

Biological characteristics of the *Trypanosoma cruzi* Arequipa strain make it a good model for Chagas disease drug discovery[☆]

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ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas disease (CD), is a genuine parasite with tremendous genetic diversity and a complex life cycle. Scientists have studied this disease for more than 100 years, and CD drug discovery has been a mainstay due to the absence of an effective treatment. Technical advances in several areas have contributed to a better understanding of the complex biology and life cycle of this parasite, with the aim of designing the ideal profile of both drug and therapeutic options to treat CD. Here, we present the *T. cruzi* Arequipa strain (MHOM/Pe/2011/Arequipa) as an interesting model for CD drug discovery. We characterized acute-phase parasitaemia and chronic-phase tropism in BALB/c mice and determined the *in vitro* and *in vivo* benznidazole susceptibility profile of the different morphological forms of this strain. The tropism of this strain makes it an interesting model for the screening of new compounds with a potential anti-Chagas profile for the treatment of this disease.

1. Introduction

Chagas disease (CD) or American Trypanosomiasis is a life-long and life-threatening disease caused by infection with the protozoan parasite *Trypanosoma cruzi*. According to the World Health Organization, CD is classified as a neglected tropical disease, the most important parasitic disease in America and, after AIDS and tuberculosis, the third most widespread infectious disease in this region. In addition, CD is the most prevalent of all the poverty-related diseases in Latin American countries (Rassi Jr et al., 2010; Urbina, 2010; Bern, 2015; DNDi. Drugs for Neglected Diseases Initiative, 2018; Lidani et al., 2019).

Although CD was limited for many decades to Latin America as a silent and silenced disease, its range has expanded due to increase mobility and migration, with a large number of infected individuals, particularly in Europe, and recorded outbreaks in Australia, New Zealand and Japan (Pérez-Molina et al., 2012; Jackson et al., 2014; Strasen et al., 2014; Lidani et al., 2019). CD is currently a global health problem, with 6–8 million infected people, 14–50 thousand deaths annually and 70–100 million people at risk of infection worldwide (Belaunzarán, 2015; PAHO. Pan American Health Organization, 2018; CDC. Centers for Disease Control and Prevention, 2019; Lidani et al., 2019; WHO.

World Health Organization, 2021).

Despite many efforts, the chronic nature and complex pathology of CD have resulted in a lack of effective treatments and vaccines. Approved treatments are limited to two nitroheterocyclic drugs, benznidazole (BNZ) and nifurtimox, which were developed more than 50 years ago and have serious drawbacks. The aim is to find a specific treatment that allows the eradication of the parasite and hence the elimination of the signs and symptoms of CD. The development of new, safer and more effective drugs that provide a shorter treatment course as well as paediatric, preferably oral, formulation is an important need (Hotez et al., 2008; Bern, 2015; Morillo et al., 2017; Aldasoro et al., 2018). In this way, the urgency of further research to discover new therapeutic alternatives, models and tools for CD drug discovery is justified.

CD is far from innocuous and, in mammalian hosts, an obligate intracellular parasite (Tyler and Engman, 2001; Kessler et al., 2017). During the initial acute-phase, parasites can be detected in the bloodstream and become widely disseminated in tissues and organs. Later, CD progresses to a long-lasting, asymptomatic chronic-phase, which is characterized by an extremely low parasite burden. Around 30% of infected people will advance to a symptomatic chronic-phase, which is

[☆] We dedicate this work to Prof. Manuel Sánchez Moreno.

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characterized by cardiomyopathy and organomegaly, outcomes for which there are few therapeutic options (Ribeiro et al., 2012; Cunha-Neto and Chevillard, 2014; Morillo et al., 2017). It is interesting to note that the outcome of the infection in a particular individual is the result of a set of complex interactions among the host's genetic background and the genetic composition of the parasite and modulated by environmental and social factors; all of which can be complicated by mixed infections and re-infections (Campbell et al., 2004).

The genetic diversity of *T. cruzi* is well characterized. *T. cruzi* belongs to a heterogeneous species consisting of a pool of strains and isolates that circulate among vectors and mammalian hosts (Rassi Jr et al., 2010; Zingales, 2018). This heterogeneity could explain the geographical differences in disease pathology, morbidity and mortality, and it has been studied extensively using biological, biochemical and molecular methods (Manoel-Caetano and Silva, 2007). Currently, *T. cruzi* is divided into seven discrete typing units (DTUs), showing different genotypes and phenotypes, evolutionary relationships, ecological and epidemiological associations, pathogenesis, tropism and drug resistance (Zingales, 2018; Martín-Escolano et al., 2020a). However, no definitive correlation between disease severity, treatment efficacy and parasite lineage has been established (Rassi Jr et al., 2010). Besides the well-known limitations of current treatments, other drawbacks are the natural resistance of the *T. cruzi* genotype to the drugs used (Mejia et al., 2012; Vela et al., 2021) and the cross-resistance for nitroheterocyclic compounds (Wilkinson et al., 2008; Mejia et al., 2012). Here, we present the *T. cruzi* Arequipa strain (MHOM/Pe/2011/Arequipa), isolated from a human from Arequipa, Peru, as an interesting model for CD drug discovery. We have determined the *in vitro* BNZ susceptibility profile of the different morphological forms of *T. cruzi* Arequipa, characterized acute-phase parasitaemia and chronic-phase tropism in BALB/c mice and analysed the *in vitro* BNZ susceptibility profile of *T. cruzi* Arequipa in both the acute and the chronic phases of CD. Some of the information related to the BNZ susceptibility profile has been compiled from previous published work (Martín-Escolano et al., 2019a, 2020b; 2020c, 2021), in which we studied this strain as a target for the discovery of new anti-Chagas compounds. The tropism of this strain makes it an interesting model for CD drug discovery.

2. Material and methods

2.1. Cultivation of *T. cruzi* Arequipa strain

2.1.1. Epimastigote forms

The human isolate, obtained by xenodiagnosis using *Rhodnius prolixus*, was provided in 2011 by the Laboratorio de Leishmaniosis y Chagas, Instituto Nacional de Salud, Lima (Peru). A polyclonal population of epimastigote forms were cultured at 28 °C in Gibco RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (hiFBS), 0.03 M hemin and 0.5% (w/v) BBL trypticase (Kendall et al., 1990).

2.1.2. Transformation to metacyclic forms

Metacyclic trypomastigotes were induced by culturing a 7-day-old culture of epimastigote forms at 28 °C in Gibco Grace's Insect Medium supplemented with 10% (v/v) hiFBS (Isola et al., 1986). Subsequently, parasites were incubated at a density of 5×10^8 mL⁻¹ for 2 h at 28 °C in TAU medium (190 mM NaCl, 17 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 8 mM phosphate buffer, pH 6.0), and later at a density of 5×10^6 mL⁻¹ for 4 days at 28 °C in TAU3AAG medium (TAU supplemented with 50 mM L-sodium glutamate, 2 mM L-sodium aspartate, 10 mM L-proline, 10 mM D-glucose) (Cardoso and Soares, 2010). Under these conditions, about 80% of the parasites in the culture are metacyclic trypomastigote forms. The percentage of metacyclic trypomastigote forms was assessed by Giemsa staining.

2.1.3. Amastigote and trypomastigote forms

Metacyclic trypomastigotes were used to infect Vero cells at 37 °C in humidified 95% air, 5% CO₂ atmosphere in Gibco RPMI 1640 medium supplemented with 10% (v/v) hiFBS (Contreras et al., 1985; Pless-Petig et al., 2012). After 5–7 days of incubation, culture-derived trypomastigotes were collected every 24 h by centrifuging the supernatant at 3,000 x g for 5 min, and used to infect new Vero cells cultures and BALB/c mice.

2.2. Genotyping

DNA from culture epimastigotes was extracted using the Wizard® Genomic DNA purification Kit (Promega). Genotyping the *T. cruzi* Arequipa was done using PCRs based on the following sequences: a) divergent domain of the 24Sα rDNA, using primers D71 (5'-AAGGTGCGTCGACAGTGTGG-3') and D72 (5'-TTTTCAGAAATGGCCGAACAGT-3') (Souto et al., 1996); b) size-variable domain of the 18S rDNA, using primers V1 (5'-CAAGCGGCTGGGTGGTTA TTCCA-3') and V2 (5'-TTGAGGGAAGGCATGACACATGT-3') (Clark and Pung, 1994), and c) non-transcribed spacer of the mini-exon genes, using primers TC1 (5'-GTGTCCGCCACCTCTTCGGGCC-3'), TC2 (5'-CCTGCAGGCACACGTGTGTGTG-3') and TC (5'-CCCCCTCCAGGCC AACTG-3') (Souto et al., 1996). Finally, the PCR amplification products were visualised by UV illumination after a 3.0% (for 24Sα and 18S rDNA) and 1.5% (for mini-exon genes) agarose gel electrophoresis for ~70 min at 90 V, containing 1:10,000 GelRed nucleic acid gel stain. The Colombian *T. cruzi* SN3 strain (IRHOD/CO/2008/SN3, DTU Tc1) (Télez-Meneses et al., 2008) was used as control.

2.3. *In vitro* BNZ activity assays

2.3.1. BNZ activity against epimastigote forms

BNZ (Sigma-Aldrich) activity against epimastigote forms was determined using the method described by Rolón et al. (2006) with some modifications. Briefly, 5×10^5 epimastigotes·mL⁻¹ were treated by adding BNZ at a concentration range from 100 to 0.4 μM (200 μL·well⁻¹ volumes) in 96-well plates at 28 °C for 72 h. Untreated growth controls were also included. 20 μL of resazurin sodium salt (0.125 mg·mL⁻¹) (Sigma-Aldrich) was added, and the plates were incubated for further 24 h. Finally, 5 μL of sodium dodecyl sulfate (10% w/v) was added, and the absorbance was measured at 570 and 600 nm. The trypanocidal activity was expressed as the concentration of drug required for 50% (IC₅₀) and 90% (IC₉₀) inhibition of parasite growth, using GraphPad Prism 6 software. Each BNZ concentration was tested in triplicate in four separate determinations.

2.3.2. BNZ activity against intracellular amastigote forms

BNZ activity against amastigote forms was performed by seeding 1×10^4 Vero cells·well⁻¹ in 24-well plates with rounded coverslips at 37 °C in humidified 95% air, 5% CO₂ atmosphere in Gibco RPMI 1640 medium supplemented with 10% (v/v) hiFBS. After 24 h of incubation, Vero cells were infected with culture-derived trypomastigotes at a multiplicity of infection (MOI) ratio of 10:1. After 24 h of infection, nonphagocytosed trypomastigotes were washed, and infected Vero cells were treated by adding BNZ at a concentration range from 100 to 0.4 μM (500 μL·well⁻¹ volumes) at 37 °C in humidified 95% air, 5% CO₂ atmosphere for 72 h. Untreated growth controls were also included. Counting of amastigotes and infected cells was performed in methanol-fixed and Giemsa-stained preparations by random analysis of 500 host cells (Martín-Escolano et al., 2020b). The infectivity index, defined as the average number of amastigote forms in infected cells multiplied by the percentage of infected cells, was also determined. The trypanocidal activity was expressed as the IC₅₀ and IC₉₀ (inhibitory concentrations 50 and 90) using GraphPad Prism 6 software. Each BNZ concentration was tested in triplicate in four separate determinations.

2.3.3. BNZ activity against extracellular trypomastigote forms

BNZ activity against culture-derived trypomastigote forms was performed by seeding 2×10^6 trypomastigotes·mL⁻¹, and after the addition of BNZ at a concentration range from 100 to 0.4 μ M (200 μ L·well⁻¹ volumes) in 96-well plates at 37 °C in humidified 95% air, 5% CO₂ atmosphere for 24 h. Untreated controls were also included. 20 μ L of resazurin sodium salt (0.125 mg·mL⁻¹) (Sigma-Aldrich) was added, and the plates were incubated for further 4 h (Martín-Escolano et al., 2019a). Finally, trypanocidal activity was determined following the same procedure as described to assess the trypanocidal activity against epimastigote forms. Each compound concentration was tested in triplicate in four separate determinations.

Since the half-life of culture-derived trypomastigotes (without host cells) was determined to be 48 h, the assay was restricted to 24 h of treatment.

2.4. Characterisation of CD infection in a murine model

2.4.1. Ethics statement

Mice were maintained under standard conditions – 12/12 h light-darkness cycle and 22 ± 3 °C temperature – with access to food and water ad libitum. Mice were handled by trained researchers in accordance with ethical considerations. Experiments were performed under the Spanish government regulations (RD1201/05) and approved by the University of Granada Ethics Committee on Animal Experimentation (RD53/2013); identification number 242-CEEA-OH-2018.

2.4.2. Characterisation of the acute CD

Female BALB/c mice – 10 weeks old and 20–25 g – were infected by intraperitoneal inoculation of 5.0×10^5 bloodstream trypomastigotes (BTs) in 0.2 mL PBS. These parasites were obtained from previously infected mice.

Five infected mice were used to characterise the acute CD, obtaining peripheral blood from the mandibular vein. 5 μ L blood was obtained every 2 days from the 3rd day post-infection (dpi) until the day parasitaemia was not detected for 10 consecutive days (Scheme 1). Blood was diluted 1:100 in PBS, and the parasitaemia was quantified using a Neubauer chamber (Martín-Escolano et al., 2018). A trypomastigotes enrichment buffer based on ammonium chloride (0.8% NH₄Cl, 0.1 mM EDTA, pH 7.2–7.6) was used for 4–10 min at RT for samples with low parasitaemia.

2.4.3. Characterisation of the chronic CD

Fifteen infected mice were used to characterise the chronic CD in a time-dependent manner, euthanizing five mice by cervical dislocation for each experimental time: 20th, 30th, and 60th dpi. Fourteen organs/tissues were harvested to evaluate the tropism: adipose tissue, bone

marrow, brain, oesophagus, heart, kidney, large intestine, liver, lung, mesenteric tissue, muscle, small intestine, spleen and stomach (Scheme 1). Hearts and spleens were also weighed to evaluate splenomegaly and cardiomegaly. These organs/tissues were immediately flushed free of blood by infusion of pre-warmed PBS to avoid contamination with BTs, and they were then thawed and ground up using a Potter-Elvehjem. Organs/tissues DNA was extracted using Wizard Genomic DNA Purification Kit (Promega). PCR was based on the Spliced Leader (SL) intergenic region sequence of *T. cruzi* (for detailed description, see Paucar et al. (Paucar et al., 2019). Finally, the PCR amplifications were visualised by UV illumination after a 2% agarose gel electrophoresis for 90 min at 90 V, containing 1:10,000 GelRed nucleic acid gel stain.

2.5. In vivo BNZ activity assays

2.5.1. Mouse infection and treatment

Infection was carried out by intraperitoneal inoculation as previously described. Treatment was performed by oral route once daily for 5 consecutive days once (a) infection was confirmed (9th dpi) for mice treated in the acute-phase and (b) it was established that the mice had entered the chronic-phase (75th dpi) for mice treated in the chronic-phase (Scheme 2).

Mice were divided into three groups ($n = 5$ per group): 0, negative control group (uninfected and untreated mice); I, positive control group (infected and untreated mice); II, BNZ group (infected and treated mice). BNZ was prepared at 2 mg·mL⁻¹ in an aqueous suspension vehicle containing 5% (v/v) DMSO and 0.5% (w/v) hydroxypropyl methylcellulose (Francisco et al., 2016). Doses of 20 mg·kg⁻¹·day⁻¹ (~200 μ L) were administered for 5 consecutive days in group II, and vehicle only was administered in groups 0 and I.

2.5.2. Parasitaemia counting

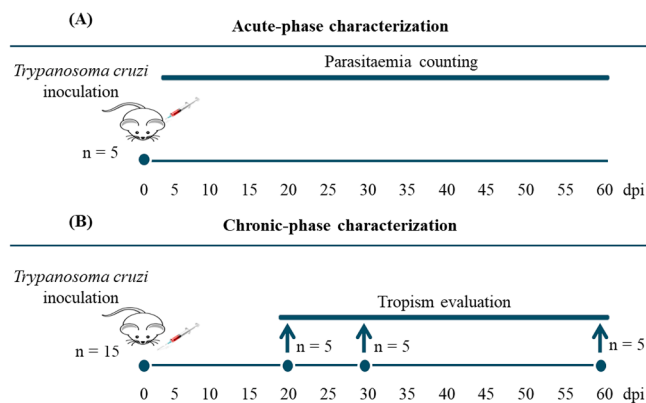
Parasitaemia counting from mice treated in the acute-phase was performed every 2 days from peripheral blood as stated above (Martín-Escolano et al., 2018) (Scheme 2).

2.5.3. Immunosuppression and parasitaemia reactivation

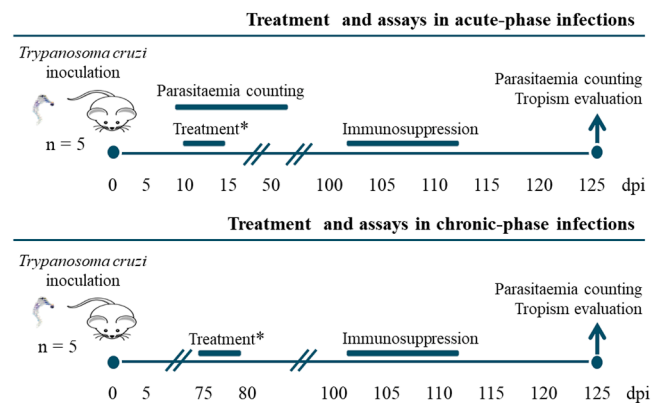
After 100th dpi, mice with significantly decreased parasitaemia and established to be in the chronic-phase of the experiment, regardless of the treatment and undetectable by blood microscopy examination, were treated with cyclophosphamide (CP) (ISOPAC) every 4 days by i.p. injection with a dose of 200 mg·kg⁻¹ for a maximum of three doses (Francisco et al., 2016) (Scheme 2). Within 1 week after the last CP injection, parasitaemia was evaluated as stated above (Scheme 2).

2.5.4. Euthanasia and PCR analysis

Mice were bled out under gaseous anaesthesia (CO₂) via heart



Scheme 1. Timeline of *in vivo* characterisation of Chagas disease on BALB/c mice infected with 5.0×10^5 bloodstream trypomastigotes of *T. cruzi* Arequipa strain. dpi, days post-infection.



Scheme 2. Timeline of BNZ activity assays on BALB/c mice infected with 5.0×10^5 bloodstream trypomastigotes of *T. cruzi* Arequipa strain. *Oral treatment at 20 mg·kg⁻¹ per day for 5 consecutive days; dpi, days post-infection.

puncture. Target organs/tissues were harvested, immediately flushed free of blood by infusion of pre-warmed PBS to avoid contamination with BTs, and they were then thawed and ground up using a Potter-Elvehjem. Organs/tissues DNA extraction and PCR was performed as stated above (Pauca et al., 2019) (Scheme 2). Finally, the PCR amplifications were visualised by UV illumination after a 2% agarose gel electrophoresis for 90 min at 90 V, containing 1:10,000 GelRed nucleic acid gel stain.

In addition, spleens and hearts were weighed to evaluate inflammation of this organ in the different groups of mice.

2.6. Statistical analyses

Analyses were performed using R-3.6 statistical software. Non-parametric tests ($P < 0.05$, 95% confidence level) were used to verify the differences between the samples. In particular, the Kruskal–Wallis test was used for multiple comparison between different conditions, and pairwise Wilcoxon rank addition tests with Holm correction were used for pairwise comparison between conditions.

3. Results

3.1. Arequipa strain genotyping

For genotyping, the protocol proposed by Brisse et al. (2001) was used. The combined analysis of the PCR products of the amplification of 24S α rDNA (Souto et al., 1996), 18S rDNA (Clark and Pung, 1994), and spacer of the mini-exon genes (Souto et al., 1996) defined that Arequipa belongs to DTU TcV (Fig. 1).

3.2. In vitro BNZ susceptibility profile

BNZ inhibitory concentrations (IC₅₀ and IC₉₀) are shown in Fig. 2. BNZ showed higher activity against the parasite forms relevant to human infection (intracellular amastigotes and trypomastigotes). The infection rate, the average number of amastigote forms per Vero cell and the infectivity index were also determined (Fig. 3A, B), providing an indication of the killing rate. It is clear that the parasite elimination, when administered as soon as possible after infection, can prevent parasite repopulation and serious disease (Rao et al., 2019). This approach has been supported, especially after the failure of the latest candidates – Posaconazole and Ravuconazole – in clinical trials (Molina et al., 2014). BNZ is a fast-acting and cidal drug; therefore, we observed a significant reduction in the infectivity index to practically zero at 50 μ M.

3.3. Natural course of CD infection in BALB/c mice

First, the course of infection was monitored during the acute-phase (Fig. 4A). Parasitaemia was detected from the 9th dpi and was undetectable from the 53rd dpi in all infected mice; peak parasitaemia was reached between the 22nd and 24th dpi, reaching more than 5 million BTs per mL of blood. Therefore, the acute-phase can be stated to end on the 53th dpi (Rassi Jr et al., 2010; Pérez-Molina and Molina, 2018). It should be noted that this level of parasitaemia corresponds to an intraperitoneal inoculum of 5×10^5 *T. cruzi* Arequipa BTs; inocula of (a) 5.0×10^3 BTs and (b) 5.0×10^4 BTs were also tested, but (a) no

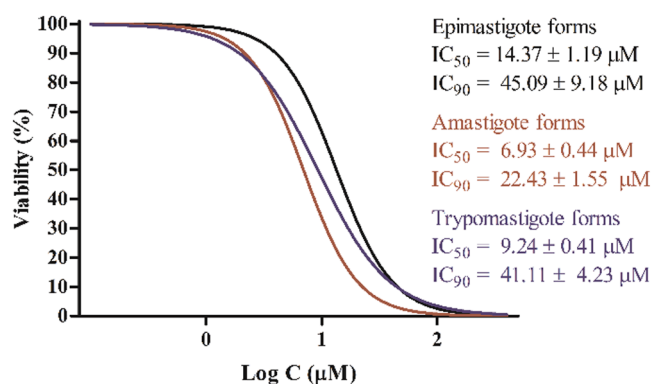


Fig. 2. Dose-response curves and inhibitory concentrations (IC) 50 and 90 of benznidazole against the three morphological forms of *T. cruzi* Arequipa plotted using GraphPad Prism 6 software.

parasitaemia was detected (by 1/100 diluted blood microscopic counting) and, (b) parasitaemia with a mean peak lower than 0.2×10^6 parasites·mL⁻¹ was shown (but infection did not occur in all mice in this group). Our experience with other strains (Olmo et al., 2015; García-Huertas et al., 2018) and a review of the literature (Rodrigues et al., 2022) suggest that the parasitaemia curve is inoculum- and strain-dependent.

Alternatively, parasites were detected at the 20st dpi in 6 out of the 14 analysed organs/tissues, and 9 of them were infected at the end of the acute-phase (40th dpi). The same organs/tissues were infected at the 60th dpi. Fig. 4B shows the PCR analysis of fourteen organs/tissues for each experimental timepoint. Therefore, this assay reveals the tropism of *T. cruzi* Arequipa for nine target organs/tissues: adipose tissue, bone marrow, brain, oesophagus, heart, lung, muscle, spleen and stomach. It should be highlighted that DTU TcV has been associated with human clinical manifestations such as chagasic cardiomyopathy and mega-oesophagus (Zingales et al., 2012; Frédérique Breniere et al., 2016; Pronovost et al., 2020; Tavares et al., 2020; Magalhães et al., 2022), as the heart and oesophagus are two organs in which *T. cruzi* Arequipa has been detected in the chronic-phase. Alternatively, analysis of a murine model has identified the gastrointestinal tract as a primary reservoir using different *T. cruzi* strains (Lewis et al., 2014, 2016; Lewis and Kelly, 2016). Hence, the intestine may also be parasitized in some areas along its length using *T. cruzi* Arequipa, but we did not detect parasitisation using the selected PCR fragments. Another reason would be that the parasite load could be below the PCR limit detection (~1 parasite per 10 mg) (Francisco et al., 2015), although three rounds of PCR were performed for all tissues/organs. Sophisticated protocols and imaging procedures are leading to a better appreciation of the spatiotemporal and quantitative dynamics of chronic infections (Canavaci et al., 2010; Lewis et al., 2014; Medeiros et al., 2017; Taylor et al., 2021), delimited using the PCR method.

It should be noted that the chronic-phase begins with the decline of parasitaemia (due to the host immune response), which in contrast to the acute-phase, remains as an intermittent and subpatent event (Guarner et al., 2001; Rassi Jr et al., 2010; Pérez-Molina and Molina, 2018). Here, the chronic-phase starts at the ~50th dpi, but parasites were already found in 6 out of the 9 target organs/tissues at the 20th dpi: the parasitaemia reflects intracellular parasitism and thus parasites are found in both acute- and chronic-phases of CD. The parasite load, however, will depend on the control of parasitism in each clinical phase.

In summary, acute-phase treatment should be performed once parasitaemia is confirmed, that is, at the ~9th dpi; and parasitaemia should be monitored until the ~55th dpi, when parasitaemia is not detected. For chronic-phase treatment, it should be performed from the 70th dpi, when this phase is established. Thereafter, assessment of cure should be performed by PCR of the 9 target organs/tissues in the late chronic-

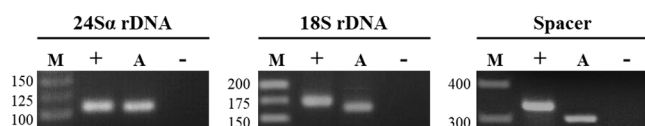


Fig. 1. PCR amplification products of 24S α rDNA, 18S rDNA, and spacer of the mini-exon genes. Lanes: (M), base pair marker; (+), PCR positive control (TcI strain); (A), *T. cruzi* Arequipa strain; (-), PCR negative control.

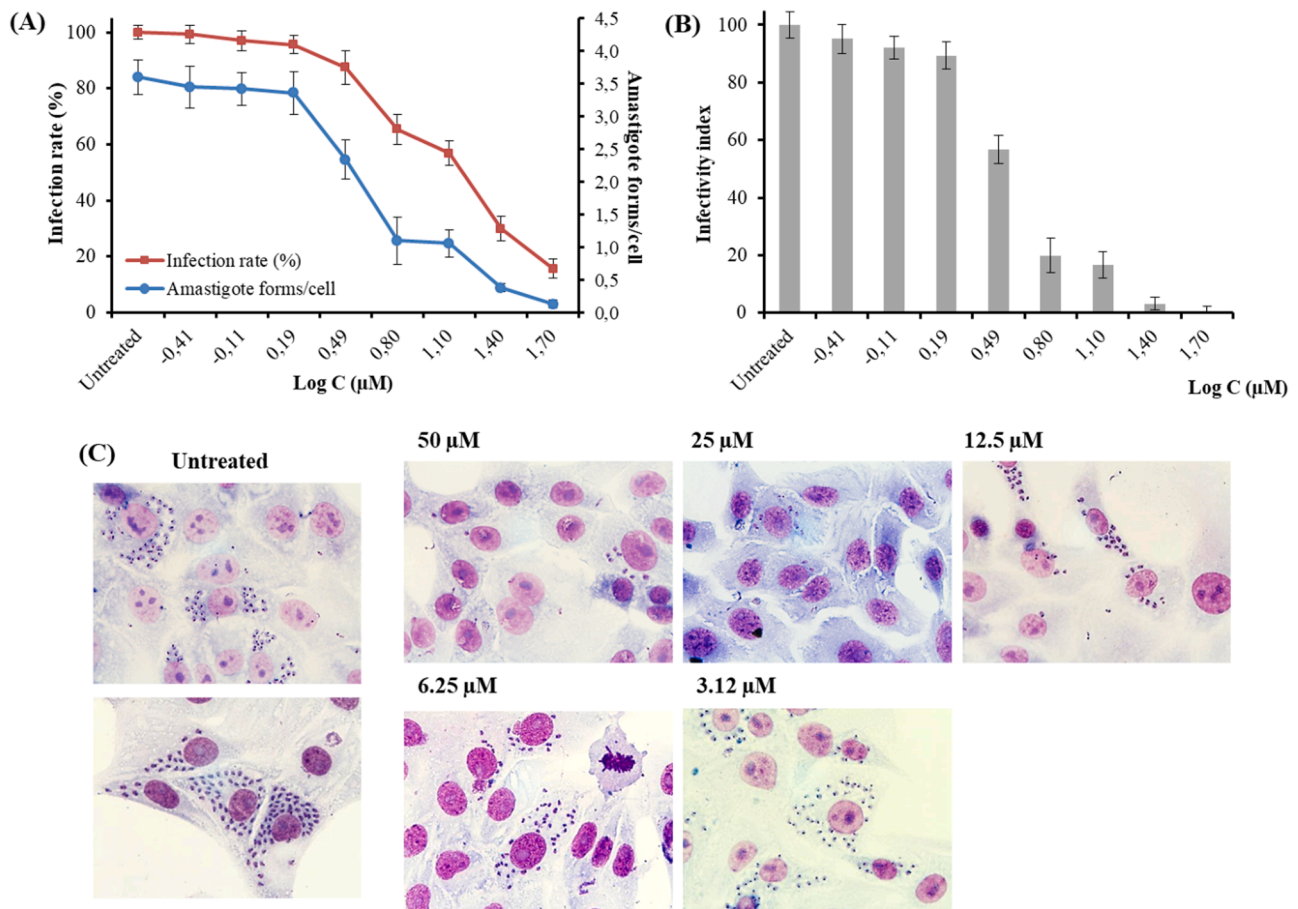


Fig. 3. (A) Percentage of infected Vero cells and average number of intracellular amastigote forms of *Trypanosoma cruzi* Arequipa per Vero cell of benznidazole-treated infected cultures. (B) Infectivity index (average number of amastigote forms in infected cells multiplied by the percentage of infected cells) of benznidazole-treated infected cultures. (C) Representative images of infected, benznidazole-treated and Giemsa-stained Vero cells.

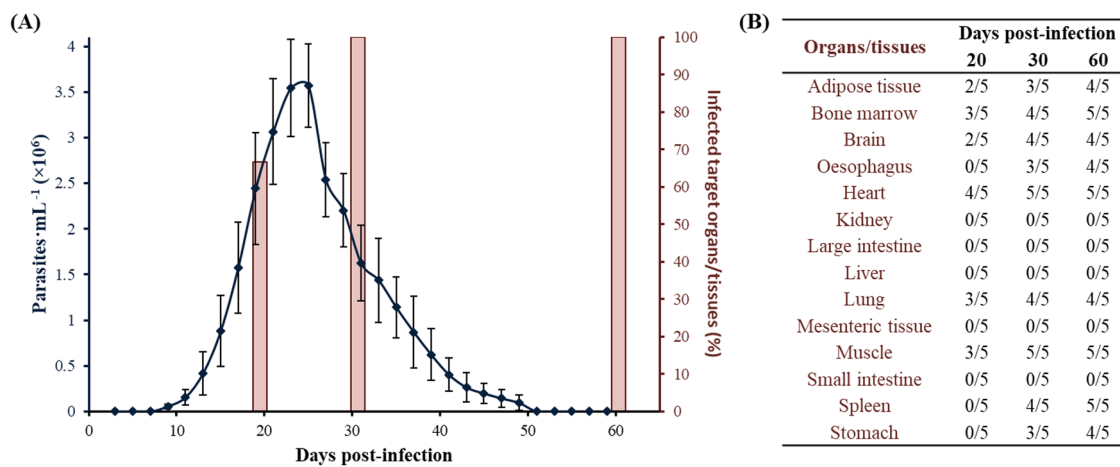


Fig. 4. (A) Acute-phase parasitaemia (blue) and tropism (red) in BALB/c mice after intraperitoneal inoculation of 5×10^5 *T. cruzi* Arequipa bloodstream trypomastigotes. Values constitute means of five mice \pm standard deviation. Tropism bars represent the percentage of organs/tissues with parasites with respect to the nine target organs/tissues. (B) Tropism evaluation by PCR analysis of fourteen potential target organs/tissues with the Spliced Leader (SL) intergenic region sequence of *T. cruzi*. The table shows the mice that were positive for each organ/tissue; $n = 5$ for each experimental timepoint.

phase.

3.4. In vivo BNZ susceptibility profile

First, parasitaemia profiles were monitored during the acute-phase (Fig. 5A). Significant differences were observed between untreated

and BNZ-treated mice, showing a remarkable parasitaemia reduction even at subcurative BNZ doses: (a) peak parasitaemia was reduced ~ 3.5 -fold, and (b) parasitaemia was eliminated or depleted to undetectable levels on the 45th dpi, that is, 8 days before that of untreated mice.

Second, the experimental cure was determined using a double-checking method widely used in *in vivo* trypanocidal assays:

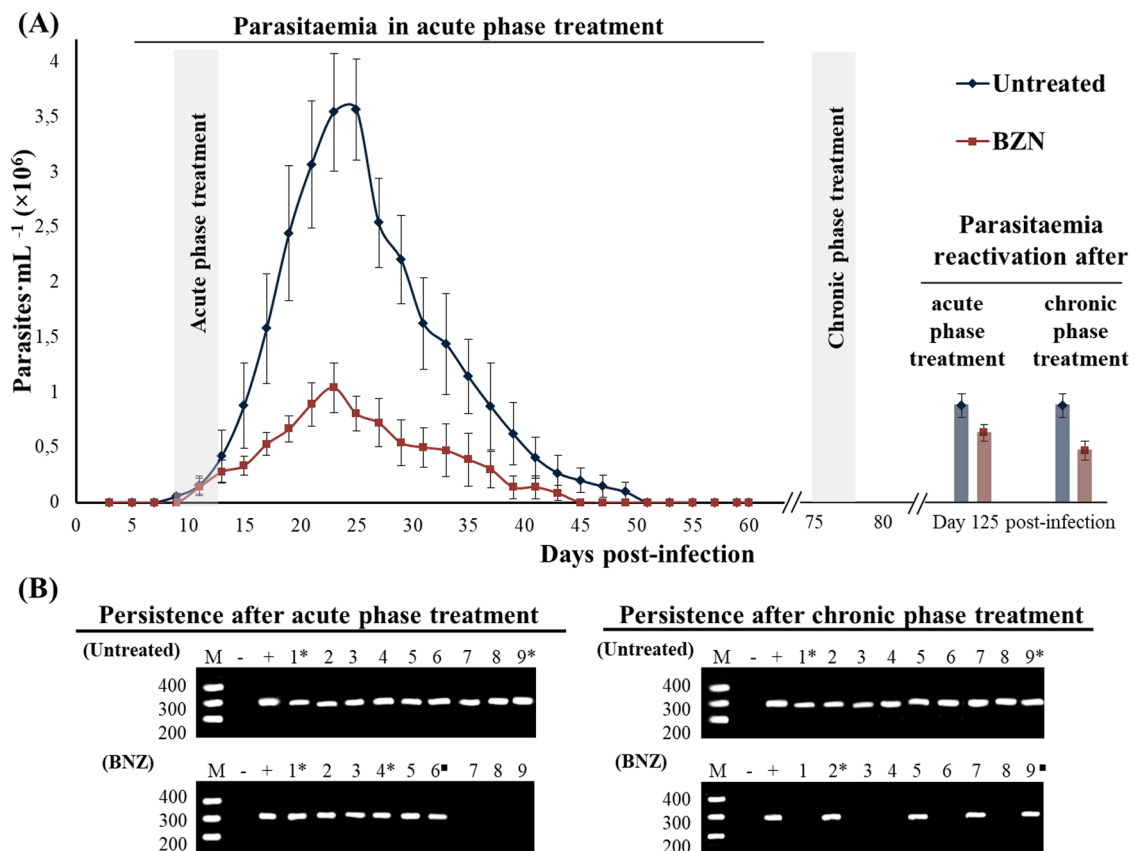


Fig. 5. (A) Parasitaemia profiles of untreated and benznidazole (BNZ)-treated mice during the acute-phase of Chagas disease over 60 days and parasitaemia reactivation after immunosuppression of untreated and BNZ-treated mice during the acute and chronic phases of Chagas disease. Treatment days are represented in grey. Values are the means of five mice \pm standard deviation. Significant differences between untreated and treated mice ($P < 0.05$) in both parasitaemia profile and reactivation. (B) Tropism evaluation by PCR analysis of the target organs/tissues of untreated and BNZ-treated mice with the Spliced Leader (SL) intergenic region sequence of *T. cruzi* during both the acute and the chronic phase of Chagas disease. Lanes: (M), base pair marker; (-), PCR negative control; (+), PCR positive control; (1-9), target organs/tissues: (1), adipose; (2), bone marrow; (3), brain; (4), esophagus; (5), heart; (6), lung; (7), muscle; (8), spleen; (9), stomach. * 4/5 of the corresponding organ/tissue PCR amplifications showed a 300 bp band on electrophoresis; ■ 3/5 of the corresponding organs/tissue PCR amplifications showed 300 bp band on electrophoresis.

immunosuppression and PCR (Fig. 5A, B) (Santos et al., 2010; Francisco et al., 2016; Martín-Escolano et al., 2020b). Mice that remained negative for parasitaemia and by PCR after immunosuppression (enhancing the reactivation of any residual infection) are considered cured. Immunosuppression enhances the reactivation of any residual infection, which is proportional to the parasite survival rate (Bustamante et al., 2014). Fig. 5A shows the reactivation of infection in both untreated and BNZ-treated mice, with significant reduction in reactivations after treatment in both CD phases. Fig. 5B shows the PCR results for the target organs/tissues, which were positive in 6/9 and 4/9 after acute- and chronic-phase treatments, respectively. Consistent with reactivation, BNZ exhibited higher efficacy in the chronic- than in the acute-phase of CD; it is widely known that BNZ is more effective in chronic than in acute CD in mouse models (Francisco et al., 2016), probably because the parasitic load is low and limited to a few locations.

Finally, organomegaly of the spleen and heart was measured (Fig. 6). Significant splenomegaly and cardiomegaly occurred in experimentally infected mice in both phases of CD. Splenomegaly is widely used as an indirect measure of infection and treatment efficacy: since the spleen is an organ implicated in the fight against infection, and those from experimentally infected mice commonly showed twice the mass of those from uninfected mice, the rate of splenomegaly could be associated with that of infection (Martín-Escolano et al., 2018). Importantly, treatment with BNZ reduced infection-induced splenomegaly in both the acute and the chronic phase of CD, even at subcurative doses and in the absence of a parasitological cure, since it is linked to a reduction of the parasite load

(Francisco et al., 2015). Cardiomegaly was also evaluated since it is one of the main pathologies causing death. A significant reduction in cardiomegaly was observed in acute-phase-treated mice, but no significant differences in cardiomegaly were observed between untreated and chronic-phase-treated mice.

4. Discussion

It is widely known that, besides the limitations of current treatments, other drawbacks are the natural drug resistance of some *T. cruzi* strains and the cross-resistance to nitroheterocyclic compounds (Wilkinson et al., 2008; Mejia et al., 2012; Vela et al., 2021). Here, the genotypes and *in vitro* susceptibility of the three morphological forms of *T. cruzi* Arequipa to the reference drug BNZ were determined. *T. cruzi* Arequipa (DTU TcV) shows moderate resistance to BNZ compared to other strains used for CD drug discovery. In fact, under the same assay conditions, *T. cruzi* SN3, a DTU TcI strain, considered resistant to BNZ, shows higher IC₅₀ values for epimastigotes, amastigotes and trypomastigotes (Martín-Escolano et al., 2019b) than those here obtained for *T. cruzi* Arequipa.

CD has quite a variable clinical presentation, ranging from asymptomatic to severe chronic disease. Although the reason for that is not fully understood, parasite (Andrade et al., 1999) and host (Andrade et al., 2002) genetics are certainly involved. The preferential tropism of *T. cruzi* strains in humans and experimental animals has been discussed by researchers for a century (Vianna, 1911). Experimental *T. cruzi*

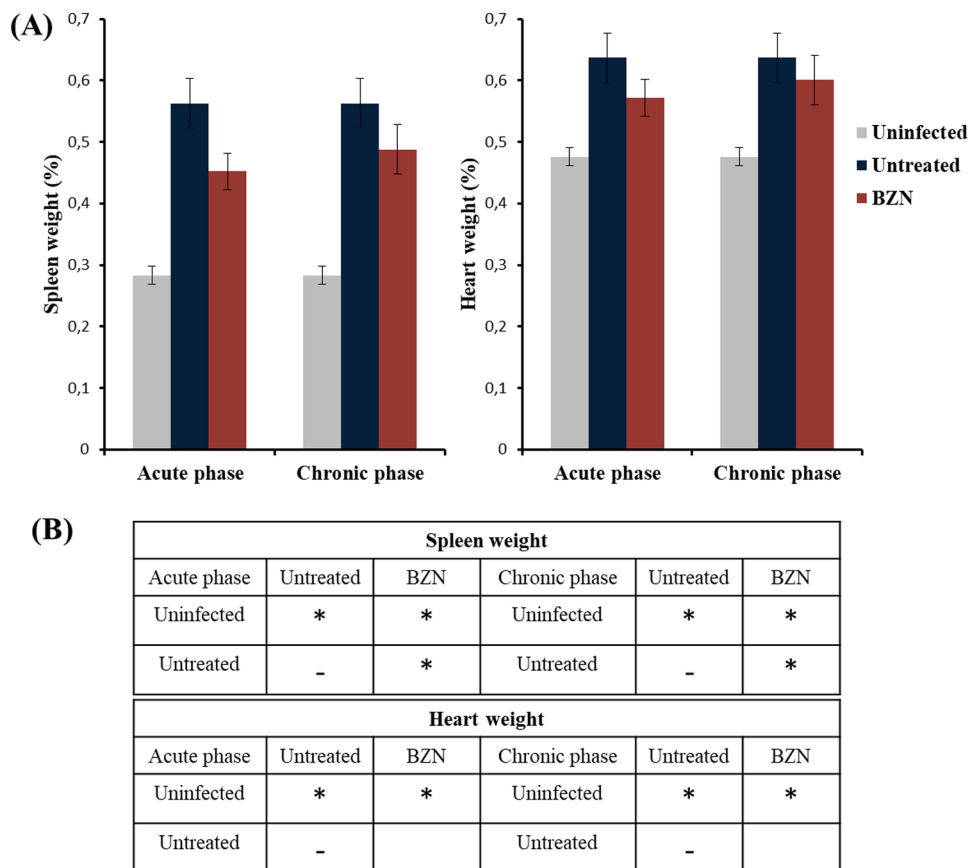


Fig. 6. (A) Organomegaly of spleens and hearts of uninfected, untreated and benznidazole (BNZ)-treated mice on the day of euthanasia. Values are the means of five mice \pm standard deviation. (B) Pairwise Wilcoxon rank addition tests with Holm correction for pairwise comparison of weight percentage of spleens and hearts between conditions. *Significant differences between conditions ($P < 0.05$). Blank cells indicate the absence of significant differences between conditions. Dashes indicate that comparisons are not applicable.

infection depends on the genetic composition of the infecting *T. cruzi* strain (Toledo et al., 2004; Santos et al., 2010; Rodriguez et al., 2014) and the genetic background of the animal model used as a host (Andrade et al., 2002; Caldas et al., 2008), that is, the host-parasite interaction. Hence, it is important to know the infective capacity of the strains used for CD drug discovery, that is, virulence (quantitative) and tropism (qualitative) in the mouse (the animal most commonly used for first *in vivo* trials), prior to *in vivo* treatments (Rodriguez et al., 2014). *T. cruzi* is able to parasitize a large variety of cells (de Souza et al., 2010; Rodriguez et al., 2014; Lewis and Kelly, 2016), and its tissue-homing ability has been reported to be strain specific (Tibayrenc and Telleria, 2010). Here, the chronic-phase tropism of *T. cruzi* Arequipa was evaluated in BALB/c mice in order to use this strain as a suitable model for CD drug discovery. This host-parasite model shows an attractive tropism for the evaluation of potential new compounds for the treatment of CD. *T. cruzi* Arequipa shows tropism towards (1) the brain, which allows us to evaluate the ability of potential compounds to cross the blood-brain barrier; (2) adipose tissue, which allows us to evaluate the solubility of compounds in lipid rich environments; and (3) the heart, which allows us to use this strain as a model for cardiac CD (one of the main fatal pathologies). It should be highlighted that these organs/tissues are the ones in which infection has been the most persistent after treatment using current potential candidates for the treatment of CD (including BNZ), showing parasites in them (Ferreira et al., 2011; Tanowitz et al., 2016). Lesser drug accessibility or parasite susceptibility in these environments could be the reasons for the lower efficacy (Nagajothi et al., 2013; Tanowitz et al., 2016); it has been postulated that inappropriate pharmacokinetics/pharmacodynamics between current drugs and tissue location of parasites is linked to the inability to reliably cure chronic infections (Urbina, 2002; Perin et al., 2017).

It should be noted that most *in vivo* chemotherapy has focused on acute-phase infections, partially because it is simpler to monitor the

course of parasitaemia (Canavaci et al., 2010; Romanha et al., 2010; Buckner, 2011; Chatelain and Konar, 2015). However, the ability to cure chronic-phase infections is the highest priority from a clinical viewpoint. The effectiveness of current drugs is especially limited during the chronic-phase of CD (Wilkinson et al., 2008; Chatelain, 2014; Scarim et al., 2018), so chronic-phase infections should be the main research focus in animal models (DNDi. Drugs for Neglected Diseases Initiative, 2018). Notably, this strain does not cause mortality: neither the infected mice used to evaluate the natural course of infection nor those used to evaluate the BNZ susceptibility profile died during the 60 and 125 days of the experiment, respectively. It is ultimately useful for comparing the *in vivo* trypanocidal efficacy of potential compounds and the reference drug BNZ in late chronic-phase.

Currently, drugs against CD present variable activity in the acute- and chronic-phases of the disease, and the effectiveness of treatments, especially during the chronic-phase, is not as high as it should be (Martín-Escolano et al., 2020a). For these reasons, the *in vivo* susceptibility of *T. cruzi* Arequipa in both the acute- and the chronic-phase was evaluated. The treatment strategy using BALB/c mice was as follows: (1) Drugs were administered orally because the oral route of administration is the preferred for the treatment of parasitic diseases in developing countries, it leads to better patient compliance and it has a low cost (Espuelas et al., 2012; DNDi. Drugs for Neglected Diseases Initiative, 2018); (2) Given that a compound showing a reasonable parasitaemia reduction following 5 days of treatment can be defined as a lead compound (Chatelain, 2014), the treatment guideline was for 5 consecutive days in order to evaluate the susceptibility of *T. cruzi* Arequipa to oral BNZ treatment. Moreover, to evaluate if potential compounds (for further research) show higher trypanocidal activity than BNZ in the first *in vivo* screening phase (Romanha et al., 2010), the treatment was performed at subcurative doses of BNZ (20 mg·kg⁻¹ per day). As expected, mice treated in the chronic-phase showed lower infection levels than

those treated in the acute-phase. It is widely known that BNZ is more effective in the chronic phase, probably because the parasite burden is significantly lower and limited to far fewer locations (Francisco et al., 2016).

5. Conclusions

CD is still considered a global health problem with significant epidemiological and socioeconomic implications. Hence, the urgency of further research to discover new therapeutic alternatives, models and tools for CD drug discovery is justified. In recent years, technical advances in several areas have contributed to a better understanding of the biology and life cycle of this parasite, which will make it possible to design the ideal profile of both drugs and therapeutic options for treating CD. Here, we have performed a biological characterisation of the *T. cruzi* Arequipa strain: we have evaluated the (a) genotype (DTU TcV), (b) acute-phase parasitaemia, (c) chronic-phase tropism, and (d) *in vitro* and *in vivo* BNZ susceptibility profile. This characterisation makes this strain a good model for the evaluation of potential compounds for CD drug discovery. For *in vitro* assays and in face of *T. cruzi* genetic variability, different strains should be used to evaluate the spectrum of action of potential compounds (Zingales et al., 2014). However, an initial *in vivo* evaluation of the activity of potential anti-Chagas compounds should be performed with a single strain in order to comply with the 3Rs (in this case, reduction in the number of experimental animals). For this, we suggest that *T. cruzi* Arequipa is a promising strain for this after complete characterisation (sequencing, pathology, and curative treatment regimen).

The next objective will be to sequence the genome of a clone of this Arequipa polyclonal strain (DTU TcV), similar to what was done with CL Brener, a clone derived from the CL strain (DTU TcVI) (Zingales et al., 1997; El-sayed et al., 2005). To date, the genome sequence of only two DTU TcV hybrid strains is available (Callejas-hernández et al., 2018; Decuir et al., 2022).

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CRediT authorship contribution statement

Rubén Martín-Escolano: Conceptualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **María José Rosales:** Resources, Methodology, Validation, Writing – review & editing. **Clotilde Marín:** Conceptualization, Resources, Methodology, Validation, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

Data availability

Data will be made available on request.

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