vaccines and new drugs against Chagas disease**
The complex lifecycle of *Typanosoma cruzi* and its diverse population structures, along with the various forms of infection and evasion mechanisms used by the parasite are major hurdles for the development of vaccines and new therapies (purple and green circles). The long-lasting nature of the chronic asymptomatic form, and the challenges associated with disease diagnosis pose additional challenges for therapeutic intervention (orange and blue circles). Effective vaccines that target the various parasite forms and subpopulations and induce lasting memory immune responses are crucial for preventing infection. The development of new drugs, drug regimens, and immunotherapeutic approaches are crucial for treating the large contingent of individuals already infected, aiming to achieve a cure or to prevent the progression to severe forms of disease. Identifying point-of-care diagnostic methods and clinical, molecular, or cellular biomarkers, or a combination of these, is essential to enable timely treatment and measure treatment effectiveness and cure. Dashed arrows denote some of the challenges, and solid arrows indicate the solutions and outcomes. The parasitaemia graph and cell graphics have been reproduced from previous publications by our group (eg, in Magalhães and colleagues[2] and Koh and colleagues[3]).

chimeric candidates can serve as strategies to circumvent this issue.[9]

While the cellular response is not clearly defined in human acute, initial, infection, it is possible that similar mechanisms described in experimental models occur since an intense inflammatory reaction is observed in acutely infected individuals.[10] Additionally, robust antibody production is detected, leading to the generation of antibodies used in conventional serological tests, as well as lytic antibodies associated with protective activity. Although the host response effectively reduces parasitaemia to a subpatent degree at the end of the acute phase, the response is insufficient to eliminate the parasite. Parasite evasion mechanisms could enable this persistence.

Without effective treatment, Chagas disease progresses to its chronic phase. Approximately 30% of individuals with

Chagas disease develop severe cardiac or digestive symptoms, or both, with the cardiac form having the highest morbidity and mortality due to intense inflammatory reactions leading to fibrosis and heart failure.[10] Patients with the cardiac clinical form display a highly inflammatory response, whereas individuals within the indeterminate clinical form display a more balanced immune response.[3] Evidence suggests that the progression to cardiac disease is associated with a loss in the ability to control inflammation (and the consequences that come from such a loss),[3,7] and a lower production of lytic antibodies.[11] Platforms to ascertain the levels of the lytic antibodies have been developed and hold great promise as much needed measures for therapeutic effectiveness.[12] Moreover, candidates that induce lytic antibody production could be effective as prophylactic and therapeutic vaccines.[9]

Numerous vaccine candidates have been identified that can elicit a robust immune response upon challenge, thereby preventing the progression of infection by reducing parasite load and tissue damage, with the more advanced ones discussed in the following sections. These candidates need to be tested in varied settings and against diverse *T cruzi* isolates to verify their effectiveness as potential vaccines.

Vaccines against *T cruzi* infection

Over a century has passed since the discovery of Chagas disease and numerous vaccine prototypes have been proposed for the disease with varying degrees of success. From whole-killed or attenuated pathogens to recombinant protein-based or nucleic acid-based vaccines,[13,14] a consistent theme has emerged: partial protection. Partial protection denotes that most immunisation protocols tested in animal models have managed to control *T cruzi* infection or mitigate damage to some extent, but the goal of achieving sterilising immunity remains elusive.

This persistent challenge raises crucial questions. Why has sterilising immunity proven to be elusive to achieve? How can partial protection be enhanced? Is achieving sterilising immunity strictly necessary? Can concomitant immunity prevent symptomatic forms of the disease? Or, as Camargo and colleagues[15] questioned, why is a vaccine against Chagas disease still not available? The essential factors that should be considered for the development of a vaccine against *T cruzi* infection have been discussed in the literature.[16]

Although prophylactic vaccines are of great interest, the major obstacle in their development is the demonstration of their clinical efficacy, mainly due to the low incidence of the target disease and the long and variable times required for the symptomatic forms to manifest. Conversely, evaluating the effectiveness of therapeutic vaccines in individuals previously diagnosed with the disease is more feasible. Additionally, the integration of immunotherapy with antiparasitic drugs, known as vaccine-linked chemotherapy, has emerged as an attractive alternative. This integration not only complements the action of the currently available drugs but also holds great promise in reducing drug doses, thereby minimising the likelihood of adverse effects.

Many studies have aimed at developing a vaccine for Chagas disease. However, despite presenting promising results, most candidates never advanced to preclinical and clinical phases, owing to many reasons, ranging from regulatory hurdles to logistic issues, market limitations, and low profitability that hindered substantial and consistent funding. This section reviews the most advanced vaccine prototypes and strategies positioned to bridge the gap between the preclinical and clinical phases. A summary of the main candidates is presented in table 1.

Vaccine candidates

Tc24, also known as flagellar calcium-binding protein, is a 24-kDa calcium-binding protein located mainly at the flagellar pocket of the parasite. The expression of Tc24 across all parasitic stages, high degree of conservation among different *T cruzi* strains,[35,36] and robust immunogenicity in experimental and natural infection have rendered Tc24 an enticing vaccine candidate. Prophylactic and therapeutic vaccines based on DNA coding for Tc24 have shown efficacy in reducing the parasite load and preventing heart muscle damage in murine and dog models. Coimmunisation with plasmids coding for Tc24 and trypomastigote surface antigen 1 has also conferred protection in prophylactic and therapeutic settings. Recombinant Tc24 (rTc24) plus adjuvant has protected against a challenge with *T cruzi* trypomastigotes. rTc24 has also shown efficacy in a therapeutic approach, eliciting a T-helper-1 response capable of decreasing parasitaemia during the acute phase and reducing the parasite load and inflammatory infiltrates in the heart at 50 days after infection.[17]

However, protein aggregates of rTc24 mediated by disulphide bonds after expression in *Escherichia coli* pose a substantial challenge that hinders production that is compliant with good manufacturing practices. To circumvent this problem, Seid and colleagues[18] designed a new version of the immunogen, called Tc24-C4, with all cysteines mutated to serines and the his-tag deleted. This modification enhanced the immunogen's acceptability to regulatory authorities for potential clinical studies. Tc24-C4 has shown non-inferiority to the wild-type version in terms of immunogenicity and efficacy. The authors also showed the robustness of the production process and the long-term stability of the immunogen.[19]

The safety and immunogenicity of Tc24-C4 have been tested in non-human primate models as well. Rhesus macaques immunised with three doses of Tc24-C4 and E6020-SE displayed a strong humoral and T-helper-1 cell-mediated response.[20] The clinical efficacy in this animal model remains to be tested. Additionally, Poveda and colleagues[21] evaluated the immunogenicity of an mRNA coding for Tc24 in a mouse model, with heterologous prime-boost protocols (mRNA followed by subunit vaccine or subunit vaccine followed by mRNA) resulting in a strong humoral, CD4, and CD8 immune response.

	Description	Key findings	References
Tc24	24-kDa calcium-binding protein, highly conserved, immunogenic.	Reduced parasite load, prevented heart damage, displayed efficacy upon coimmunisation with TSA-1, displayed efficacy with recombinant Tc24 plus monophosphoryl lipid A, and showed promising results with Tc24-C4 and E6020-SE during the chronic phase. Safety and immunogenicity tested in non-human primates. Upcoming clinical trials by Baylor College of Medicine, TX, USA and the Autonomous University of Yucatán, Mexico.	17–21
Traspain	Trivalent chimeric antigen engineered with domains of cruzipain, trans-sialidases, and ASP-2, as part of the CRUZIVAX project, showed promising results in mice.	CRUZIVAX focuses on preclinical evaluation in multiple animal models, production compliant with good manufacturing practices, health economics analysis, and phase 1 trials. Needle-free intranasal immunisation with recombinant Traspain and the adjuvant CDA reduced parasite burden and prevented muscle damage.	22,23
Trans-sialidases	Active trans-sialidases and ASP-2 successful as preclinical vaccine candidates.	Reduced parasitaemia, increased survival, and prevented cardiac injury. Prophylactic intranasal immunisation with trans-sialidase plus CDA showed protection. Trans-sialidase and ASP-2 used widely for monocomponent or multicomponent vaccine prototypes.	24-26
Multicomponent vaccines and chimeric antigens	Various combinations explored for increased immune response.	Combination of antigens more effective than individual vaccinations. Traspain chimeric protein showed efficacy with the adjuvant CDA. Combination of Tc24-C4 with $CD8^+$ T-cell polyepitope sequence showed higher survival. The heterologous antigen NCz-SEGN24A provided protection.	22,24,27–32
Vaccine-linked chemotherapy	Therapeutic vaccines combined with antiparasitic drugs.	Combined treatment with a trans-sialidase vaccine followed by benznidazole showed improvements in pathology parameters. A bivalent vaccine (TSA-1, Tc24) combined with low doses of benznidazole in the early chronic phase partly prevented cardiac fibrosis and activated $CD8^+$ T-cell responses. Concurrent approach with adjunct therapies proposed for preserving cardiac structure and function.	32–34

ASP-2=amastigote surface protein 2. CDA=cyclic di-AMP. TSA-1=trypomastigote surface antigen 1.

Table 1: **Vaccine candidates for *Trypanosoma cruzi* infection**

Another candidate is Traspain, a breakthrough in vaccine design, comprising a trivalent chimeric antigen crafted from three pivotal *T cruzi* antigens, the N-terminal domain of cruzipain (a prominent *T cruzi* cysteine protease), an α-helix linker inspired by a trans-sialidase sequence and the central region of the amastigote surface protein-2 (ASP-2). Needle-free intranasal immunisation of mice with recombinant Traspain and the adjuvant cyclic di-AMP induced strong humoral and cell-mediated immunity, which conferred protection against challenges with different *T cruzi* strains.[22] Mice intranasally immunised with recombinant Traspain and cyclic di-AMP showed reduced parasitaemia and a higher survival rate than the non-immunised mice, after a lethal challenge with the RA strain of *T cruzi*. Additionally, when challenged with a sublethal dose of the same *T cruzi* strain, vaccinated mice showed lower serum levels of enzymes associated with tissue damage (creatine kinase, creatine kinase-MB, lactate dehydrogenase, glutamic oxaloacetic transaminase) in the chronic phase and lower inflammatory infiltrates in the cardiac and skeletal muscles.

The promising outcomes of this work spurred the establishment of a multinational consortium comprising 11 institutions, culminating in the inception of the CRUZIVAX project in 2019, currently funded by a Horizon 2020 grant from the EU. The main objective of CRUZIVAX is to bridge the gap between preclinical and clinical development by performing preclinical and clinical phase 1 studies of a needle-free vaccine against *T cruzi*. This ongoing project covers (1) preclinical assessment of Traspain plus cyclic di-AMP in three animal models (mice, dogs, and non-human primates) via prophylactic and therapeutic approaches, (2) development and manufacturing of the immunogen and adjuvant under standards that are compliant with good manufacturing practices, (3) a health economics analysis to identify the crucial parameters of the target product profile, and (4) a phase 1 vaccine clinical trial in healthy volunteers. The design of the health economics study has been published.[23]

For more on the **CRUZIVAX project**, see https://www.cruzivax.eu/en/

Trans-sialidases stand out as pivotal virulence factors of *T cruzi*, with various members of this gene superfamily proposed and tested as vaccine candidates.[37,38] Particularly promising are the catalytic domain of active trans-sialidases and ASP-2, an inactive trans-sialidase primarily expressed during the intracellular stage of the parasite. Mice immunised with adenoviral vectors encoding trans-sialidases and ASP-2 presented reduced parasitaemia and enhanced survival rates than the mice in the control groups upon *T cruzi* challenge.[24] Pacini and colleagues[25] showed that prophylactic intranasal immunisation with trans-sialidases coupled with cyclic di-AMP elicited robust humoral and cell-mediated immunity, conferring protection against oral *T cruzi* challenges. Therapeutic vaccination with recombinant adenoviruses encoding ASP-2 plus trans-sialidases during the chronic phase of infection not only arrested disease progression but also ameliorated pre-existing cardiac injury.[26]

Multicomponent vaccines and chimeric antigens

Researchers have explored the combination of multiple parasite antigens in their pursuit of increasing the breadth of the immune response, to ensure its ability to control parasite proliferation. A preventive vaccine candidate

comprising an oral multicomponent vaccine, such as cruzipain, Tc52, and Tc24, showed strong systemic and mucosal immune responses, leading to reduced parasite load and enhanced survival rates after a challenge.[27] Similarly, a vaccine candidate containing two antigens, TcG2 and TcG4, encoded in plasmids of a eukaryotic expression-based vaccine, showed high effective immunity and efficacy in mice.[28] Likewise, Machado and colleagues[24] combined adenoviral vectors encoding ASP-2 and trans-sialidases and obtained similar results.

An alternative strategy involved a therapeutic DNA vaccine fusing cruzipain and its natural inhibitor chagasin with granulocyte-macrophage colony-stimulating factor as an adjuvant. Upon being administered orally and delivered by an attenuated *Salmonella* spp strain during the acute phase of *T cruzi* infection, this bicomponent vaccine not only enhanced the protection afforded by each antigen as a monocomponent therapeutic vaccine but also triggered a robust cellular response characterised by interferon gamma secretion. This response curbed parasitaemia in the acute phase of infection and mitigated tissue damage in the chronic stage.[39] In all instances, the combination of antigens proved more effective than the individual vaccinations. However, the authors noted that utilising vaccination protocols with multiple antigens might not be cost-effective.

In response to the challenge of attacking the parasite from different angles yet avoiding the production of multiple antigens, the chimeric recombinant protein Traspain was developed.[40] Traspain adjuvanted with a stimulator of interferon genes agonist cytidine deaminase (CDA) elicited a robust humoral and cellular immune response. A prime-boost combination of recombinant Traspain plus CDA with the *Salmonella* spp Traspain DNA-delivery system further increased the cellular response, enhancing protection.[40] In another study, a chimeric antigen containing segments of trans-sialidases and ASP-2, along with the C-terminal repeats of trans-sialidases (known as shed acute phase antigen), adjuvanted with the Toll-like receptor (TLR)-3 and MDA5 ligand, polyinosinic–polycytidylic acid–poly-L-lysine carboxymethylcellulose, was shown to reduce tissue parasitism and parasitaemia in mice, as well as heart inflammation in dogs.[25]

Mice immunised with Tc24-C4 fused to a $CD8^+$ T-cell polyepitope sequence (Tc24-C4.10E) as a therapeutic vaccine without adjuvant during the acute phase of the infection, showed higher survival and lower parasitaemia, than those immunised with Tc24-C4.[30]

A heterologous antigen comprising a non-toxic modified bacterial superantigen and the N-terminal domain of cruzipain was also developed.[31] This chimeric antigen NCz-SEGN24A adjuvanted with CpG- oligodeoxynucleotide reduced parasitaemia, tissue parasitism, and inflammation, improving the performance of non-conjugated Nt-Cz and SEGN24A antigens. An oral version of NCz-SEGN24A DNA immunisation vectored by attenuated *Salmonella enterica*, or a prime-boost immunisation with both components, was particularly effective in inducing a strong cellular response that reduced the parasite load throughout the acute phase and maintained 100% mouse survival with a substantial reduction in tissue damage (Antonoglou MB and colleagues, Universidad de Buenos Aires, Buenos Aires, Argentina, personal communication).

Vaccine-linked chemotherapy

The progression of Chagas disease to chronic phase is predominantly attributed to the inconspicuous acute infection that often goes unnoticed, preventing early therapeutic intervention. Although the drugs benznidazole and nifurtimox have been used successfully during the acute phase, their effect on the clinical progression of the disease remains uncertain. The lower effectiveness observed in the chronic phase could be explained in part by the difficulty of the drugs to reach the niches in which metabolically quiescent parasites that are resistant to treatment could persist.[33]

Therapeutic vaccines have emerged as a promising alternative, prompting investigations into their synergy with antiparasitic drugs in murine models. Although therapeutic vaccines might work through variable mechanisms that need to be determined for each candidate, their use could contribute to more efficient elimination of the parasite, reducing parasite-triggered inflammation. Moreover, therapeutic vaccines could allow for the use of lower drug doses, thereby reducing the incidence of adverse effects. Some of the different vaccine-linked chemotherapy schemes found in the literature are described in the following paragraphs.

One study used a vaccine formulation based on the N-terminal fragment of trans-sialidases and an immunostimulant particle adjuvant followed by benznidazole treatment. Although the administration of the vaccine as monotherapy reduced electrocardiographic alterations, cardiac tissue damage, and parasite load, vaccination associated with benznidazole treatment further improved electrocardiographic parameters in animals infected with the strain Tulahuen cl/2.[40]

A bivalent therapeutic vaccine, blending two *T cruzi* antigens (trypomastigote surface antigen 1 and Tc24), a synthetic TLR-4 agonist-adjuvant, and low doses of benznidazole (25 mg/kg daily) administered on days 72–79 post-infection, partly prevented cardiac fibrosis and strongly activated $CD8^+$ T-cell responses.[32] A subsequent study utilising Tc24 and the TLR-4 adjuvant showed that vaccine-linked chemotherapy effectively ameliorates the changes in cardiac structure and function induced by *T cruzi* infection.[41] However, using a similar approach, other studies found that treatment with low doses of benznidazole plus vaccine preserved the liver health better but did not reduce the parasite burden.[42]

Given the absence of a vaccine to prevent Chagas disease and the considerable number of individuals already infected with *T cruzi*, new and better therapeutic interventions are of paramount importance. The challenges and novel

perspectives for treating Chagas disease will be discussed further in the following sections.

Therapeutic approaches for Chagas disease

Main challenges in developing new drugs

The availability of only two antiparasitic drugs to treat *T cruzi* infection underscores the classification of Chagas disease as one of the 25 neglected tropical diseases defined by WHO. Benznidazole and nifurtimox are both prodrugs metabolised by parasite nitroreductases, which convert them into intermediate products with antiparasitic activity by generating reactive oxygen species. Currently, both drugs are indicated for acute cases, congenital infections, reactivations, and individuals in the chronic phase either without clinical symptoms or with mild cardiac or digestive involvement.[43] The recommended standard-of-care regimens are 300 mg per day of benznidazole, divided into two-three doses for 60 days, and 8 mg/kg per day of nifurtimox, divided into three doses for 60 days.

Although highly effective when administered during the acute phase of the disease, the effectiveness of benznidazole and nifurtimox reduces in the chronic stage. Nevertheless, benznidazole and nitroheterocyclic drugs remain the preferred therapeutic option over all the compounds tested and their various combinations.[44–49] Of note, treating *T cruzi*-seropositive women with benznidazole before pregnancy effectively prevents the transmission of infection from mother to child.[50,51]

Even decades after the discovery of their effectiveness in treating *T cruzi* infection, some key challenges persist in the use of benznidazole and nifurtimox. A series of published studies showed that the presence of the parasite, linked to an unbalanced immune response in some individuals, is sufficient to trigger a sustained inflammatory response that underlies the organ damage observed in chronic Chagas disease.[52]

Demonstrating the long-term effectiveness of current medicines and the development of new drugs poses serious challenges. The success of therapeutic response is measured in terms of achieving negative results in all conventional serological tests. In chronic cases, this positive specific conventional serology could persist for 8–10 years under optimal circumstances. Moreover, given the extended period required to observe clinical complications in the chronic stages of the infection, together with the absence of disease progression markers, little information is available on the long-term effectiveness of the current drugs, which hampers the evaluation of new molecules.

T cruzi possesses a diploid genome containing up to 22 000 coding sequences, which have key roles in parasite invasion and antigenicity. These coding sequences are frequently subject to recombination events.[53] Additionally, *T cruzi* is genetically categorised into seven major lineages, referred to as discrete typing units, namely, TcI–TcVI and TcBat. These lineages show distinct geographical distributions and varying degrees of virulence and resistance to drugs.[16] The genetic variability of *T cruzi* infection adds complexity to the development of new therapeutic options.

Of late, a subset of metabolically quiescent *T cruzi* amastigotes has been identified, which persist as quiescent or persister parasites, replicating at a slow rate and displaying reduced susceptibility to nitroheterocyclic drugs.[54,55] These quiescent parasites retain the ability to become metabolically active at some point, compromising the effectiveness of standard-of-care regimens of current medicines, as well as the evaluation of new molecules.[39]

Another crucial factor is the high incidence of adverse drug reactions such as dermatologic, gastrointestinal, and neurological reactions associated with the use of benznidazole and nifurtimox in adults. Although most adverse drug reactions are mild and can be effectively managed with symptomatic treatment, the occurrence of adverse drug reactions results in permanent discontinuation of treatment in 9–31% of cases.[44–47,56] The challenges in administering benznidazole and nifurtimox, including managing adverse drug reactions, coupled with the previously mentioned challenges, contribute to the current scenario in which fewer than 1% of individuals with *T cruzi* infection worldwide have received etiological treatment.[57]

Current status of drug pipeline

The therapeutic approaches for *T cruzi* infection have substantially changed in the past 10 years, with ongoing phase 2 clinical trials of two molecules (fosravuconazole and fexinidazole),[47,49] and a third therapeutic option, which is based on repurposing an antifungal compound with activity against *T cruzi* (posaconazole).[44] However, none of these options have shown higher efficacy than benznidazole at the doses and regimens tested. Additionally, when tested in combination with benznidazole, the efficacy of the combination did not surpass that of benznidazole monotherapy in the standard-of-care regimen.[45,47] Regarding safety and tolerability, fosravuconazole generally causes mild to moderate adverse effects, primarily gastrointestinal. Fexinidazole has been associated with mild side-effects, including headache and nausea, with few severe reactions reported. Common side-effects include gastrointestinal issues, liver enzyme elevations, and headaches. Although generally well-tolerated, long-term use might require monitoring for liver toxicity and other potential complications.

Hence, efforts to provide improved treatment options have focused on two main areas, namely, (1) enhancing the utilisation of benznidazole and nifurtimox by investigating different treatment regimens for both the drugs, and (2) continuing the search for novel chemical entities that are active against *T cruzi* infection with alternative modes of action beyond those already explored.

With respect to the clinical trials evaluating the different regimens of benznidazole and nifurtimox, publications on population pharmacokinetic experimental models report that the current regimens achieve serological concentrations

	Experimental arms	Design	Countries	Follow-up period	Study population	Status
BETTY NCT0367248	Benznidazole 150 mg per day for 30 days	Phase 3 non-inferiority	Argentina	10 months	Postpartum women with chronic *Trypanosoma cruzi* infection	Active
EQUITY NCT02369978	Benznidazole 150 mg per day for 120 days; nifurtimox 240 mg per day for 120 days	Phase 3 non-inferiority	Colombia, Argentina	12–18 months	Adults with chronic *T cruzi* infection	Concluded, under analysis
MULTIBENZ NCT03191162	Benznidazole 400 mg per day for 15 days; benznidazole 150 mg per day for 60 days	Phase 2 non-inferiority	Argentina, Brazil, Colombia, Spain	12 months	Adults with chronic *T cruzi* infection	Recently published[20]
TESEO NCT03981523	Benznidazole 150 mg per day for 30 days; benznidazole 300 mg per day for 60 days; benznidazole 150 mg per day for 90 days; nifurtimox 240 mg per day for 30 days; nifurtimox 480 mg per day for 30 days; nifurtimox 240 mg per day for 90 days	Phase 2 non-inferiority	Bolivia	36 months	Adults with chronic *T cruzi* infection	Active
NuestroBen NCT04897516	Benznidazole 300 mg per day for 2 weeks; benznidazole 300 mg per day for 4 weeks	Phase 3 non-inferiority	Argentina	12 months	Adults with chronic *T cruzi* infection	Active

Table 2: **Current clinical trials evaluating new regimens involving benznidazole or nifurtimox, or both**

of benznidazole within the effective range (nifurtimox, 1–3 μg/mL; benznidazole, 3–6 μg/mL).[58] Additionally, reducing the daily dosing of benznidazole and nifurtimox in murine models yielded parasitological efficacy similar to that of standard regimens.[58]

An observational study showed that 20% of the patients who did not complete the entire treatment with benznidazole, but completed at least 10 days of treatment, displayed negative serological results.[59] Comparison of fosravuconazole (E1224) with the placebo and benznidazole in another study revealed parasite clearance in all E1224 treatments during the treatment phase but the absence of a sustained response. Animal studies showed that E1224 could be an ideal candidate for combination therapy with benznidazole and nifurtimox, to enhance therapeutic response and shorten the treatment duration. Thus, the BENDITA trial was designed to evaluate E1224 in combination with benznidazole, and shorter regimens of benznidazole in monotherapy (300 mg daily for 15 and 30 days),[47] which revealed that 80% or more of the patients in all treatment groups had a sustained parasitological response at 12 months after the end of treatment. Shorter regimens of benznidazole in monotherapy warranted further investigation.

Based on the aforementioned information, a series of studies have been initiated since 2019 to investigate different regimens of benznidazole or nifurtimox, or both. These studies explore lower doses administered over shorter or longer durations, aiming to enhance the effectiveness or safety, or both, of the regimens that are currently considered standard-of-care. A brief overview of each study, pending the analysis of their results, is shown in table 2.

Publications from the MULTIBENZ trial[60] reveal that upon comparing the low-dose (150 mg daily for 60 days) and short-course (400 mg daily for 15 days) regimens with the standard treatment group, there were no discernible differences in the sustained parasitological negativity, in line with the results of the BENDITA trial.[47] In an observational study, an intermittent treatment schedule with benznidazole administered at the recommended daily doses every 5 days for a total of 60 days yielded similar antiparasitic efficacy as the standard schedule at the end of the treatment, in addition to substantially reducing benznidazole-related adverse events.[61]

The second strategy to enhance the therapeutic options for *T cruzi* infection that has been pursued since 2004 involves the exploration of new chemical entities. Identifying new molecules that are active against *T cruzi* is an intensive, lengthy, expensive, and multidisciplinary process.[62] In this context, the requirement to adequately address the aforementioned persistent forms of the parasite that are currently poorly characterised and cannot be reproduced in vitro, as well as the widespread tissue distribution of *T cruzi* in vivo and their localisation within host cells, further complicates the drug development process. This complication is owing to the need for compounds with specific physicochemical properties to effectively reach these sites. Drug candidates need to achieve sterile cure in a chronically infected Chagas murine model, in addition to an appropriate degree of preclinical safety for clinical development. The most advanced clinical and preclinical drug candidates that meet these criteria are discussed in the following section.

Fexinidazole, a nitroimidazole approved for the treatment of both the peripheral nervous system and CNS stages of human African trypanosomiasis, has shown a curative efficacy in a murine model of *T cruzi* infection.[63] Although the exact mechanism of action of fexinidazole remains unknown, nitroreductases encoded by trypanosomes appear to activate fexinidazole and its metabolites through reduction to form reactive intermediates that are most likely to act against and damage multiple DNA and protein targets within the parasite.[64,65] In a phase 2 study in Bolivia, the drug revealed a quick and sustained antiparasitic effect in patients with chronic *T cruzi* infection, but the trial was halted due to safety events.[48] A multicentre study carried out in Spain with lower and shorter doses of fexinidazole showed low efficacy (19% *vs* 13% in the control group) with monotherapy after 12 months of follow-up.[49]

Following the discovery of a selective proteasome inhibitor, GNF6702, which showed in-vitro and in-vivo activity against *T cruzi*, *Trypanosoma brucei*, *Leishmania donovani*,

and *Leishmania major*,[66] a newly derived structural analogue LXE408 showed conserved pan-kinetoplastid activity on the same proteasome target.[67] LXE408 showed potent growth inhibition activity against intracellular *T cruzi* and was non-cytotoxic to mammalian cells up to the highest concentration tested (Rao S and colleagues, Novartis, personal communication). Based on unpublished results of preclinical and phase 1 studies, a phase 2 study to compare the efficacy, safety, pharmacokinetics, and pharmacodynamics of LXE408 to those of placebo and benznidazole in participants with chronic Chagas disease without severe cardiac or gastrointestinal dysfunction is under evaluation to be implemented in 2024 (Rao S and colleagues, Novartis, personal communication).

The other two potential therapeutic options that are currently in preclinical and phase 1 stage of development are benzoxaboroles. One of them is AN15368, a benzoxaborole that targets the kinetoplast mRNA processing factor, cleavage and polyadenylation specificity factor 3,[68] which has shown in-vitro activities in a range of *T cruzi* discrete typing units as well as full parasite clearance in non-human primates naturally infected with *T cruzi*.[69] The other option is DNDI-6148, a second benzoxaborole identified in a lead optimisation programme targeting visceral leishmaniasis. In addition to the combined impressive efficacy of DNDI-6148 against *L donovani* in vitro and in vivo, DNDI-6148 was fully curative in a murine model with chronic *T cruzi* infection. DNDI-6148 was part of the completed phase 1 study with a single oral ascending dose in healthy volunteers (EudraCT: 2018–004023-37); a phase 1 multiple ascending dose trial will be the next step for the compound, after which DNDI-6148 will be lined up for further development of a drug for visceral leishmaniasis.[70] DNDI-6148 has also been nominated as a clinical candidate for the treatment of Chagas disease (Pinazo MJ, DNDi, personal communication).

Cyanotriazoles, which are topoisomerase inhibitors, were originally identified as potent inhibitors of *T brucei* growth in a whole-cell high-throughput screening of the Novartis compound collection and further proven to be potent growth inhibitors of a panel of kinetoplastid parasites, including *T cruzi* and *L donovani*, before being further optimised to specifically improve their solubility, oral availability, and brain penetration for targeting human African trypanosomiasis and Chagas disease.[71] The optimised candidate CT3 identified in this programme delivered sterile cure in a bioluminescence mouse model with chronic *T cruzi* infection over a remarkably short period of only 5 days, thereby highlighting the rapid onset of drug action. The mechanism of action of cyanotriazoles was elucidated with the help of cryoelectron microscopy approaches that confirmed a selective, irreversible inhibition of trypanosomal topoisomerase II via stabilisation of the double-stranded DNA–enzyme cleavage complexes.[71] The covalent interaction with the topoisomerase target can be reasonably associated with the rapid and complete sterilising activity profile of cyanotriazoles.

From a related discovery and lead optimisation effort, Novartis additionally reported a series of amidobenzimidazoles as pan-kinetoplastid protein kinase inhibitors that can achieve a nanomolar potency range for *T brucei*, *Leishmania* spp, and *T cruzi* in cellular assay systems.[72] Target deconvolution using overexpressed *T brucei* mutants identified the dual-specificity protein kinase CDC-like kinase 1, a kinetochore component essential for mitosis, as the primary target of this series. The lack of specificity for human CLK1 is conferred via the irreversible binding to an ATP pocket that is not present in human CLK1, as shown by biochemical and co-crystallisation studies.[72] The amidobenzimidazole lead has shown encouraging results in achieving sterile cure in chronically infected murine models of Chagas disease (Rao S and colleagues, Novartis, personal communication), tentatively adding another preclinically validated mechanism of action and associated chemical series to the small research and development portfolio of Chagas disease.

This overview of preclinical candidates can be completed by mentioning the University of Washington series, a novel series of *T cruzi* inhibitors identified by Buckner and colleagues at the University of Washington, Seattle, WA, USA. A partnership with the University of Dundee Drug Discovery Unit, GlaxoSmithKline, and DNDi is currently further optimising this scaffold with the aim of nominating a preclinical candidate to advance to the next stage of development.[73] So far, the so-called UW series has shown promising efficacy and drug metabolism and pharmacokinetics profiles and is associated with a novel (but still confidential) mode of action. Of note, a few lead candidates emerging from this chemical series are among the few compounds that can provide single-compound cure in an in-vivo model of chronic Chagas disease. A few chemically unrelated chemical series putatively working with the same mode of action have so far been identified and are being optimised in parallel as part of a back-up strategy.

Future of natural products

Natural products have been well documented to cover a broader and more complex chemical space than compounds produced by means of chemical synthesis[74] and have so far been largely underexplored in the discovery of drugs for Chagas disease. Natural products can be considered as biologically competent molecules engineered through evolution to address biological targets, and part of their complexity has been inherited from these interactions.[75] Privileged natural product-based structures can also serve as chemical templates to identify novel mechanisms of action for the development of drugs for Chagas disease. Despite the substantial efforts that have resulted in the publication of over 1000 distinct scientific papers since 1994 in the field of drug discovery for Chagas disease using natural products, only a few of these research findings have been translated into drug development assets. No advanced preclinical drug candidate of natural origin has progressed

beyond proof-of-concept of reduction of an established infection in an acute preclinical model,[76] and no new mechanism of action addressing the chronic stage of the disease has been validated so far.

The following reasons explain this disappointing outcome. First, a high percentage of the published literature describes small-scale screening of a small number of natural products that are mainly evaluated as complex mixtures, which is presumably due to insufficient assaying technology (ie, low testing capacity, only one citation of a library with more than 5000 samples has been found),[77] as well as the limited availability of natural products that are globally available for testing as pure, well-characterised chemical entities. Second, a large portion of *T cruzi* hits resulting from screening of natural products remain structurally unresolved (mainly complex mixtures reported for *T cruzi* activity) or associated to recurrently appearing non-developable compounds. This overrepresentation in the literature undermines drug development based on natural products by presenting invalid bioactive starting points as lead candidates for further progression. A well-documented analysis of such misleading outcomes identified 39 panacea natural products proven to act via various assay interference mechanisms; these account for an overwhelming 5–8% of the reported activities and occurrences for natural products in the NAPRALERT database, but represent less than 0·002% of the total natural product content.[78] Finally, there is insufficient follow-up on structurally characterised and validated natural product hits, mainly due to development bottlenecks related to compound tractability, structural complexity, inadequate analytical, pharmacokinetics, and medicinal chemistry expertise, as well as funding constraints. Publication of research results also contribute to disincentivise further development. The unavailability of a clearly defined drug development pathway that relies upon specific criteria of compound progression and reliable standardised assay systems with adequate activity translation value needs to be mentioned as well.

Looking ahead, the miniaturisation of *T cruzi* screening assay systems, particularly with the 384-well plate format becoming a laboratory standard, and advancements in analytics and data processing for natural product compound dereplication and pre-identification of novel structures and mechanisms of action directly within complex mixtures, will enable more efficient identification of natural products of interest. Advances related to tandem mass spectroscopy-based molecular networking[79,80] and imaging-based[81] technologies already contribute to this progress by focusing the bio-guided fractionation laboratory work on material worth this effort. The integration of web-based protocols,[82] fast developing artificial intelligence technologies, and open access databases to support (ie, store, organise, standardise, annotate, compare, and categorise) the analysis of large and complex data files issued from natural product drug discovery programmes, including metabolomics and imaging, will be instrumental in the process.

There is an urgent need to explore new therapeutic strategies against *T cruzi* infection, encompassing host-directed drugs, novel chemical entities, and potentially repurposed compounds with activity against *T cruzi*. Combining various options could prove to be an effective strategy, alongside efforts to optimise the utilisation of current drugs. Equally crucial is the research into treatment response and progression biomarkers, closely intertwined with pathological pathways.

Conclusions

The development of effective vaccines and therapies for Chagas disease remains a formidable challenge that is hindered by the complex lifecycle and genetic diversity of *T cruzi*, in addition to its ability to evade host immune responses. Tackling these obstacles requires a multifaceted approach that encompasses innovative vaccine design, novel therapeutic strategies, and a deeper understanding of host–parasite interactions and the resulting immune response.

In this Series paper we focused predominantly on advanced vaccine and drug candidates, which could introduce a bias towards more developed therapeutic options while potentially overlooking earlier-stage or less conventional approaches. Additionally, the inclusion criteria were limited to peer-reviewed published literature and reported information, which could result in a selection bias by excluding emerging research or unpublished data. Furthermore, the focus on clinical applicability could limit the discussion on preclinical innovations that are yet to translate into clinical settings.

Vaccine research efforts need to overcome the antigenic and geographical variance among *T cruzi* populations to stimulate broad and lasting immune defences, ensuring protection against all strains of the parasite and across different modes of transmission. Furthermore, identifying strategies to neutralise the parasite's immune evasion mechanisms is crucial for the effectiveness of any potential vaccine. Developing therapeutic vaccine candidates in combination with drugs for Chagas disease (vaccine-linked chemotherapy) presents overlapping challenges encountered in both vaccine development as well as drug development. Additionally, identifying effective drug-vaccine combinations requires extensive research to ensure both safety and effectiveness, considering the diverse genetic strains of the parasite. Regulatory hurdles, funding limitations, and the need for comprehensive clinical trials further impede progress. The goal is to create a synergistic approach that effectively clears the parasite and enhances the immune response, necessitating a multifaceted and collaborative effort.

On the therapeutic front, the limitations of the existing treatments highlight the urgent need for novel therapeutic agents with improved effectiveness, safety, and shorter treatment times. Advancing therapeutic innovation requires the capability to address the challenges posed by the asymptomatic nature of early-stage infections and absence

Search strategy and selection criteria

References for this review were identified through searches of PubMed for articles published between Jan 1, 2010, and Dec 31, 2023, using the terms "*Trypanosoma cruzi* AND vaccines", "*Trypanosoma cruzi* AND chimeric vaccines", "*Trypanosoma cruzi* AND immunotherapy", "*Trypanosoma cruzi* AND therapeutic vaccine", "*Trypanosoma cruzi* AND vaccine-linked chemotherapy", "Chagas disease AND vaccines", "*Trypanosoma cruzi* AND immune response AND human", "*Trypanosoma cruzi* AND cardiomyopathy AND inflammation AND human", "*Chagas disease* AND human AND immune response", "Chagas disease AND human AND cytokines", "*Trypanosoma cruzi* OR *Chagas disease* AND *clinical trials* AND *antiparasitic treatment*", "*Trypanosoma cruzi* OR *Chagas disease* AND *treatment*", "*Chagas disease AND drug development AND natural products*", "*Trypanosoma cruzi AND natural products*", and "*Trypanosoma cruzi AND natural products AND in vivo*". Relevant articles published outside the timeframe chosen were identified through searches in the personal files of the authors, and in Google Scholar, as well as the Google search engine. Articles published in English, Spanish, and Portuguese were included. We did not include abstracts from meetings. Citations were chosen within the limits permitted by the journal.

of reliable methods for confirming a cure. Yet, despite the progress in the search for biomarkers to identify the cure for the disease or halt its progression, or both, and to assist in evaluating the therapeutic effectiveness,[83–86] a dependable marker remains elusive.

Collaborative efforts among community health-care representatives, researchers, clinicians, industry partners, and funding agencies are crucial for accelerating progress in the research on Chagas disease. The COVID-19 pandemic has unequivocally showed the potential for rapid scientific progress through collaborative endeavours. By fostering interdisciplinary collaborations and pooling resources, the research community can leverage collective expertise and infrastructure to address key challenges and accelerate the development of new interventions. Partnerships with endemic regions are also essential to ensure that research and development efforts are aligned with local needs and realities, enhancing the relevance and applicability of new treatments. Public–private partnerships can also drive innovation by integrating academic research with industrial capabilities, and advocacy and patient organisations can raise awareness and help to secure funding. Such comprehensive, multistakeholder collaborations are important for overcoming the scientific, logistical, and financial barriers to developing effective vaccines and therapies for Chagas disease.

Furthermore, sustained funding support is essential for driving research efforts forward and ensuring that promising discoveries are translated into practical health-care solutions. Prioritising research and development for Chagas disease not only stands to benefit the millions of individuals currently affected by this disease but also aligns with global health objectives aimed at addressing neglected tropical diseases. Through dedicated efforts and sustained support, substantial strides could be made in mitigating the effect of Chagas disease on a worldwide scale.

Contributors

MJP, EM, J-RI, AB, KJG, and WOD did the literature search, wrote the initial draft, and revised and edited the manuscript. WOD created the figure. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests. MJP and J-RI are affiliated with DNDi, which oversees the development of Chagas disease treatments, some of which are highlighted in this Series paper.

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