Serological evaluation of a cohort of patients with chronic Chagas disease treated with benznidazole in a low endemic area of Central Brazil

ABSTRACT

Background: The most widely accepted criterion for assessing the cure of chronic Chagas disease after etiological therapy is the negativization of anti-*Trypanosoma cruzi* antibodies long after the completion of treatment.

Materials and Methods: We employed various serological antibody detection techniques to ascertain whether benznidazole treatment successfully cured chronic Chagas disease in a retrospective cohort of patients in a low-endemic area in central Brazil. Antibodies were assessed using indirect immunofluorescence (IIF) and enzyme immunoassay (ELISA) before treatment and 1-18 years post-treatment. A reevaluation of 89 patients was conducted in 2018, which included indirect hemagglutination (IHA), chemiluminescence (CL), and polymerase chain reaction (PCR) analyses.

Results: Seventeen patients were monitored for 15 years or more, seven for 10-15 years, 50 for 6-10 years, and 17 for 1-5 years. Only two patients who received treatment tested PCR-positive at reevaluation, indicating treatment failure. Both patients discontinued treatment early due to benznidazole's side effects. The incidence of serological test negativization was 10.1%. However, antibody levels determined by ELISA decreased with the increased duration of follow-up (p=0.002). Similarly, antibody titers of IIF-positive sera were lower in post-treatment reevaluations than those in pre-treatment titers, indicating reduced levels of anti-*T. cruzi* antibodies. These levels decreased in a duration-dependent manner during follow-up.

Conclusion: The results reveal a low cure rate based on antibody negativization, but antibody titers significantly decreased in all conventional serological tests. These findings suggest that decreased antibody levels may be associated with parasitological cure in patients with chronic Chagas disease after etiological treatment with benznidazole. Further prospective studies should be conducted to confirm and validate these findings.

Keywords: Chagas disease, Treatment, Benznidazole, Cure criterion, Conventional serology.

Introduction

The efficacy of etiological treatment of chronic Chagas disease has been evaluated using anti-*T. cruzi* antibody screening, parasite recovery in blood culture and xenodiagnosis, and parasite DNA detection using molecular techniques [1]. The cure criteria for the treatment of chronic *T. cruzi* infection are the conventional serology seroconversion and the clearence of parasitological tests, such as polymerase chain reaction (PCR) [2-4]. However, these methods do not provide reliable results for continuous evaluation of therapeutic response due to unstable antibody behavior and poor sensitivity of parasitological tests [5,6]. The lack of reliable methods is a major challenge for cure evaluation after treating patients with chronic Chagas disease [3,7]. To address this challenge a recent and promising study demonstrates how the use of the parasite's cell-free DNA and the extracellular vesicles from the parasite's secretome, present in the serum of chronic patients, can serve as 'containers' for transporting parasite DNA and be utilized for the molecular diagnosis of Chagas disease [8].

An adequate evaluation of treatment response of chronic Chagas disease requires long-term patient follow-up, typically for periods longer than 20 years, to confirm anti-*T. cruzi* negativization [4]. However, decreased antibody titers over time may be a predictor of favorable therapeutic response, as they may become negative over time without further treatment [9,10]. Antibody titer negativization is characterized by persistent and progressive decline at more than three dilutions. Negativization is determined using the IIF test at dilutions lower than 1:40 and by ELISA when the optical density of the reaction is lower than cutoff [1,11,12]. Although CL has not been evaluated as a technique to determine negativization, some studies have shown that CL is highly sensitive, which suggests that it may be a good measure of negativization at patient follow-up after antiparasitic treatment [13,14].

The presence of parasitic DNA in blood samples of treated patients with chronic Chagas disease indicates therapeutic failure. However, negative PCR results are not sufficient to confirm that the protozoan was completely eliminated [14-16]. Because PCR analysis results in a high rate of false negative results, it is not considered adequate for monitoring the efficacy of chronic disease treatment.

Although no conventional serological tests are considered ideal to monitor the response of Chagas disease to treatment [12], general consensus is that the chronic Chagas disease response to treatment should be monitored by quantitative anti-*T. cruzi* antibody screening using sensitive methods such as ELISA, IIF, and IHA, which allow for determination of antibody kinetics over time following treatment [4,12]. Chemiluminescence is considered a promising method to monitor Chagas disease treatment efficacy due to its high sensitivity. Chemiluminescence should be used in conjunction with other conventional serological tests and PCR analysis [13,14].

Few studies have focused on chronic Chagas disease follow-up in patients treated with benznidazole for long durations. Detection of circulating parasites by microscopy or PCR has not produced satisfactory results compared to conventional serological methods for monitoring patients treated for chronic Chagas disease [12,17]. Potential biomarkers of early therapeutic responses have been tested in different clinical contexts. A decrease in IgG1 antibodies suggests a successful cure, whereas a sustained or increased level of IgG1 may serve as a potential indicator of treatment failure. However, due to its limited sensitivity, IgG1 should not be relied upon as a primary diagnostic marker. Instead, it holds promise as a potential biomarker of cure with further development [18]. An ELISA using a 29 kDa flagellar recombinant F29 antigen of the parasite (ELISA F29) has been successfully assessed as an early biomarker of response to treatment with benznidazole in children and adults treated with antitrypanosomal therapy [19]. However, such innovation

is not yet available for widespread use and has also not been validated in an appropriate cohort of treated patients.

Thus, use of conventional serology as a tool to monitor therapeutic response should be further studied. Our study evaluated the results of several serological techniques for detection of anti-*T. cruzi* antibodies following etiological treatment with benznidazole to determine new tools to evaluate therapeutic response of patients with chronic Chagas disease.

Materials and methods

This was a retrospective, descriptive, and epidemiological cohort study to evaluate the antibody response of 89 patients with chronic Chagas disease previously treated with benznidazole. The patients were treated from 2001 to 2017 at the Infectious Diseases Reference Center, Júlio Müller University Hospital, Cuiabá (MT), Brazil. Demographic, clinical, and laboratory data for each patient prior to treatment and results of anti-*T. cruzi* antibody screening during follow-up were collected from medical charts. Etiological treatment of chronic Chagas disease followed the Brazilian Chagas Disease Consensus, which recommends 5 mg/kg/day benznidazole orally for 60 days. The Consensus also indicates systematic clinical and laboratory reevaluations for a period longer than 15 years.

Anti-*T. cruzi* antibodies were screened using IIF and ELISA before treatment and at follow-up one to 18 years posttreatment. Reevaluation conducted in 2018 included IHA, CL, and PCR analyses, which were not used prior to treatment, to increase the sensitivity and specificity of cure determination for Chagas disease.

Indirect immunofluorescence was performed on a suspension of *T. cruzi* antigens fixed on a slide using the Immuno-COM commercial kit (Wama Diagnóstica, São Paulo, Brazil). PBS samples were diluted 1:30, 1:60, 1:120, and 1:240, as recommended by the manufacturer.

The antigens used for IHA (Wama Diagnóstica, São Paulo, Brazil) were produced in bird erythrocytes stabilized and sensitized with highly purified *T. cruzi* antigenic components. Sera were diluted 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, and 1:4096 in 2-mercaptoethanol diluent solution, which removes interfering antibodies.

Chemiluminescence analysis was performed using an enzyme-linked immunoassay against recombinant *T. cruzi* proteins (Alinty Chagas Reagent commercial kit, Abbott, Wiesbaden, Germany). Fluorescence of CL samples was expressed as relative light units (RLU).

A combination of recombinant antigen and purified protozoan epimastigote lysates (Brazilian strains) was added to polystyrene plates as a solid phase for ELISA, and analysis was performed using the commercial kit Gold Elisa Chagas (REM Indústria e Comércio Ltd., São Paulo, Brazil).

S35/S36 primers, which amplify a repetitive sequence of 330 base pairs of parasite kinetoplast DNA, were used for PCR analysis [20] of pure and diluted DNA samples at dilutions of 1:10 and 1:100. Blood samples were used as positive controls and distilled water was used as a negative control for *T. cruzi* infection. Reactions were amplified using 10 µL of PCR Master Mix (Promega Corporation, Wisconsin, USA) with 2 µL each of the S35 (AAATAATGTACGGGTGAGATGCATGA) (Integrated DNA Technologies, City, Country) and S36 (GGGTTCGATTGGGGTTGGTGT) (Integrated DNA Technologies, City, Country) primers.

In this study, the definition of cure of chronic Chagas disease was based on negative results from at least two different antibody tests during patient reevaluation [12]. The geometric mean reciprocal titers (GMRT) of anti-*T. cruzi* antibodies were used for analysis of antibody levels using IIF and IHA. Reciprocal titers were log transformed to compare antibody levels before and after treatment. Spearman's non-parametric correlation was used to analyze the correlation of antibody levels with all techniques used and follow-

up time after benznidazole treatment. Finally, the anti-*T. cruzi* antibody levels obtained in pre- and posttreatment evaluations were compared using the Wilcoxon's non-parametric paired test. Stata statistical package version 12.0 (Stata Corp, Texas, USA) was used for all analyses, with an alpha level of 0.05.

Results

Fifty-seven (64.0%) of the 89 patients were female, with mean (SD) age of 52.9 (10.8) years. Most patients were from the endemic Southeast (21.4% of cases) and Central regions (20.2% of cases) of Brazil. Only 10.1% of the patients were likely victims of autochthonous transmission in the study area. The remaining 89.9% of the cases were imported from other Brazilian endemic states. The most common occupations of the patients were homemaker (51.7%), commerce (29.0%), construction (7.9%), and agriculture (4.5%) (Table 1).

The undetermined clinical form of Chagas disease was the most frequently observed (68.6%). Cardiac (16.3%), digestive (3.1%), and cardiodigestive (12.0%) complications were less frequent in these patients. All patients received benznidazole treatment. Treatment duration was 60 days for 66 (75.0%) patients, 40 to 60 days for eight (9.1%) patients, and less than 40 days for 14 (15.9%) patients. Follow-up duration ranged from 15.0 to 17.9 years for 16.8% of patients, 10.0IS to 14.9 years for 7.9% of patients, 5.0 to 9.9 years for 50.2% of patients, and 1.0 to 4.9 years for 19.1% of patients (Table 1).

Results of laboratory reevaluations conducted one to 18 years after etiological benznidazole treatment show a low cure rate (Table 2). Polymerase chain reaction analysis of 51 patients in 2018 showed that only two (3.9%) were positive for *T. cruzi* at reevaluation. Each of these patients discontinued benznidazole treatment prior to 60 days due to side effects.

Indirect immunofluorescence was negative in only three (3.4%) of the 89 patients treated. Of 73 patients reevaluated using ELISA, five (6.8%) were negative for anti-*T. cruzi* antibodies. Of the 51 patients reevaluated using IHA and CL, three (5.9%) and two (3.9%) were negative, respectively. The analysis of negative results using two serological methods showed that the anti-*T. cruzi* antibody negativization occurred in only 10.1% at follow-up one to 18 years after treatment (Table 3).

Comparison of anti-*T. cruzi* antibody results produced using the different assays used showed no association between antibody level and assay type. Furthermore, antibody levels were similar in patients who received benznidazole for 60 days and in patients treated for less than 60 days. However, mean serum antibody levels decreased with increased follow-up duration, as determined using ELISA ($p=0.005$) (Table 3).

Follow-up time after etiological treatment and antibody levels showed a negative and statistically significant correlation only for ELISA results ($p=0.002$) (Figure 1). Although the other techniques showed slightly decreased anti-*T. cruzi* antibody levels with increased follow-up time, this decrease was not significant. Indirect immunofluorescence analysis of antibody titers showed a statistically significant decrease between pre- and posttreatment with benznidazole ($p<0.0001$) (Figure 2A). Anti-*T. cruzi* antibody levels were also decreased with ELISA detection ($p<0.0001$) following treatment with benznidazole (Figure 2B).

Discussion

The results of this study showed a low cure rate, as determined by serological negativization, in patients with chronic Chagas disease one to 18 years after etiological treatment with benznidazole in an area of low disease prevalence in Central Brazil. The different serological techniques used did provided different levels of therapeutic response

monitoring efficacy. However, all conventional methods used for analysis showed that etiological treatment significantly decreased anti-*T. cruzi* antibody levels. Unambiguous therapeutic failure due to positive PCR at reevaluation was seen in only two patients, both of whom discontinued benznidazole treatment due to side effects.

The demographic characteristics of the patients analyzed in this study suggest that they were infected during childhood through the insect vector. Chagas disease transmission in Brazil has been significantly reduced by the intensive public health program implemented in the 1970s and certified in 2006 by the WHO, which aims to interrupt transmission by *Triatoma infestans* [21].

In this study, most patients were classified as having an undetermined chronic form of the disease, which is most prevalent form in older individuals [12]. Many patients treated at the Reference Center were referred by the local health system after positive anti-*T. cruzi* antibody results during blood donation screening. As such, these patients were asymptomatic and did not experience complications.

Previous studies have shown that benznidazole can effectively eliminate parasitemia and cure patients during the chronic infection stage [22]. Therefore, parasitemia clearance should be the main objective when investigating etiological treatment efficacy in the chronic phase. Different parasitological methods have been studied, such as blood culture, xenodiagnosis, and PCR. However, none of these techniques can definitely determine whether etiological treatment resulted in a cure [23].

In this study, use of PCR did not contribute to evaluation of the therapeutic response to benznidazole, since this technique was not used prior to treatment. Evaluation of chronic Chagas disease cure by PCR in isolated blood samples is controversial because due to low sensitivity detection of parasite DNA. Furthermore, although PCR has good positive predictive value of infection, the rate of false negative results is high due to several factors such as low circulating parasitemia, transient and intermittent parasitemia in

the chronic phase, and genetic variability of circulating *T. cruzi* strains [5]. Thus, PCR may be more effective with analysis of multiple samples from the same patient to increase sensitivity. However, the clinical value of PCR as a criterion of cure for chronic Chagas disease patients requires further study [4].

Of the 89 patients, 15.9% discontinued treatment before the recommended 40 days, primarily due to side effects of benznidazole treatment. Other studies reported discontinuation rates of 12% to 18% in patients treated with benznidazole, primarily due to side effects [24]. In our study, the two patients with positive PCR results during posttreatment reevaluation belonged to the incomplete treatment group.

Only nine patients in our study had negative seroconversion to anti-*T. cruzi* antibodies during follow-up, which is of great concern. However, it is important to consider that a serological follow-up period of 10 years or more is essential for evaluation of response to chronic Chagas disease etiological treatment [12] Many of the patients in our study were evaluated less than 10 years after treatment.

The serological criterion for determination of therapeutic response to benznidazole is considered a reference and requires evidence of immunological negativization to characterize a patient as cured [12,25]. Benznidazole efficacy for treatment of chronic Chagas disease ranges from 8% to 40% [1]. This large variability in efficacy is likely due to heterogeneous follow-up durations across studies [1, 25]. Some studies that monitored therapeutic responses using serological criteria showed low rates of anti-*T. cruzi* antibody negativization following benznidazole treatment. These studies showed that a conclusive evaluation of treatment efficacy requires an extremely long follow-up period due to slow clearance of these antibodies [12,26]. For this reason, we used reduction of antibody levels at reevaluation to determine therapeutic response to chronic Chagas disease treatment.

In the evaluation carried out by ELISA, most patients treated with benznidazole had significantly decreased antibody titers even after short follow-up durations. A similar

finding was reported by Niborski et al. (2016) at three-year follow-up [9]. Anti-*T. cruzi* antibody levels decreased with all techniques used in this study, with significant decreased observed using IIF and ELISA. These results agreed with those in previous studies that evaluated treatment efficacy using similar serological evaluations [26]. Another study, which had a much shorter follow-up period, also showed decreased serum anti-*T. cruzi* IgG levels as determined using IIF, and significantly decreased mean optical density using ELISA, which suggested that continued follow-up was required to determine whether the patients were cured [27]. However, it is important to highlight a recent study used a gPhage platform of unbiased library of antigens, which is different from conventional serological assays that rely on predetermined antigens. These new antigens reacted with antibodies in high correlation with PCR [28]. Although preliminary, this result may represent a strategy to reduce patient follow-up time with the aim of verifying the therapeutic response.

A justification that decreased antibody levels indicates a favorable therapeutic response is that patients with decreased antibody titers achieved parasite load reduction or complete parasite elimination [29]. In addition, this argument is consistent with the observation of better clinical outcomes in response to benznidazole in patients who showed reduced serological responses than in those who did not experience an antibody titer change [30]. In addition, a meta-analysis that combined data from both observational cohorts and small randomized trials showed that benznidazole had significant activity against *T. cruzi* when evaluated using seroconversion or significantly decreased antibody titers as criteria [22].

In summary, benznidazole treatment in patients with chronic Chagas disease can result in a cure, but with low frequency. The low frequency of positive PCR one to 18 years after treatment of chronic Chagas disease with benznidazole did not contribute to evaluation of treatment response, which suggested that this technique is not suitable for

detection of the parasite under low parasitemia conditions in isolated blood samples. In this way, we formulated the hypothesis that the reduction in antibody levels observed in the patients analyzed here suggests that serial measurement of antibody titers after the treatment of chronic Chagas disease may be useful as a marker of an adequate response to etiological treatment. Thus, in addition to complete seroconversion, as determined by negative results using two tests, decreased titers in two or three conventional serological tests could also be used as a criterion to evaluate the response to treatment of chronic Chagas disease treatment, as suggested by Viotti et al. (2011) [29].

Our study was limited by a small number of patients, observational design, short follow-up period, and lack of PCR results prior to benznidazole administration. In addition, spontaneous cure of chronic Chagas disease although rare, has been documented in untreated patients followed-up for long periods of time [29], and should also be considered in uncontrolled studies such as ours. However, spontaneous cure likely did not influence our study due to the short follow-up period. These limitations did not appear to impact the main finding of this study.

Conclusions

Benznidazole treatment in patients with chronic Chagas disease can result in a cure, but with low frequency. The reduction in *T. cruzi* antibody levels after a follow-up period of one to 18 years after treatment suggested that this decrease could be used as a marker of adequate therapeutic response. The low frequency of positive PCR one to 18 years after treatment of chronic Chagas disease with benznidazole did not contribute to evaluation of treatment response, which suggested that this technique is not suitable for detection of the parasite under low parasitemia conditions in isolated blood samples.

Ethical Approval

The research project was approved by the Ethical Committee of the Júlio Müller University Hospital in 2001, at the beginning of the cohort, and again on June 28, 2018, under the CAAE number 86630518.8.0000.5541 and opinion number 2.744.199, respectively.

References

1. Sosa-Estani S; Viotti R Segura EL. Therapy, diagnosis and prognosis of chronic Chagas disease: insight gained in Argentina. Mem Inst Oswaldo Cruz. 2009; 104: 167-180.
2. Sguassero Y, Cuesta CB, Roberts KN, Hicks E, Comandé D, Ciapponi A, Sosa-Estani S. Course of chronic *T. cruzi* infection after treatment based parasitological and serological tests: a systematic review of follow-up studies. PLoS One. 2015;10 e0139363.
3. Murcia L, Carrilero B, Ferrer F, Roig M, Franco F, Segoiva M. Success of benznidazole chemotherapy in chronic *T. cruzi*-infected patients with a sustained negative PCR result. Eur J of Clin Microbiol Infect Dis. 2016; 35:1819-1827.
4. PAHO. Pan-American Health Organization (PAHO). Guidelines For the Diagnosis and Treatment of Chagas disease. United States of America, Washington, DC (2018). Nov (updated June 2019); accessible at URL: https://iris.paho.org/handle/10665.2/49653.
5. Coura JR, Castro SL. A Critical Review on Chagas Disease Chemotherapy. Mem Inst Oswaldo Cruz. 2002; 97: 3-24.
6. Britto CC. Usefulness of PCR-based assays to asses drug efficacy in Chagas disease chemotherapy: value and limitations. Mem Inst Oswaldo Cruz. 2009; 104:122-135.

7. Alonso-Padilla J, Abril M, Alarcon de Noya B, Almeida IC, Angheben A, Araujo-Jorge T, et al. Target product profile for a test for the early assessment of treatment efficacy in Chagas disease patients: an expert consensus. PLoS Negl Trop Dis. 2020;14: e0008035.

8. Lozano N, Samblas MG, Calabuig E, Giménez Martí MJ, Gómez Ruiz MD, Arce JM, Sequera-Arquelladas S, Moreno JM, Trelis M, Osuna A. Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease. Plos one. 2023;18(9): e0282814.

9. Niborski LL, Grippo V, Lafon SO, Levitus G, Garcia-Bournissen F, Ramirez JC, Burgos JM, Bisio M, Juiz NA, Ayala V, Coppede M, Herrera V, Lopez C, Contreras A, Gomez KA, Elean JC, Mujica HD, Schijman AG, Levin MJ, Longhi SA. Serological based monitoring of a cohort of patients with chronic Chagas disease treated with benznidazole in a highly endemic area of northern Argentina. Mem Inst Oswaldo Cruz. 2016; 111: 365–371.

10. Albareda MC, Natale MA, De Rissio AM, Fernandez M, Serjan A, Alvarez MG, Cooley G, Shen H, Viotti R, Bua J, Castro Eiro MD, Nunez M, Fichera LE, Lococo B, Scollo K, Tarleton RL, Laucella SA. Distinct treatment outcomes of antiparasitic therapy in Trypanosoma cruzi-infected children are associated with early changes in cytokines, chemokines, and t-cell phenotypes. Frontiers in Immunology. 2018; 9: 1958.

11. Hasslocher-Moreno A, Brazil PEAA, Sousa AS, Xavier SS, Chambela MC, Silva GMS. Safety of benznidazole in the treatment of chronic Chagas' disease. J Antimicrob Chemother. 2012; 67:1261-1266.

12. Dias JCP, Ramos Jr NA, Dias GE, Luquetti A, Shikanai-Yasuda MA, Coura JR. II Brazilian Consensus on Chagas Disease, 2015. Epidemiol Serv Saúde. 2016; 25: 7-8

13. Abras A, Gallego M, Llovet T, Tebar S, Blacksmith M, Berenguer P, Ballart C, Marti C, Munoz CABR. Serological Diagnosis of Chronic Chagas Disease: Is It Time for a Change? J Clin Microbiol. 2016; 54: 1566–1572.

14. Torrico F, Gascon J, Ortiz L, Alonso-Vega C, Pinazo MJ, Schijman A, Almeida IC, Alves F, Strub-Wourgaft N, Ribeiro I. Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomized, placebo-controlled trial. The Lancet Infectious Diseases. 2018; 18: 419-4

15. Sánchez-Valdéz FJ, Padilla A, Wang W, Orr D, Tarleton RL. Spontaneous dormancy protects Trypanosoma cruzi during extended drug exposure. Elife. 2018;7: e34039.

16. Machado de Assis GF, Diniz GA, Montoya RA, Dias JCP, Coura JR, Machado-Coelho GLL, Albajar-Viñas P, Torres RM, de Lana M. A serological, parasitological and clinical evaluation of untreated Chagas disease patients and those treated with benznidazole before and thirteen years after the intervention. Mem Inst Oswaldo Cruz. 2013; 108:873-880.

17. de Lana M, Martins-Filho OA. Revisiting the Posttherapeutic Cure Criterion in Chagas Disease: Time for New Methods, More Questions, Doubts, and Polemics or Time to Change Old Concepts? Biomed Res Int. 2015; 2015: 652985

18. Murphy N, Cardinal MV, Bhattacharyya T, Enriquez GF, Macchiaverna NP, Alvedro A, Freilij H, Martinez de Salazar P, Molina I, Mertens P, Gilleman Q, Gürtler RE, Miles MA. Assessing antibody decline after chemotherapy of early chronic Chagas disease patients. Parasit Vectors. 2021;14(1):543.

19. Rivero R, Esteva MI, Huang E, Colmegna L, Altcheh J, Grossmann U, Ruiz AM, CHICO and CHICO SECURE Study Groups. ELISA F29–A therapeutic efficacy biomarker in Chagas disease: Evaluation in pediatric patients treated with nifurtimox and followed for 4 years post-treatment. PLoS Negl Trop Dis. 2023; 17(6):e0011440.

20. Barrera YK, Guevara JM, Pavia PX, Montilla M, Nicholls RS, Vine E, Gate CJ. Evaluation of the TcH2AF-R and S35-S36 PCR tests for the detection of *T. cruzi* in mouse cardiac tissue. Biomedical 2008; 28: 616-626.

21. Arias AR, Monroy C, Guhl F, Sosa-Estani S, Santos WS, Abad-Franch F. Chagas disease control-surveillance in the Americas: the multinational initiatives and the practical impossibility of interrupting vector-borne Trypanosoma cruzi transmission. Mem Inst Oswaldo Cruz. 2022; 117: e210130.

22. Perez-Molina, JA, Perez-Ayala A, Moreno S, Fernandez-Gonzalez MC, Zamora J, Lopez-Velez R. Use of benznidazole in the treatment of chronic Chagas' disease: a systematic review with meta-analysis. J Antimicrob Chemother. 2009; 64: 1139-1147.

23. Oliveira MF, Nagao-Dias AT, Pontes VMO, Souza Jr. AS, Coelho HLL, Coelho ICB. Etiological treatment of Chagas disease in Brazil. Rev Patol Trop. 2008; 37: 209-228.

24. Álvarez MG, Vigliano C, Lococo B, Petti M, Bertocchi G, Viotti R. Seronegative conversion after incomplete benznidazole treatment in chronic Chagas disease. Trans Royal Soc Trop Med Hyg. 2012; 106: 636-638.

25. Sangenis LHC. Chagas disease in people from the state of Rio de Janeiro: clinical-epidemiological aspects, molecular parasitological characterization and eco-epidemiological study of autochthonous cases. Rio de Janeiro: Oswaldo Cruz Foundation. 2013. Doctoral Thesis in Tropical Medicine.

26. Fabbro D, Arias E, Streiger M, del Barco M, Amicone N, Miglietta H. Evaluation of specific chemotherapy in adult chagasic infections in an undetermined phase with more than five years of follow-up. Rev. de la Fed. Argentina de Cardiol. 2001; 30:496-503.

27. Andrade MC, Rocha EA, Costa AL, Lima DA, Silva Filho JD, Bezerra FSM, Barbosa WGS, Oliveira MF. Electrocardiographic and serological evolution of patients with chronic Chagas disease followed-up for six years after treatment with benznidazole. Relampa. 2016; 29:141-149.

28. Rodriguez Carnero LA, Kuramoto A, Campos de Oliveira L, Monteiro JS, Setubal JC, Cunha-Neto E, Cerdeira Sabino E, Giordano RJ. Anti-Trypanosoma cruzi antibody profiling in patients with Chagas disease treated with benznidazole assessed by genome phage display. PLoS Negl Trop Dis. 2023;17(1): e0011019.

29. Viotti R, Vigliano C, Álvarez MG, Lococo B, Petti M, Bertocchi G, Armenti A, Rissio AM, Cooley G, Tarleton R, Laucella S. Impact of aetiological treatment on conventional and multiplex serology in chronic Chagas disease. PLoS Negl Trop Dis. 2011; 5: e1314.

30. Viotti R, Vigliano C, Lococo B, Petti M, Bertocchi G, Armenti A. Clinical predictors of chronic chagasic myocarditis progression. Rev Esp Cardiol. 2005; 58: 1037-1044.

Table 1 - Demographic and clinical characteristics of patients with chronic Chagas disease etiologically treated with benznidazole from 2001 to 2018.

CARACTERISTIC		n	%
Age (years)	*20 ⊢ 40*	13	14.6
	40 ⊢ 60	48	53.9
	60 ⊢ 80	28	31.5
	Mean (SD): 52.9 (10.8)		
Sex	*Female*	57	64.0
	Male	32	36.0
Origin	*Minas Gerais*	80	89.9
	Other Brazilian states	9	10.1
Occupation	*Homemaker*	46	51.7
	Commerce	25	28.0
	Construction	7	7.9
	Agriculture	4	4.5
	Others	7	7.9
Clinical form (n=86)	*Undetermined*	59	68.6
	Cardiac	14	16.3
	Digestive	3	3.5
	Cardiodigestive	10	11.6
Treatment duration (days) (n=88)	*60*	66	75.0
	40⊢ 60	8	9.1
	<40	14	15.9
	Mean (SD): 51.0 (17.28)		
Cohort duration (years)	*1⊢5*	17	19.1
	5⊢10	50	56.2
	10 ⊢15	7	7.9
	15 ⊢18	15	16.8
	Mean (SD): 8.0 (4.7)		

SD: standard deviation

Table 2 - Parasitemia and anti-*T. cruzi* antibody results in a cohort of patients with chronic Chagas disease one to 18 years after etiological treatment with benznidazole.

CHARACTERISTIC		n	%
PCR after treatment (n=51)	*Positive*	2	3.9
	Negative	49	96.1
Indirect immunofluorescence (n=89)	*Positive*	86	96.6
	Negative	3	3.4
Indirect hemagglutination (n=51)	*Positive*	48	94.1
	Negative	3	5.9
Chemiluminescence (n=51)	*Positive*	49	96.1
	Negative	2	3.9
Enzyme immunoassay (n=73)	*Positive*	68	93.2
	Negative	5	6.8
Cure as determined by serological negativization (n=89)	*Yes*	9	10.1
	No	80	89.9

Table 3 - Anti-*T. cruzi* antibody screening results in patients with chronic Chagas disease one to 18 years after etiological benznidazole treatment using different serological techniques

		Serological methods			
		IIF GMRT (95% CI)	**IHA** GMRT (95% CI)	**CL** S/CO (mean) (95% CI)	**ELISA** OD (mean) (95% CI)
Clinical form	*Undetermined*	155 (127, 189)	160 (118, 219)	7.4 (5.6, 9.8)	0.862 (0.725, 1.027)
	Cardiac	231 (194, 275)	223 (134, 372)	10.2 (7.7, 13.5)	0.729 (0.467, 1.138)
	Cardiodigestive	182 (118, 282)	323 (108, 967)	6.2 (2.9, 13.3)	0.530 (0.286, 0.985)
	p	**0.163**	**0.162**	**0.330**	**0.142**
Treatment duration (days)	*60*	154 (128, 184)	183 (136, 247)	7.4 (5.8, 9.6)	0.730 (0.611, 0.873)
	30 – 59	220 (179, 270)	304 (110, 841)	9.3 (5.6, 15.4)	0.866 (0.452, 1.256)
	2 – 29	154 (128, 184)	161 (54, 482)	8.2 (4.5, 15.2)	0.809 (0.452, 1.446)
	p	**0.067**	**0.423**	**0.488**	**0.619**
Follow-up duration (years)	*1 ⊢ 5*	217 (159, 297)	181 (84, 388)	7.7 (3.0, 20.2)	0.901 (0.530, 1.532)
	5 ⊢ 10	167 (134, 207)	222 (148, 332)	9.3 (7.8, 11.2)	0.899 (0.778, 1.038)
	10 ⊢ 15	144 (74, 278)	172 (121, 1411)	8.5 (6.6, 10.0)	0.695 (0.461, 1.047)
	15 ⊢ 18	158 (115, 218)	171 (115, 254)	5.5 (3.0, 9.9)	0.458 (0.306, 0.685)
	p	**0.204**	**0.839**	**0.286**	**0.005**

IIF: Indirect immunofluorescence (GMRT)
IHA: Indirect hemagglutination (GMRT)
CL: Chemiluminescence
ELISA: Enzyme immunoassay
GMRT: Geometric mean of reciprocal titers
S/CO: Relative light unit (sample/cutoff)
OD: Optical density
P values obtained using the non-parametric Kruskal-Wallis test.

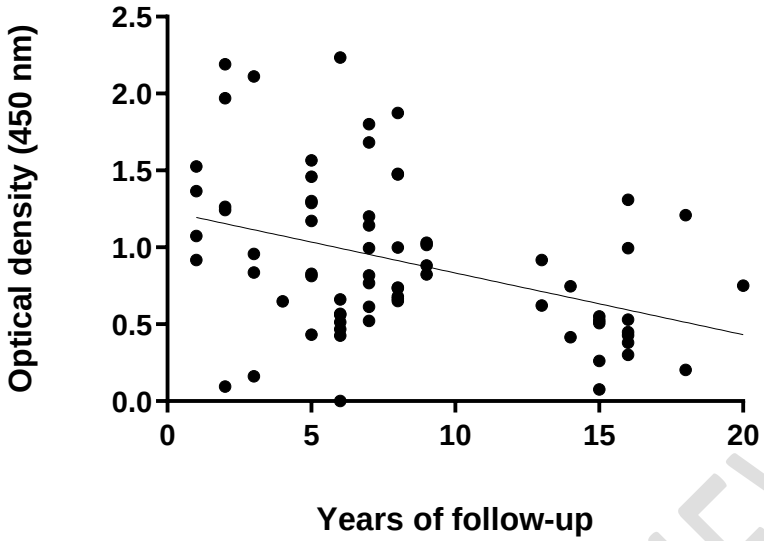

Figure 1 Legend.

Figure 1 - Correlation analysis of anti-*T. cruzi* antibody levels determined using enzyme immunoassay and time of patient follow-up after benznidazole treatment for chronic Chagas disease. [p= 0.002; Spearman's correlation test].

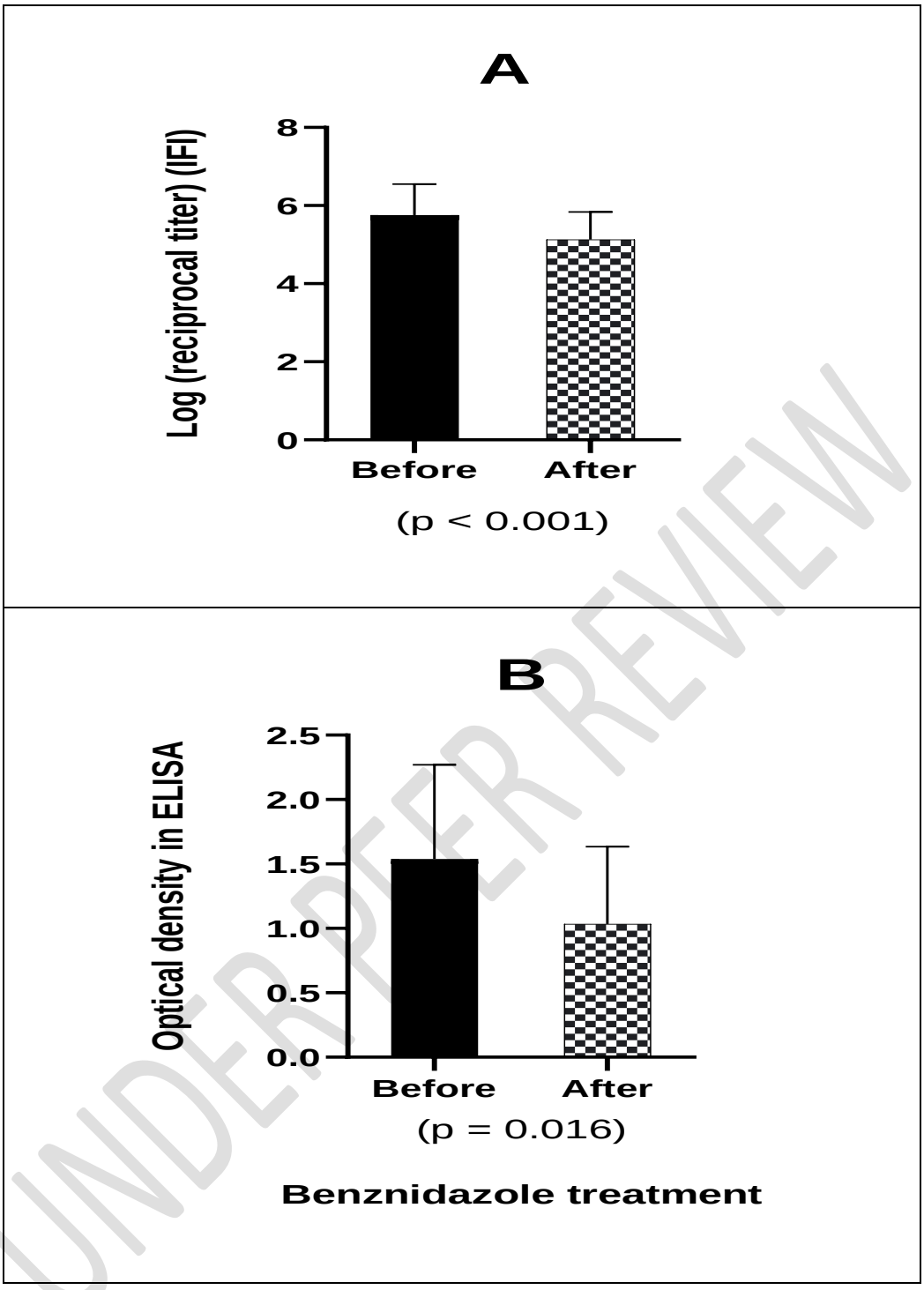

Figure 2 Legend.

Figure 2: Anti-*T. cruzi* antibody levels as determined using indirect immunofluorescence (**A**) and ELISA (**B**) in patients with chronic Chagas disease before benznidazole treatment, and at follow-up.

* Wilcoxon Test.