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Post-therapeutic cure criterion in chronic Chagas disease using *Trypanosoma cruzi* chimeric proteins

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Abstract: Chagas disease (CD) is a neglected disease caused by *Trypanosoma cruzi* Chagas, 1909. Causative treatment can be achieved with two drugs: benznidazole or Nifurtimox. There are some gaps that hinder progress in eradicating the disease. There is no test that can efficiently assess cure control after treatment. Currently, the decline in anti-*T. cruzi* antibody titres is assessed with conventional serological tests, which can take years. However, the search for new markers of cure must continue to fill this gap. The present study aimed to evaluate the decline in serological titres using chimeric proteins after treatment with benznidazole in chronic patients diagnosed with CD. It was a prospective cross-sectional cohort study between 2000 and 2004 of *T. cruzi*-positive participants from the Añatuya region (Argentina) treated with benznidazole. Serum samples from ten patients were collected before treatment (day zero) and after the end of treatment (2, 3, 6, 12, 24 and 36 months). For the detection of anti-*T. cruzi* antibodies, an indirect ELISA was performed using two chimeric recombinant proteins (IBMP-8.1 and IBMP-8.4) as antigens. The changes in reactivity index within the groups before and after treatment were evaluated using the Friedman test. All participants experienced a decrease in serological titres after treatment with benznidazole, especially IBMP-8.1. However, due to the small number of samples and the short follow-up period, it is premature to conclude that this molecule serves as a criterion for sustained cure. Further studies are needed to validate tests based on these or other biomarkers to demonstrate parasitological cure.

Keywords: Chagas disease, benznidazole, serological test, seroconversion, antibody titre, biomarker

This article contains supporting tables (Tables S1) online at http://folia.paru.cas.cz/suppl/2024-71-004.pdf

Chagas disease (CD) is a neglected infectious disease caused by the haemoprotozoan *Trypanosoma cruzi* Chagas, 1909 that affects ~5.7 million people in 21 endemic countries in Latin America, resulting in 7,500 deaths annually (WHO 2015). This parasite is transmitted by several routes, including infected haematophagous insects (triatomine vectors), consumption of contaminated food and beverages, from mother to child, blood transfusions, organ transplants and, less commonly, laboratory accidents. In recent decades, population shifts and migration flows have favoured the spread of *T. cruzi*-infected individuals from Latin America to non-endemic countries in Europe, North America, Oceania and Asia (Antinori et al. 2017, Lidani et al. 2019).

The initial infection is followed by an acute phase characterised by high parasitaemia, which is asymptomatic in most cases. However, non-pathognomonic symptoms, such as self-limiting febrile illness, occur in 10% of infected individuals (Murphy et al. 2019). Without specif-

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ic treatment, the disease enters a lifelong, asymptomatic, indeterminate phase. However, after years or decades, 20–30% of individuals infected with *T. cruzi* enter an advanced, life-threatening, debilitating chronic phase with severe cardiac complications characterised by arrhythmias or heart failure, which in severe cases can lead to sudden death (Rassi et al. 2010), with or without complications in the digestive tract, especially in the oesophagus and colon, leading to mega-oesophagus and megacolon, respectively (Stanaway and Roth 2015), and with neurological (Useche et al. 2022) or mixed changes.

Despite the knowledge of CD accumulated over the last 114 years, there are still some gaps that need to be filled, such as (1) the description of a marker that can predict the prognosis of the disease and (2) markers to evaluate therapeutic success (criterion of cure). The last gap is the demonstration of complete elimination of *T. cruzi* in treated individuals (including tissue and circulating blood forms), which consistently show negative results in reproducibly performed parasitological, molecular and serological tests (Añez et al. 2015).

Indeed, the lack of a feasible criterion for parasitological cure is the main challenge for the accurate and reliable assessment of post-treatment cure and the evaluation of new drugs in clinical trials (Alonso-Padilla et al. 2021), even when different methods with satisfactory specificity and sensitivity are used. Therefore, the applicability of parasitological, serological and molecular methods as criteria for post-treatment cure in different stages of infection and clinical forms of CD has been criticised (Machado-de-Assis et al. 2012, De Lana and Martins-Filho 2015). Nevertheless, there is no consensus among authors on the criterion of cure in CD, which has led to controversy about the usefulness of specific treatment, making this an interesting open field of investigation (Espinoza 2003).

Chimeric recombinant *T. cruzi* proteins can be used as antigens in non-conventional methods (Santos et al. 2016). Chimeric molecules consist of repeated and conserved fragments of amino acid sequences of epitopes from multiple antigenic proteins of the parasite. In the last decade, several chimeric antigens have been proposed to improve the diagnosis of chronic CD with high accuracy (Houghton et al. 1999, Camussone et al. 2009, Hernández et al. 2010, Peverengo et al. 2018). Among them, IBMP antigens (IBMP-8.1, IBMP-8.2, IBMP-8.3 and IBMP-8.4) have shown high diagnostic potential. They have been tested in both endemic and non-endemic areas and have shown high performance despite differences in the geographical origin of the samples (Santos et al. 2017b, Del-Rei et al. 2019, Dopico et al. 2019).

Furthermore, all four IBMP antigens have shown negligible cross-reactivity with visceral and American cutaneous leishmaniasis, toxoplasmosis and other pathogens (Santos et al. 2017b, Daltro et al. 2019, Dopico et al. 2019). Considering the high diagnostic performance of IBMP-8.1 and IBMP-8.4 antigens in previous studies (Santos et al. 2017b, Silva et al. 2020, Daltro et al. 2022), we sought to evaluate their ability to detect a decrease in anti-*T. cruzi* IgG titre in serum samples from benznidazole (Bz)-treated individuals.

MATERIALS AND METHODS

Ethics statement

This study followed the principles of the Declaration of Helsinki and the guidelines set out in Resolution N°1480/11 of the Argentine Ministry of Health. Approval was obtained from the Institutional Review Board (IRB) for Human Research at the Zonal Hospital of Añatuya (Añatuya, Santiago del Estero). We used samples from the biorepository of the Laboratory of Molecular Biology of Chagas Disease (Institute for Research on Genetic Engineering and Molecular Biology, INGEBI, CONICET-UBA). To maintain the confidentiality of patient data, samples were anonymised so that investigators did not have access to individual patient data. Informed consent was obtained prior to blood collection.

Clinical samples and study design

We used anonymised human sera obtained from INGEBI-CONICET serum bank, Buenos Aires, Argentina. The sera came from a prospective study conducted in 2000–2004 in Añatuya (Fig. 1), a town in a highly CD-endemic area in Santiago del Estero province, Argentina (Niborski et al. 2016). We collected sera from 10 *Trypanosoma cruzi*-infected adult volunteers aged 26–53 years. Selection of clinical samples was based on positivity of at least two serological tests (ELISA and/or indirect haemagglutination test) according to international guidelines (Dias et al 2016, PAHO 2019). During this period, all serum samples underwent analysis through three in-house ELISA assays, which employed epimastigote lysate (CL-Brener strain; DTU Tc VI), JL7, and B13 as antigens, as outlined by Niborski et al. (2016). The CO/DO or reactivity index can be found in S1 Table.

Exclusion criteria included visceral damage, systemic arterial hypertension, hepatic or renal insufficiency, severe cardiac lesions associated with other cardiac diseases, pregnancy, lactation, alcoholism and hypersensitivity to the drug. Benznidazole (Bz) was administered three times daily for 60 days at a total dose of 5 mg/kg/day (100 mg/tablet; Radanil®, Roche). During treatment, patients were clinically examined to detect side effects. Blood samples were collected before treatment (T0) and at the following time points after the start of treatment: two months (T1), three months (T2), six months (T3), one year (T4), two years (T5) and three years (T6). Each sample was assigned an identification code in the laboratory to ensure blinded analysis. Publicly available digital maps were extracted from the Brazilian Institute of Geography and Statistics (IBGE) cartographic database in shapefile format (.shp), reformatted and processed using QGIS version 3.22.16 (Geographic 140 Information System, open-source Geospatial Foundation Project. Freely available at http://qgis.osgeo.org). This open-source software package was used for data processing, analysis and presentation of the mapped data.

Recombinant proteins synthesis

Synthetic genes of *T. cruzi* optimised for expression in a prokaryotic system were acquired from a commercial supplier (GenScript, Piscataway, New Jersey, USA). The synthetic genes acquired in pUC57 were subcloned in-house into the pET28a expression vector. The chimeric recombinant proteins (IBMP-8.1 and IBMP-8.4) were expressed as soluble proteins in *Escherichia coli*-Star (DE3) cells grown in lysogenic broth supplemented with

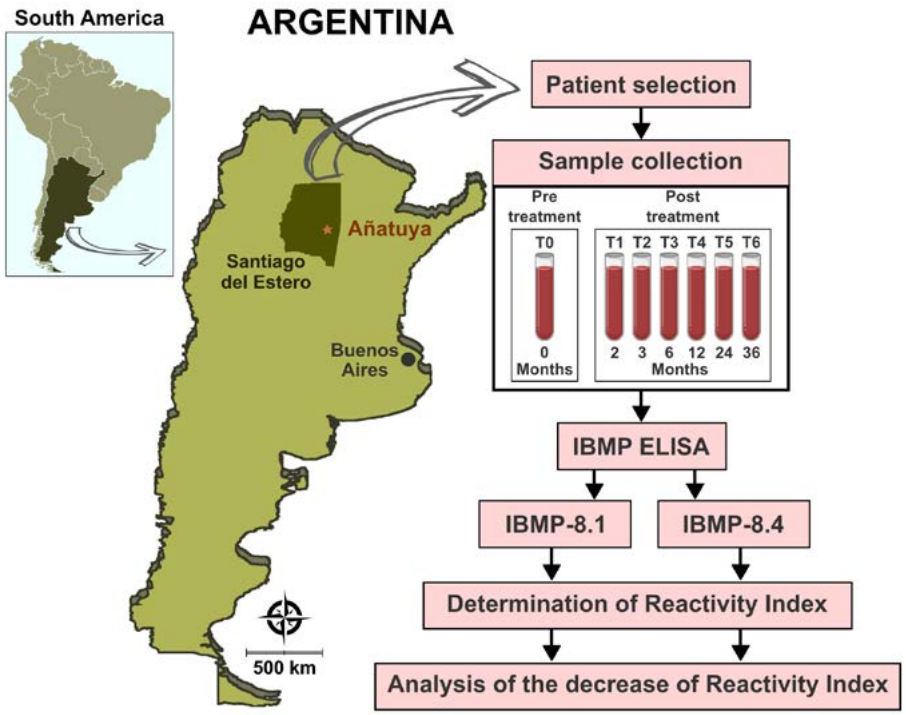

Fig. 1. Design of a study procedure to evaluate chimeric IBMP *Trypanosoma cruzi* antigens as a post-therapeutic cure criterion in chronic Chagas disease. The digital map in the public domain (publicly available) was obtained from the IBGE cartographic database in shapefile format (.shp), which was subsequently reformatted and analysed using QGIS version 3.22.16. The authors note that this figure is licensed under CC BY 4.0.

0.5 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). Subsequently, the proteins were purified by both affinity and ion-exchange chromatography and then quantified by a fluorometric assay. The plasmidial construct was previously described in Santos et al. 2016.

Serological tests

Anti-*T. cruzi* immunoassays were performed by ELISA according to previous reports (Santos et al. 2017b, Del-Rei et al. 2019, Dopico et al. 2019). In brief, polystyrene "Maxisorp" microplates with 96 wells (Nunc, Roskilde, Denmark) were coated with 25.0 ng IBMP-8.1 or IBMP-8.4 per well diluted in coating buffer (0.05 M carbonate bicarbonate, pH 9.6). The microtitre plates were blocked with Well Champion reagent (Kem-En-Tec, Taastrup, Denmark) according to the manufacturer's instructions. Serum samples were diluted in 0.05 M phosphate buffered saline (pH 7.2)-0.5% Tween 20 (PBS-T) and 100 µl was added to each well. After 60 minutes incubation at 37 °C, the microtitre plates were washed with PBS-T to remove unbound antibodies. HRP-conjugated goat anti-human IgG (Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, Brazil) was diluted 1 : 40,000 in PBS-T, 100 µl was added to each well and microtitre plates were incubated at 37 °C for 30 minutes. The wells were washed five times and the immune complexes were detected by adding 100 µl of TBM substrate (tetramethylbenzidine; Kem-En-Tec, Taastrup, Denmark).

After another incubation step (10 minutes at room temperature in the dark), the reaction was stopped by adding 50 µl 5N H_2SO_4 and absorbance was measured at 450 nm in a VersaMax microplate reader with a 450 nm filter (Molecular Devices, San Jose, California, USA). The background values (buffer dilution) were subtracted from the measurement experiments. In order to determine relevant cut-off values (CO) for the IBMP antigens, ten *T. cruzi*-positive and ten *T. cruzi*-negative samples in all microtitre plates were analysed in parallel. These samples had previously been characterised as *T. cruzi*-positive or negative by two serological tests according to the World Health Organisation diagnostic consensus (WHO 2011). Cut-off point analysis was used to determine the optimal optical density value (OD) that distinguishes negative from positive samples. The threshold was defined by the largest distance from the diagonal line of the Receiver Operating Characteristic Curve (ROC).

DNA extraction and amplification

Blood samples were mixed with an equal volume of 6 M guanidine-HCl/0.2 M EDTA buffer pH: 8.0. Guanidine-EDTA blood (GEB) was heated in boiling water for 15 minutes and total DNA was purified from 500 µl GEB with phenol-chloroform-isoamyl alcohol (25 : 24 : 1, V/V), as previously reported (Niborski et al. 2016). The 330-bp variable regions of the *T. cruzi* kinetoplastid minicircle genome were amplified with 121 [5′-AAATAATGTACGG G(T/G)GAGATGCATGA-3′] and 122 [5′-GGTTCGATTGGGGTTGGTGTAATATA-3′] primers by conventional PCR, as previously described (Niborski et al. 2016).

Data analysis

First, the data were coded and analysed using computer graphics software (GraphPad Prism version 9.5.0, San Diego, California, USA). The results were expressed as an index representing the ratio between the OD of the samples and the OD of the thresh-

Table 1. Sociodemographic characteristics of patients used to evaluate the reduction in reactivity index values for two recombinant chimeric *Trypanosoma cruzi* antigens after treatment with benznidazole

Patient code	Gender classification	Age (years)	Area of residence	Cardiac involvement (Pinazo et al. 2011)
704-817 (P1)	Male	40	Urban	Kushnir 1
705-815 (P2)	Male	45	Urban	Kushnir 0
711-826 (P3)	Female	53	Urban	Kushnir 0
716-857 (P4)	Male	35	Rural	Kushnir 1
719-811 (P5)	Male	42	Urban	Kushnir 0
721-831 (P6)	Female	43	Urban	Kushnir 0
726-870 (P7)	Male	33	Urban	Kushnir 0
739-884 (P8)	Male	33	Rural	Kushnir 1
748-852 (P9)	Male	26	Urban	Kushnir 0
751-900 (P10)	Female	37	Urban	Kushnir 0

Table 2. Results of polymerase chain reaction (PCR) of patients to evaluate the reduction of reactivity index values for two recombinant chimeric *Trypanosoma cruzi* antigens after treatment with benznidazole.

Patient code	Before treatment (T0)	Time of treatment (months) 2 (T1)	3 (T2)	6 (T3)	12 (T4)	24 (T5)	36 (T6)
P1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
P2	Pos	Neg	Neg	Neg	Neg	Neg	Neg
P3	Pos	Pos	Neg	Neg	Neg	Neg	Neg
P4	Pos	Pos	Pos	Neg	Neg	Neg	Neg
P5	Pos	Pos	Neg	Neg	Neg	Neg	Neg
P6	Pos	Pos	Neg	Neg	Neg	Neg	Neg
P7	Pos	Neg	Neg	Neg	Neg	Neg	Neg
P8	Pos	Pos	Neg	Neg	Neg	Neg	Neg
P9	Neg	Neg	Neg	Neg	Neg	Neg	Neg
P10	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Table 3. Interpretation of ELISA results for patients used to evaluate the reduction in reactivity index values for two recombinant chimeric *Trypanosoma cruzi* antigens after treatment with benznidazole

Patient code	Situation number LYS	JL7	B13	IBMP-8.1	IBMP-8.4
P1	2	1	2	1	1
P2	2	2	2	1	1
P3	3	3	3	2	3
P4	3	2	3	2	3
P5	3	2	2	3	3
P6	2	NP	NP	2	2
P7	3	2	2	3	4
P8	2	2	2	3	3
P9	2	2	2	2	2
P10	3	2	2	1	1

Legend: Situation 1 – the signal decreased and become negative); Situation 2 – the signal decreased but did not become negative; Situation 3 – the signal remained constant over time; Situation 4 – the signal grew stronger over time; NP (not performed).

old. This index is referred to as the reactivity index (RI), and all results ≥ 1.00 were considered positive. RIs were calculated at baseline and two, three, six, 12, 24 and 36 months after treatment. Longitudinal data were then entered into SPSS version 16 and descriptive analysis required for data analysis was performed. Data were skewed according to the Kolmogorov-Smirnov test ($p < 0.05$). RI values were grouped according to the time period in which the samples were collected and analysed using the box-and-whisker plot, a technique for exploratory data analysis. The Friedman test was used to assess the changes in reactivity index within the groups before and after treatment. All analyses were two-sided tests and a p-value below 5% was considered significant ($p < 0.05$). A flow chart was created to illustrate the design of the study (Fig. 1).

RESULTS

The post-therapeutic reactivity index was measured in a total of 10 patients with two recombinant chimeric *Trypanosoma cruzi* antigens, with 70 samples (7 per patient) obtained before and after treatment with Bz. The median age of the study population was 38.5 years (IQR, 33.0–43.5 years), and the male-to-female ratio was ~2.3 : 1 (Table 1). Most patients lived in urban areas, while only 20% lived in a rural area of Añatuya. Cardiac involvement according to Pinazo et al. (2011) included seven (70%) patients in stage 0 (reactive serology, normal electrocardiogram, normal chest X-ray or echocardiogram without left ventricular dilatation) and three (30%) in stage 1 of the Kushnir classification (reactive serology, abnormal electrocardiogram, normal chest X-ray or echocardiogram without left ventricular dilatation). No patient was classified as stage 2 (reactive serology, abnormal electrocardiogram, chest radiograph or echocardiogram with dilated left ventricle, without clinical or radiological heart failure) and stage 3 (reactive serology, abnormal electrocardiogram, chest radiograph or echocardiogram with dilated left ventricle, heart failure). Gastrointestinal involvement was not investigated.

None of the patients who participated in the present study voluntarily stopped or discontinued treatment due to death, pregnancy, non-compliance with the physician's prescription or adverse effects. Blood samples were collected from all ten patients before (T0) and after treatment with Bz (T1–T6). *Trypanosoma cruzi* PCR in peripheral blood was positive in eight (80%) patients at T0, six (60%) at T1, two (20%) at T2 and one (10%) at T3–T6. Two patients were PCR negative throughout the study period, while only one patient was positive at all study time points (Table 2).

Anti-*T. cruzi* antibodies were measured with two chimeric recombinant antigens (IBMP-8.1 and IBMP-8.4) to assess the reduction in reactivity index values after treatment with Bz (individual RI values are available in Table S1). First, the RI values were grouped according to the period during which the samples were collected and analysed using the boxplot. The plots in Fig. 2 show that the RI values do not vary across the seven periods analysed but show a large spread at each sampling point. The boxplot analysis shows no trend towards decreasing reactivity index values.

When we analyse the distribution of points over time for each patient, we find four different situations (Fig. 3): (1) The signal decreased and become negative. In patients P1, P2 and P10, a decrease in signal intensity for the molecules IBMP-8.1 and IBMP-8.4 is observed over time, indicating a possible serological negativity after 36 months of treatment (RI < 1.0) in P1 and P2. It is important to note that from the initial evaluation at T0, the patients P1, P2 and P10 consistently demonstrated lower reactivity to both antigens. Although P10 became negative by ELISA 36 months after treatment, PCR remained positive, indicat-

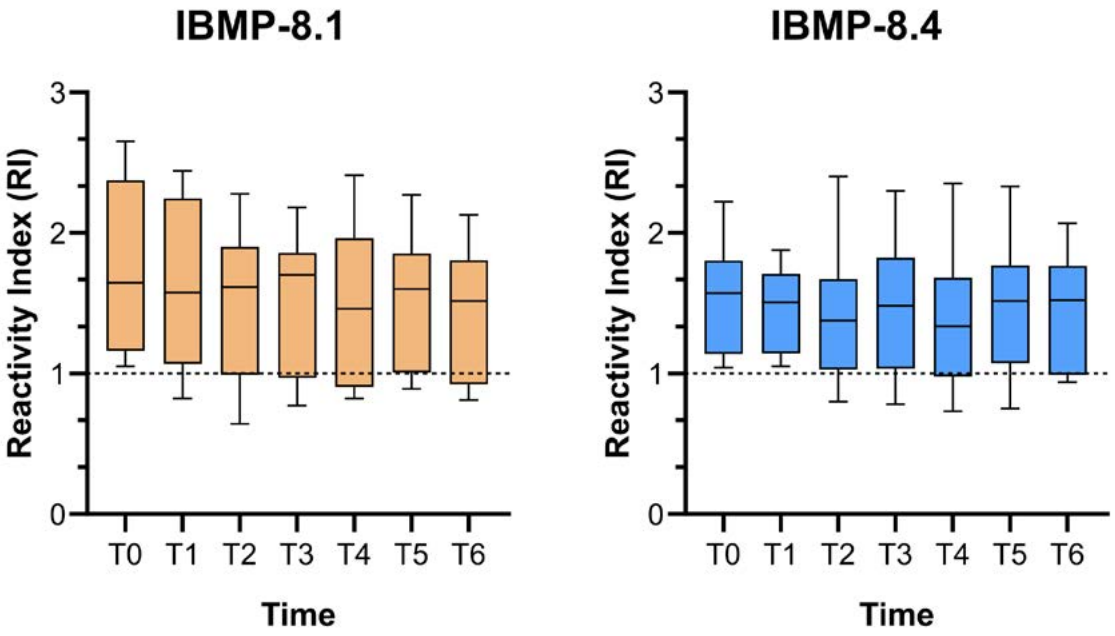

Fig. 2. Boxplot of reactivity index values as a function of the time period during which samples were collected. T0 – before treatment; T1 – two months after treatment; T2 – three months after treatment; T3 – six months after treatment; T4 – one year after treatment; T5 – two years after treatment; T6 – three years after treatment.

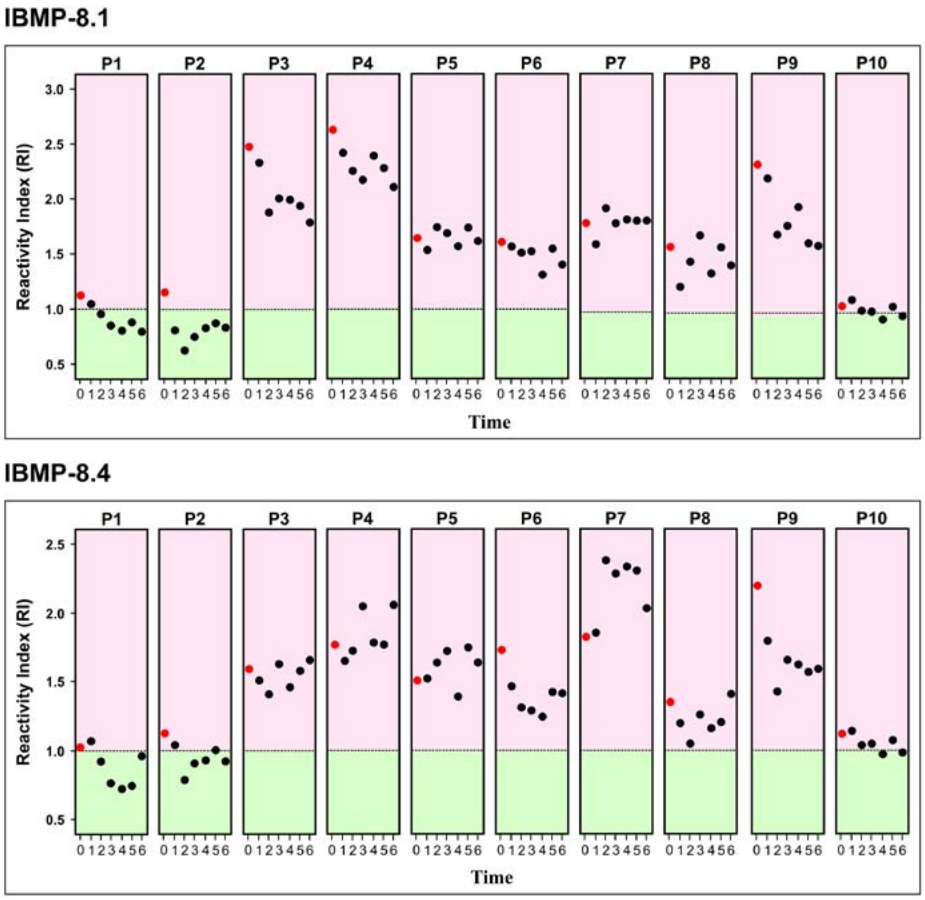

Fig. 3. Distribution of reactivity index values according to the time of sample collection for each patient. T0 – before treatment; T1 – two months after treatment; T2 – three months after treatment; T3 – six months after treatment; T4 – one year after treatment; T5 – two years after treatment; T6 – three years after treatment.

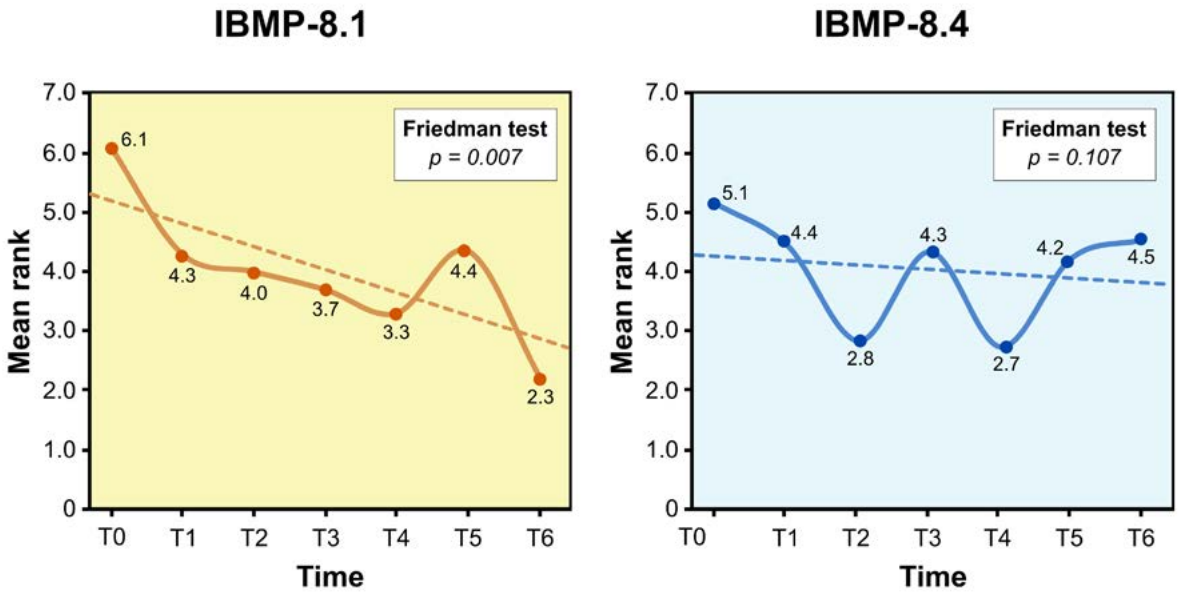

Fig. 4. Mean rank of reactivity index values according to Friedman analysis. T0 – before treatment; T1 – two months after treatment; T2 – three months after treatment; T3 – six months after treatment; T4 – one year after treatment; T5 – two years after treatment; T6 – three years after treatment.

ing treatment failure; (2) The signal decreased but did not become negative. In patients P3 (IBMP-8.1), P4 (IBMP-8.1), P6 (IBMP-8.1 and IBMP-8.4) and P9 (IBMP-8.1 and IBMP-8.4), the signal decreased over time but was not sufficient for ELISA negativity (RI remained > 1.0); (3) The signal remained constant over time. In patients P3 (IBMP-8.4), P4 (IBMP-8.4), P5 (IBMP-8.1 and IBMP-8.4), P7 (IBMP-8.1) and P8 (IBMP-8.1 and IBMP-8.4), the signal remained almost constant during the study period; (4) The signal grew stronger over time. Surprisingly, when P7 was analysed with IBMP-8.4, it showed an increase in the reactivity index signal. Compared to the three in-house ELISA assays used as reference tests (Table S1), which were performed when the samples were collected between 2000 and 2004, we observed a reduction in signal for P1, P2, and P10. It is important to emphasize that the results decreased but did not turn negative, except in the case of patient P1 when assayed with the JL7 antigen.

For the remaining seven patients, the signal either remained constant or decreased but without reaching negativity in the reference tests. Notably, no sample exhibited an increase in the signal over time, except as previously described for P7 when tested with IBMP-8.4. A summary of the interpretation of all five ELISA assays performed for patients P1 to P10 is available in Table 3.

The Friedman test (Fig. 4) revealed significant differences in the reactivity index values for the samples tested with IBMP-8.1 (p = 0.007). However, no changes were found for the samples tested with IBMP-8.4 (p = 0.107).

DISCUSSION

Currently, there is no universal marker that confirms a parasitic cure of CD after aetiological treatment. Although there are several newer, different and sophisticated methods that can be used in post-therapeutic surveillance of CD, studies on this topic are controversial and contradictory because of the different methods of assessment, the elapsed time of infection and the assumed criteria for cure. Whenever a new method is proposed, there is a lack of a plausible short-term 'gold standard' that can be validated and is sufficiently sensitive and specific to demonstrate elimination of the parasite (De Lana and Martins-Filho 2015). In the present study, we investigated the use of serological non-conventional assays using chimeric recombinant *Trypanosoma cruzi* antigens (IBMP-8.1 and IBMP-8.4) to detect the decrease in IgG anti-*T. cruzi* titres in ten patients after treatment of CD with Bz. These two antigens were selected based on their previously demonstrated exceptionally high diagnostic performance, irrespective of the geographic origin of the sample and the diagnostic platform (Santos et al. 2017a,b, 2022, Daltro et al. 2019, 2022, Del-Rei et al. 2019, Dopico et al. 2019, Silva et al. 2020, Freitas et al. 2022).

In our study, reactivity values were first grouped according to the time period in which the samples were collected and analysed together. No trend towards signal reduction was observed for both IBMP-8.1 and IBMP-8.4 across all study time points. We believe that the follow-up period of 36 months is not sufficient to detect a significant signal reduction. A study examining 94 patients over a 10-year follow-up period after Bz treatment found that only 8.5% of patients were considered cured when tested with a recombinant ELISA (Chagatest kit; recombinant ELISA v.3.0; Wiener Laboratorios, Rosario, Argentina) (Machado-de-Assis et al. 2012).

The same cure rate was observed when the authors simultaneously demonstrated negativity with three conventional serological tests (ELISA, HAI and IFF). The time required for seroreversion of conventional serology is very long, even when parasitological or molecular methods have

been persistently negative, leading to continued dissatisfaction in the scientific community and especially among physicians involved in the treatment of human or experimental aetiologies (De Lana and Martins-Filho 2015).

In contrast, when we analysed the reactivity index values for each patient, we found negativity in three (30%) patients tested for the antigens IBMP-8.1 and IBMP-8.4. Unfortunately, one patient remained positive in the PCR assay, indicating probable treatment failure and a false-negative result in the ELISA. However, in the case of P2, while their reactivity remained consistently low throughout the evaluations and turned negative at 36 months post-treatment, it is noteworthy that the general trend showed an increase in reactivity, with the exception of T6. These data suggest that the follow-up period of 36 months was not sufficient to confirm seroconversion in 70% of the patients studied (P3–P9).

Our results partially align with the in-house ELISA assays conducted between 2000 and 2004 when the samples were collected. During that period, three different antigenic compositions were employed. The conventional ELISA, utilising the epimastigote lysate of the CL-Brener strain (DTU Tc VI), which represents the predominant circulating strain in northern Argentina, did not exhibit a reduction in reactivity over the time. In fact, the signal either remained constant or decreased but did not turn negative.

Similar behaviour was observed for the two non-conventional in-house ELISA assays using JL7 and B13 recombinant antigens. These findings suggest that reactivity to different antigens may vary depending on the antigenic composition used for detecting anti-*T. cruzi* antigens. The differential protein expression (De Pablos et al. 2012) and antigenic variability of *T. cruzi* (Zingales 2018) can potentially interfere with the immune response in infected individuals, leading to the possibility of false-negative results. This complexity further complicates the search for a consensus marker to ensure the cure of treated individuals.

The Friedman test showed a significant decrease in reactivity index scores for samples tested with IBMP-8.1; however, no changes were observed in samples tested with IBMP-8.4. This difference could be due to the unequal amino acid composition of these antigens. IBMP-8.4 has greater epitope diversity compared to IBMP-8.1, which increases the chance of identifying circulating anti-*T. cruzi* antibodies (Santos et al. 2018, Celedon et al. 2021, Santos et al. 2021, 2022). This is especially true when the values of the performance parameters are compared. In all studies using both antigens, IBMP-8.4 was found to be more sensitive compared to IBMP-8.1 (Santos et al. 2017a,b, Del-Rei et al. 2019, Dopico et al. 2019). However, despite the signal reduction observed with IBMP-8.1 in the Friedman assay, we cannot conclude the ability of this molecule to serve as a cure criterion due to the small number of samples. For this purpose, we need to evaluate a larger number of samples and observe patients over a longer period of time.

In the last decades, several antigens have been studied as a tool to monitor cure with promising results after Bz treatment, notably kinetoplastid membrane protein 11 (KMP11), paraflagellar rod protein 2 (PFR2), heat shock protein 70 (HSP70) (Pinazo et al. 2015, Fernandez-Villegas et al. 2016), the immunodominant repetitive epitope 3973 (Buschiazzo et al. 1992, Fernandez-Villegas et al. 2016), the *T. cruzi* Ca^{2+}-binding flagellar protein F29 (Sosa Estani et al. 1998, Fabbro et al. 2013) and a group of peptide antigens forming the MultiCruzi assay, as part of a multiplex assay for diagnosis: 60S ribosomal protein L19, KMP11, mucin-like protein, mucin TcMUCII, CRA, surface antigen 2, MAP, TcD, TSSA, TcR39, TcR69, SAPA, trans-sialidase, cruzipain and Tc40 (Granjon et al. 2016, Zrein et al. 2018).

Although we did not include a paediatric cohort in our study, it seems important to briefly discuss the results of studies that have analysed new technologies to monitor cure using this study population. Recently, the MultiCruzi test and an F2/3 ELISA serology were evaluated as predictors of cure in a cohort of treated infants and children with chronic CD in a retrospective longitudinal study with clinical, serological and parasitological follow-up (Moscatelli et al. 2019, Medina et al. 2021). It was concluded that the MultiCruzi can be used as a predictive surveillance tool to assess cure in most infants and children. Despite these promising results, seroconversion is known to occur more frequently in children than in adults (Sosa Estani et al. 1998, Moscatelli et al. 2019). Therefore, this new technology should be further explored involving a well-characterised cohort of chronic CD adults treated with Bz.

The main limitations of the present study are the short follow-up time of patients treated with Bz and the small number of patients analysed. Although no conclusion can be drawn, the results found here could be an interesting starting point for further studies comparing post-treatment behaviour with different types of chimeric antigens with different epitope compositions. Further studies with a longer follow-up period are needed to confirm the hypothesis that IBMP-8.1 and IBMP-8.4 might be useful to verify a signal lowering of serological titres and thus can be used as a post-therapeutic cure criterion in chronic Chagas disease.

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