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New scheme of intermittent benznidazole administration in patients chronically infected with *Trypanosoma cruzi*: Clinical, parasitological and serological assessment after three years of follow-up.

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Running Title: Intermittent benznidazole treatment for Chagas disease

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Abstract

Introduction. In a pilot study, we showed that intermittent administration of benznidazole in chronic Chagas disease patients resulted in a low rate of treatment suspension and therapeutic failure, as assessed by qPCR at the end of treatment. Herein, a three-year post-treatment follow-up study of the same cohort of patients is presented.

Methods. The treatment scheme consisted of 12 doses of benznidazole at 5 mg/kg/day in two daily doses every 5 days. Parasite load, *T. cruzi*-specific antibodies and serum chemokine levels were measured prior to treatment and after a median follow-up of 36 months post-treatment by kDNA and SatDNA qPCR methods, conventional serological techniques and a Luminex-based assay with recombinant *T. cruzi* protein, and a cytometric bead array, respectively.

Results. At the end of follow-up, 14 of 17 (82%) patients had negative qPCR findings, whereas three of 17 (18%) had detectable nonquantifiable findings by at least one of the qPCR techniques. A decline in parasite-specific antibodies at 12 months post-treatment was confirmed by conventional serological tests and the Luminex assays. Monocyte chemoattractant protein-1 (MCP-1) levels increased after treatment, whereas monokine induced by gamma interferon (MIG) levels decreased. New post-treatment electrocardiographic abnormalities were observed in only one patient who had cardiomyopathy prior to treatment.

Conclusions. Altogether, these data strengthen our previous findings by showing that the intermittent administration of benznidazole results in a low rate of treatment suspension, with comparable treatment efficacy to that of a daily dose of 5mg/kg for 60 days.

Keywords: *Trypanosoma cruzi*, Chagas disease, benznidazole, intermittent treatment

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Introduction

The primary clinical outcome of *Trypanosoma cruzi* infection is a chronic cardiomyopathy, which manifests in approximately 30% of infected individuals 10-20 years after the initial infection (World Health Organization, https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis). It is estimated that approximately 1.2 million individuals are living with heart disease, making Chagas disease the most frequent cause of infectious cardiomyopathy in the world (1). Although efforts have been made in previous decades, no new compounds have been approved for the treatment of Chagas disease, with benznidazole (BZ) and nifurtimox as the only two currently available medications (2–5). Adverse events are some of the main limitations of widely applicable therapies in the chronic phase in adults (6).

Although various guidelines based on randomized studies in children recommend the use of a 60-day treatment schedule with BZ in adults (7), several studies have suggested that treatment outcomes do not differ between 30 and 60 days of BZ administration (8–10). Currently, in the Benznidazole New Doses Improved Treatment and Associations (BENDITA) clinical trial, BZ-sparing regimens in monotherapy, including the 30 days vs. 60 days schemes, are being evaluated (ClinicalTrials.gov Identifier: NCT03378661).

Furthermore, pharmacokinetic studies have shown that BZ plasma concentrations in children are markedly lower than those reported in adults, whereas the therapeutic response is higher (11, 12), adding more unresolved issues in treatment of chronic Chagas disease. Additionally, in a mouse model of chronic *T. cruzi* infection, reducing the overall dosage of BZ or nifurtimox using intermittent administration every five days cured the infection (13). As part of a pilot study, we previously showed that intermittent administration of BZ resulted in a low rate of treatment suspension and therapeutic failure as assessed by qPCR at the end of treatment (14). Herein, this same cohort of patients was followed up with a median of 36 months post-intermittent administration of BZ, in which the clinical status, parasite burden, *T. cruzi*-specific humoral responses and serum levels of chemokines were assessed.

Methods

Study population, etiological treatment and clinical follow-up. Seventeen adult patients with confirmed chronic Chagas disease [i.e., positive findings on at least two of the three serological tests, including enzyme-linked immunosorbent assay (ELISA), hemagglutination (IHA) and immunofluorescence (IIF)], aged between 28 and 57 (median, 45) years, were included (Table 1). All subjects recruited in the study provided informed consent. Patients with a history or laboratory findings compatible with liver or kidney disease, blood dyscrasias or concomitant systemic illnesses were excluded from the study. Other exclusion criteria were previous etiological treatment, pregnancy or presumption of failure in contraception during the treatment period, and location of residence that could interfere with patient participation in the study. Since arterial hypertension is a very frequent comorbidity in patients with chronic Chagas disease in our health center, this factor was not considered as an exclusion criterion. BZ was administered in intermittent doses of 5 mg/kg/day, divided in two daily doses every 5 days, with a total of 12 doses, as previously reported (14). A baseline electrocardiogram (ECG) and a 2-D echocardiogram were performed to stratify the patients according to the presence or absence of cardiomyopathy (Table 1). After treatment, ECG and echocardiogram were performed yearly. Blood samples were taken prior to treatment, one week after the end of treatment, at 12 months post-treatment and yearly thereafter up to 48 months posttreatment follow up. The median time of follow up was 36 months (range 12-48 months) and the median number of samples taken per patient was five (range, two to seven samples). The study was approved by the Committee for Research and Bioethics of the Hospital Eva Peron. The latter is enrolled in the Provincial Registry for Ethics Committees accredited by the Central Ethics Committee, Ministry of Health, Buenos Aires, Argentina, dated 09/17/2010 under number 18/2010, page 54 of the Minutes Book Nº1.

Assessment of qPCR for *T. cruzi.* Five milliliters of whole blood were mixed with an equal volume of 6 M guanidine hydrochloride buffer containing 0.2 M EDTA at pH 8.00 (GEB). After 48-72 hours at room temperature, GEB samples were boiled at 100 °C for 15 minutes and stored at 4 °C until DNA extraction and subsequent analysis by qPCR. GEB samples were centralized, codified for distribution in aliquots and sent to the two laboratories that performed the qPCR assay without knowledge of clinical data or sampling time. Each laboratory analyzed the samples in duplicate by two qPCR methods based on TaqMan technology, one directed to the conservative region of the DNA minicircle kinetoplastid (kDNA) and the other to the nuclear DNA satellite sequence (SatDNA). PCR findings were considered positive if *T cruzi* DNA was detectable in both laboratories by at least one qPCR assay. DNA extraction and both qPCR methods were carried out as previously described (15). The Limit of Quantification was 0.90 par. eq./mL (parasite equivalents per mL of blood) and 1.53 par. eq./mL for the kDNA and SatDNA qPCRs, respectively (15). To minimize bias, blinded control samples from seropositive and seronegative subjects were run in parallel with the study samples.

Measurement of *T. cruzi*-specific antibodies. Serum specimens were screened for the presence of *T. cruzi*-specific antibodies by conventional serological tests (16) and by a Luminex-based assay, as previously described (17). All samples were processed simultaneously by the same technician and with the same reagent lots. To determine the serological treatment response in individual patients, conversion to negative findings in at least 2 of 3 conventional serologic tests, a 30% reduction in ELISA titers and a 2-fold dilution by IHA or IIF were considered as significant declines in *T. cruzi*-specific antibodies, as previously reported (18, 19). Likewise, in the multiplex assay, the reduction of serological response to each individual *T. cruzi* protein was considered significant if the mean fluorescence intensity (MFI) declined by 50% relative to that of a pre-therapy sample assessed concurrently (20).

Cytometric bead array (CBA). CBA assays with sera samples were conducted for IL-8, Interferon γ-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), monokine induced by gamma interferon (MIG) and regulated on activation, normal T cell expressed and secreted (RANTES), according to the manufacturer's instructions (BD Biosciences Franklin Lakes, NJ, USA). The samples were acquired on a FACSCalibur flow cytometer and were analyzed using FACSComp Software v1.4 (BD, Franklin Lakes, NJ, USA).

Statistical Analysis. The normality of the variable distribution was assessed using the Kolmogorov-Smirnov criterion. Data from descriptive statistics, such as the proportions of the total and percentages and median, were determined as appropriate. Qualitative pre- and post-treatment PCR findings were compared by the McNemar test. Differences in chemokine levels between *T. cruzi*-infected and uninfected subjects were evaluated by the Mann-Whitney U-test. Changes in *T. cruzi*-specific antibodies and chemokine levels during post-treatment follow-up were evaluated with an ANOVA for repeated measures with the available data. To analyze changes in the levels of *T. cruzi*-specific antibodies measured by IHA and IIF, the ANOVA for repeated measures was performed after log transformation of the data. Statistical analysis was conducted using the Analytical Software Statistix v8.0 (Analytical Software, Tallahassee, FL, USA) and GraphPad Prism v8.0.1 (GraphPad Software, San Diego, CA, USA).

Results

Clinical characteristics of patients in the study.

We previously published a pilot study to assess the safety and short-term efficacy of a scheme of intermittent administration of BZ in patients chronically infected with *T. cruzi* (14). Seventeen of the 20 patients recruited in our former study were followed for a median period of three years (range, 12-48 months) after BZ administration. Eleven of the 17 (65%) patients recruited had no electrocardiographic or echocardiographic alterations at baseline (Table 1). Ten of the eleven patients without cardiomyopathy remained stable during follow-up, while the remaining patient (i.e., Patient I14 of Table 1) showed basal inferior hypokinesis of the left ventricle at 36 months post-treatment without significant changes in the ECG. Five of the 17 (35%) patients showed mild cardiomyopathy prior to treatment (i.e., two subjects with conduction disturbances [i.e., Patient I8 and I9 of Table 1], one subject with both conduction and rhythm disturbances [i.e., Patient I4] and two subjects with ventricular arrythmia [i.e., Patient I3 and Patient I10]. Of note, Patient I10, who had arterial hypertension as a comorbidity, developed diastolic dysfunction as assessed by echocardiography during follow-up (Table 1). The remaining subject showed repolarization abnormalities of the inferior left ventricle wall (i.e., Patient I20) not related to Chagas disease and presented a mild mitral insufficiency at the end of follow-up.

PCR monitoring.

Fourteen of the 17 (82%) patients showed no detectable *T. cruzi* DNA either by kDNA (Fig. 1A) or SatDNA (Fig. 1B) qPCR methods at the end of follow-up, while three (18%) patients had positive qPCR results (Fig. 1A).The only patient who showed positive qPCR findings at the end of treatment had no detectable results by either qPCR method from 12 months until the end of post-treatment follow up (Fig. 1A and 1B). During post-treatment follow-up, all positive qPCR samples gave parasitic loads below the Limit of Quantification for both qPCR methods. Two of the seven patients with no detectable qPCR findings at baseline had positive results at the end of follow-up, while the remaining five patients showed no detectable qPCR results throughout the post-treatment follow-up. Fourteen out of the 15 subjects followed up until 36 months posttreatment had at least three samples tested during the follow-up period. As a whole, the proportion of subjects with positive kDNA/SatDNA qPCR of the total evaluated significantly decreased over time post-treatment (i.e., t0, 10/17 vs. t2, 1/16, P=0.0014; vs. t12, 0/16, P= 0.0002; vs. t24, 0/10, P<0.0001; vs. t36, 3/15 P=0.025).

***T. cruzi*-specific humoral response following intermittent administration of BZ.**

The levels of *T. cruzi*-specific antibodies measured by conventional techniques significantly declined 12 months following treatment by ELISA (Fig. 2A) and at 24 months post-treatment by IHA (Fig. 2B) and IIF (Fig. 2C). As defined in the Material and Methods, at an individual basis, seven of 16 (43.75%) patients had a decrease in the levels of *T. cruzi*-specific antibodies by ELISA (Fig. 2A), four of 17 (23.53%) by IHA (Fig. 2B) and seven of 17 (42.18%) by IIF (Fig. 2C). The multiplex assay to measure antibodies against a set of 10 *T. cruzi*-derived recombinant proteins was conducted in 14 of the 17 patients treated with intermittent BZ. Twelve of the 14 (85.71%) patients with baseline reactive serum by the Luminex-based multiplex assay showed significant decreases in the reactivity to one or more proteins following intermittent administration of BZ (Fig. 3, Fig. S1).

Monitoring of inflammatory cytokines. Serum levels of IL-8, IP-10, MCP-1, MIG and RANTES were measured prior to and after a median period of 36 months after intermittent administration with benznidazole. Prior to treatment, MCP-1 levels were increased, and MIG levels were decreased in *T. cruzi*-infected subjects compared with uninfected subjects (Fig. 4). These alterations in chemokine levels reverted following drug therapy, with an increase in MCP-1 levels and a decline in MIG levels. No significant changes were observed in serum levels of IL-8, IP-10 or RANTES (P > 0.05).

Discussion

Most pharmacokinetic and pharmacodynamic studies have shown that patients with lower plasma BZ levels can achieve an appropriate parasitological response (21), even among those who cannot complete treatment due to adverse events (22, 23). Herein, we report that the parasitological response in a group of patients treated with BZ administered every five days and followed for a median period of three years was in the same range as that observed with the standard daily dose (4, 24, 25). In a seven-year follow-up study of chronic Chagas disease patients living in an area non-endemic for *T. cruzi* infection treated with BZ as monotherapy, the rate of parasitological response measured by kDNA qPCR was 90% (25) measured one year after treatment. A lower rate of parasitological response was observed in the BENEFIT (Benznidazole Evaluation For Interrupting Trypanosomiasis Trial) clinical trial, in which chronic Chagas disease patients with cardiomyopathy received BZ monotherapy, with 55.4% of patients with negative qPCR findings at two years post-treatment and 46.7% at five or more years (24). In another study assessing the efficacy of three oral E1224 (a water-soluble ravuconazole prodrug) regimens and benznidazole versus placebo in adult chronic indeterminate Chagas disease, the rate of parasitological response with BZ was 82% at 12 months post-treatment by SatDNA qPCR (4).

In our study, one patient presented early therapeutic failure at the end of treatment but achieved no detectable PCR results at later time points. Of the remaining 16 patients, 13 had no detectable parasite DNA throughout the post-treatment follow up period by both qPCR methods, and three showed detectable PCR at the end of follow-up. These findings, and concordant results from other authors showing a high rate of undetectable levels by PCR after one year post-treatment (3, 4, 25), raise the question about the optimal time length required to assess treatment efficacy. It is worth noting that, in addition to implementing a longer follow-up period, we applied two qPCR methods that use two different molecular targets as recommended for confirmatory purposes (26, 27), and the sample analysis was performed in two different laboratories.. We observed 6% discordancy between the two qPCR methods and 6% discordancy between the laboratories. Discordancy occurred between samples in which the parasitic loads were near the limit of detection of the corresponding methods. This can be expected since qPCR precision diminishes at low parasitic loads (15). One limitation of this study is that the patients were not followed during the same time interval, and as consequence the number of follow-up samples was also different among patients.

Recently, non-replicating intracellular *T. cruzi* amastigotes, considered dormant, were demonstrated to be resistant to extended drug treatment *in vivo* and *in vitro* and could re-establish a growing infection after drug exposure (28). Dormancy could be a factor for treatment failure since BZ requires metabolic activation to exert its trypanocidal effect. Therefore, it is important to keep the plasma concentrations of the drug within the accepted therapeutic range and to guarantee that all dormant parasites are eliminated (12, 28). The fact that intermittent BZ administration allowed a five-fold reduction of the standard daily dose of 5 mg/kg/day per 60 days (5) opens the possibility of extending the length of drug administration, eventually targeting dormant parasites which might re-initiate replication (28). It is also likely that the lower frequency of dosing every five days avoids the accumulation of toxic metabolites of BZ, thus reducing the severity of adverse events (14).

The efficacy of intermittent BZ administration was also reflected by the decrease in *T. cruzi*-specific antibodies, either by conventional serologic tests or the multiplex assay, to the same extent as that observed in our previous study with daily BZ doses for 30 days (18). Although 36 months is a short period of time to assess disease progression, it is of note that the two patients who showed echocardiographic changes during follow-up had presented cardiac alterations prior to treatment. Of note, one of these patients had arterial hypertension which is one of the most frequent comorbidities (29, 30) and a risk factor for heart failure (31) in chronic Chagas disease.

Chronic *T. cruzi* infection leads to an inflammatory process that keeps the parasite under control but can also induce tissue damage. Patients with chronic Chagas disease with cardiac involvement show a higher level of pro-inflammatory cytokines compared with patients without cardiac disease (32). Moreover, *T. cruzi*-infected patients with chronic heart disease and arterial hypertension have increased plasma levels of pro-inflammatory cytokines compared with patients without hypertension (33). Chemokines have been identified as regulators of leukocyte trafficking during the different phases of both innate and adaptive immune responses (34). MCP-1, which participates in the recruitment of monocytes, memory T cells and dendritic cells, exerts its effects through binding to G-protein-coupled receptors on the surface of activated leukocytes. The decreased levels of MCP-1 found in untreated *T. cruzi*-infected subjects compared with uninfected controls may reflect an increased consumption of this chemokine during chronic infection (35), while the restoration of its levels following treatment reflects a decrease in leukocyte activation. In agreement with these findings, a decrease in macrophage activation following treatment with BZ has been reported (36, 37). In contrast, serum levels of MIG, which induces migration of activated T cells (38), decreased after the intermittent administration of BZ compared to pretreatment levels. This result is consistent with the early decrease of IFN-γ-producing cells observed in *T. cruzi*-infected subjects treated with the standard BZ scheme that can be followed by a reemergence of polyfunctional IFN-γ-producing cells (19, 20, 39, 40). In summary, the findings of this pilot study provide a basis for further exploration of treatment schemes with intermittent administration of BZ.

References

1. WHO. 2015. Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Wkly Epidemiol Rec 90:33–43.

2. Molina I, Goḿez I Prat J, Salvador F, Treviño B, Sulleiro E, Serre N, Pou D, Roure S, Cabezos J, Valerio L, Blanco-Grau A, Sánchez-Montalv́a A, Vidal X, Pahissa A. 2014. Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. N Engl J Med 370:1899–1908.

3. Morillo CA, Waskin H, Sosa-Estani S, del Carmen Bangher M, Cuneo C, Milesi R, Mallagray M, Apt W, Beloscar J, Gascon J, Molina I, Echeverria LE, Colombo H, Perez-Molina JA, Wyss F, Meeks B, Bonilla LR, Gao P, Wei B, McCarthy M, Yusuf S, Diaz R, Acquatella H, Lazzari J, Roberts R, Traina M, Taylor A, Holadyk-Gris I, Whalen L, Bangher MC, Romero MA, Prado N, Hernández Y, Fernandez M, Riarte A, Scollo K, Lopez-Albizu C, Gutiérrez NC, Berli MA, Cáceres NE, Petrucci JM, Prado A, Zulantay I, Isaza D, Reyes E, Figueroa A, Guzmán Melgar I, Rodríguez E, Aldasoro E, Posada EJ, Serret N, Sánchez-Montalvá A, López-Vélez R, Reyes-López PA. 2017. Benznidazole and Posaconazole in Eliminating Parasites in Asymptomatic *T. Cruzi* Carriers: The STOP-CHAGAS Trial. J Am Coll Cardiol 69:939–947.

4. Torrico F, Gascon J, Ortiz L, Alonso-Vega C, Pinazo MJ, Schijman A, Almeida IC, Alves F, Strub-Wourgaft N, Ribeiro I, Santina G, Blum B, Correia E, Garcia-Bournisen F, Vaillant M, Morales JR, Pinto Rocha JJ, Rojas Delgadillo G, Magne Anzoleaga HR, Mendoza N, Quechover RC, Caballero MYE, Lozano Beltran DF, Zalabar AM, Rojas Panozo L, Palacios Lopez A, Torrico Terceros D, Fernandez Galvez VA, Cardozo L, Cuellar G, Vasco Arenas RN, Gonzales I, Hoyos Delfin CF, Garcia L, Parrado R, de la Barra A, Montano N, Villarroel S, Duffy T, Bisio M, Ramirez JC, Duncanson F, Everson M, Daniels A, Asada M, Cox E,

Wesche D, Diderichsen PM, Marques AF, Izquierdo L, Sender SS, Reverter JC, Morales M, Jimenez W. 2018. Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomised, placebo-controlled trial. Lancet Infect Dis 18:419–430.

5. Scarim CB, Jornada DH, Machado MGM, Ferreira CMR, dos Santos JL, Chung MC. 2019. Thiazole, thio and semicarbazone derivatives against tropical infective diseases: Chagas disease, human African trypanosomiasis (HAT), leishmaniasis, and malaria. Eur J Med Chem 162:378–395.

6. Viotti R, Vigliano C, Lococo B, Alvarez MG, Petti M, Bertocchi G, Armenti A. 2009. Side effects of benznidazole as treatment in chronic Chagas disease: Fears and realities. Expert Rev Anti Infect Ther 7:157–63.

7. Bern C, Montgomery SP, Herwaldt BL, Rassi A, Marin-Neto JA, Dantas RO, Maguire JH, Acquatella H, Morillo C, Kirchhoff L V., Gilman RH, Reyes PA, Salvatella R, Moore AC. 2007. Evaluation and treatment of Chagas disease in the United States: A systematic review. J Am Med Assoc 298:2171–81.

8. Barclay CA, Cerisola JA, Lugones H, Ledesma O, Lopez Silva J MG. 1978. Aspectos farmacológicos y resultados terapéuticos del benznidazol en el tratamiento de la infección chagásica. La Prensa Médica Argentina 65:239–244.

9. Viotti R, Vigliano C, Lococo B, Bertocchi G, Petti M, Alvarez MG, Postan M, Armenti A. 2006. Long-term cardiac outcomes of treating chronic Chagas disease with benznidazole versus no treatment: A nonrandomized trial. Ann Intern Med 144:724–34.

10. De Suasnábar DF, Arias E, Streiger M, Piacenza M, Ingaramo M, Del Barco M, Amicone N. 2000. Evolutive behavior towards cardiomyopathy of treated (nifurtimox or benznidazole) and

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untreated chronic chagasic patients. Rev Inst Med Trop Sao Paulo 42:99–109.

11. Altcheh J, Moscatelli G, Mastrantonio G, Moroni S, Giglio N, Marson ME, Ballering G, Bisio M, Koren G, García-Bournissen F. 2014. Population Pharmacokinetic Study of Benznidazole in Pediatric Chagas Disease Suggests Efficacy despite Lower Plasma Concentrations than in Adults. PLoS Negl Trop Dis 8:e2907.

12. Soy D, Aldasoro E, Guerrero L, Posada E, Serret N, Mejía T, Urbina JA, Gascón J. 2015. Population pharmacokinetics of benznidazole in adult patients with Chagas disease. Antimicrob Agents Chemother 59:3342–9.

13. Bustamante JM, Craft JM, Crowe BD, Ketchie SA, Tarleton RL. 2014. New, combined, and reduced dosing treatment protocols cure *Trypanosoma cruzi* infection in mice. J Infect Dis 209:150–162.

14. Álvarez MG, Hernández Y, Bertocchi G, Fernández M, Lococo B, Ramírez JC, Cura C, Albizu CL, Schijman A, Abril M, Sosa-Estani S, Viotti R. 2016. New scheme of intermittent benznidazole administration in patients chronically infected with *Trypanosoma cruzi*: A pilot short-term follow-up study with adult patients. Antimicrob Agents Chemother 60:833–7.

15. Ramírez JC, Cura CI, Da Cruz Moreira O, Lages-Silva E, Juiz N, Velázquez E, Ramírez JD, Alberti A, Pavia P, Flores-Chávez MD, Muñoz-Calderón A, Pérez-Morales D, Santalla J, Marcos Da Matta Guedes P, Peneau J, Marcet P, Padilla C, Cruz-Robles D, Valencia E, Crisante GE, Greif G, Zulantay I, Costales JA, Alvarez-Martínez M, Martínez NE, Villarroel R, Villarroel S, Sánchez Z, Bisio M, Parrado R, Maria Da Cunha Galvão L, Da Câmara ACJ, Espinoza B, De Noya BA, Puerta C, Riarte A, Diosque P, Sosa-Estani S, Guhl F, Ribeiro I, Aznar C, Britto C, Yadón ZE, Schijman AG. 2015. Analytical validation of quantitative real-time PCR methods for quantification of *Trypanosoma cruzi* DNA in blood samples from

Chagas disease patients. J Mol Diagnostics 17:605–15.

16. TDR/WHO. 2012. Research Priorities for Chagas Disease, Human African Trypanosomiasis and Leishmaniasis.WHO Technical Report Series.

17. Cooley G, Etheridgel RD, Boehlke C, Bundy B, Weatherly DB, Minning T, Haney M, Postan M, Laucella S, Tarleton RL. 2008. High throughput selection of effective serodiagnostics for *Trypanosoma cruzi* infection. PLoS Negl Trop Dis 2:e316.

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

19. Albareda MC, Natale MA, De Rissio AM, Fernandez M, Serjan A, Alvarez MG, Cooley G, Shen H, Viotti R, Bua J, Castro Eiro MD, Nuñez M, Fichera LE, Lococo B, Scollo K, Tarleton RL, Laucella SA. 2018. Distinct treatment outcomes of antiparasitic therapy in *Trypanosoma cruzi*-infected children is associated with early changes in Cytokines, Chemokines, and T-Cell Phenotypes. Front Immunol 9:1958.

20. Laucella S a, Mazliah DP, Bertocchi G, Alvarez MG, Cooley G, Viotti R, Albareda MC, Lococo B, Postan M, Armenti A, Tarleton RL. 2009. Changes in *Trypanosoma cruzi*-specific immune responses after treatment: surrogate markers of treatment efficacy. Clin Infect Dis 49:1675–1684.

21. Fernández ML, Marson ME, Ramirez JC, Mastrantonio G, Schijman AG, Altcheh J, Riarte AR, Bournissen FG. 2016. Pharmacokinetic and pharmacodynamic responses in adult patients with Chagas disease treated with a new formulation of benznidazole. Mem Inst Oswaldo Cruz 111:218–21.

22. Álvarez MG, Vigliano C, Lococo B, Petti M, Bertocchi G, Viotti R. 2012. Seronegative conversion after incomplete benznidazole treatment in chronic Chagas disease. Trans R Soc Trop Med Hyg 106:636–8.

23. Fernández ML, Marson ME, Ramirez JC, Mastrantonio G, Schijman AG, Altcheh J, Riarte AR, Bournissen FG. 2016. Pharmacokinetic and pharmacodynamic responses in adult patients with Chagas disease treated with a new formulation of benznidazole. Mem Inst Oswaldo Cruz 111:218–221.

24. Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A, Rosas F, Villena E, Quiroz R, Bonilla R, Britto C, Guhl F, Velazquez E, Bonilla L, Meeks B, Rao-Melacini P, Pogue J, Mattos A, Lazdins J, Rassi A, Connolly SJ, Yusuf S. 2015. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. N Engl J Med 373:1295–1306.

25. Murcia L, Carrilero B, Ferrer F, Roig M, Franco F, Segovia M. 2016. Success of benznidazole chemotherapy in chronic *Trypanosoma cruzi*-infected patients with a sustained negative PCR result. Eur J Clin Microbiol Infect Dis 35:1819–1827.

26. Schijman AG, Bisio M, Orellana L, Sued M, Duffy T, Mejia Jaramillo AM, Cura C, Auter F, Veron V, Qvarnstrom Y, Deborggraeve S, Hijar G, Zulantay I, Lucero RH, Velazquez E, Tellez T, Leon ZS, Galvão L, Nolder D, Rumi MM, Levi JE, Ramirez JD, Zorrilla P, Flores M, Jercic MI, Crisante G, Añez N, de Castro AM, Gonzalez CI, Viana KA, Yachelini P, Torrico F, Robello C, Diosque P, Chavez OT, Aznar C, Russomando G, Büscher P, Assal A, Guhl F, Estani SS, DaSilva A, Britto C, Luquetti A, Ladzins J. 2011. International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. PLoS Negl Trop Dis 5:e931.

27. Cura CI, Ramírez JC, Rodríguez M, Lopez-Albízu C, Irazu L, Scollo K, Sosa-Estani S. 2017.

Comparative Study and Analytical Verification of PCR Methods for the Diagnosis of Congenital Chagas Disease. J Mol Diagnostics 19:673–681.

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

29. Alves RMDA, Thomaz RP, De Almeida EA, Wanderley JDS, Guariento ME. 2009. Chagas' disease and ageing: The coexistence of other chronic diseases with Chagas' disease in elderly patients. Rev Soc Bras Med Trop 42:622–628.

30. Mordini OD, Bavio E, Beloscar J, Tognoni G, Sosa FJ, Reyes O, Pairone E, Lacunza D, Manzur R, Redondo M, Hernández D, Mujica H, Olavegogeoescoechea P, Antero A, María CA, Susana C, Lucía V, Mariana G, Beatriz JM, Patricia M, Verónica P, Janet G, Doris P, Carina A, Susana M, Belén M, Marcelo O, Georgina P, Emilia A, Lumila L, Osvaldo R, Del Carmen BM, Federico NB, Victorino F, Luis SJ, Martin V, Graciela S, Marcelo S, Héctor C, Luisa G, Jorge M, Karina P, Farez B, Angel AM, Antonio A, Virginia B, Néstor C, Fernando P, Jorge P, María B, Marianela G, Altina E, Pezzotto S. 2016. Chagas disease in Argentina. "National registry of Chagas disease of the 'Federación Argentina de Cardiología'". RENECH study. Rev la Fed Argentina Cardiol 45:84–92.

31. Guariento ME, Ramos MC, Gontijo JA, Carvalhal S dos S. 1993. Chagas disease and primary arterial hypertension. Arq Bras Cardiol 60:71–75.

32. Poveda C, Fresno M, Gironès N, Martins-Filho O a., Ramírez JD, Santi-Rocca J, Marin-Neto J a., Morillo C a., Rosas F, Guhl F. 2014. Cytokine profiling in Chagas disease: Towards understanding the association with infecting *Trypanosoma cruzi* discrete typing units (A benefit trial sub-study). PLoS One 9:1–8.

33. Bestetti RB, Dellalibera-Joviliano R, Lopes GS, Faria-Jr M, Furlan-Daniel R, Lopes KC,

Batista DR. 2019. Determination of the Th1, Th2, Th17, and Treg cytokine profile in patients with chronic Chagas heart disease and systemic arterial hypertension. Heart Vessels 34:123–133.

34. Ridiandries A, Tan JTM, Bursill CA. 2018. The role of chemokines in wound healing. Int J Mol Sci 19:3217.

35. Yadav A, Saini V, Arora S. 2010. MCP-1: Chemoattractant with a role beyond immunity: A review. Clin Chim Acta 411:1570–9.

36. Marañón C, Egui A, Fernández-Villegas A, Carrilero B, Thomas MC, Segovia M, López MC. 2013. Benznidazole treatment reduces the induction of indoleamine 2,3-dioxygenase (IDO) enzymatic activity in Chagas disease symptomatic patients. Parasite Immunol 35:180–7.

37. Perez-Mazliah DE, Alvarez MG, Cooley G, Lococo BE, Bertocchi G, Petti M, Albareda MC, Armenti a. H, Tarleton RL, Laucella S a., Viotti R. 2013. Sequential combined treatment with allopurinol and benznidazole in the chronic phase of *Trypanosoma cruzi* infection: A pilot study. J Antimicrob Chemother 68:424–437.

38. Groom JR, Luster AD. 2011. CXCR3 ligands: Redundant, collaborative and antagonistic functions. Immunol Cell Biol 89:207–15.

39. Alvarez MG, Bertocchi GL, Cooley G, Albareda MC, Viotti R, Perez-Mazliah DE, Lococo B, Castro Eiro M, Laucella SA, Tarleton RL. 2016. Treatment Success in *Trypanosoma cruzi* Infection Is Predicted by Early Changes in Serially Monitored Parasite-Specific T and B Cell Responses. PLoS Negl Trop Dis 10:e0004657.

40. Mateus J, Pérez-Antón E, Lasso P, Egui A, Roa N, Carrilero B, González JM, Thomas MC, Puerta CJ, López MC, Cuéllar A. 2017. Antiparasitic Treatment Induces an Improved CD8 +

T Cell Response in Chronic Chagasic Patients. J Immunol 198:3170–3180.

422 **Tables and Figure Legends**

423 **Table 1. Clinical characteristics of the study population at baseline and following**
424 **intermittent administration of BZ.**

ID	Gender	Age	Basal ECG	ECG at the end of follow-up	Basal Echocardiogram	Echocardiogram at the end of follow-up	Cardiac disease Progression [A]
I2	F	53	Normal	Normal	Normal	Normal	No
I3	F	42	VA	VA	LVSD 60/apical dyskinesis	LVSD 50/apical dyskinesis	No
I4	F	45	LAFB/VA	LAFB/VA	Normal	Normal	No
I6	F	32	Normal	Normal	Normal	Normal	No
I8	M	57	RRBB	RRBB	Normal	Normal	No
I9	M	55	RRBB/LAFB	RRBB/LAFB	Septal hypertrophy, LVH	Septal hypertrophy, LVH	No
I10	F	41	VA	VA	MMI	MMI and diastolic dysfunction	No [B]
I11	F	46	Normal	Normal	Normal	Normal	No [B]
I12	F	49	Normal	Normal	Normal	Normal	No [B]
I13	M	49	Normal	Normal	Normal	Normal	No
I14	M	46	Normal	Normal	Normal	Inferior basal hypokinesis	Yes (basal inferior hypokinesis)
I15	M	28	Normal	Normal	Normal	Normal	No

I16	F	40	Normal	Normal	Normal	Normal	No
I17	M	35	Normal	Normal	Normal	Normal	No
I18	M	45	Normal	Normal	Normal	Normal	No
I19	F	33	Normal	Normal	Normal	Normal	No
I20	M	43	Repolarization abnormalities	Repolarization abnormalities	Normal	MLAD	Yes (not related with Chagas disease)

[A] Disease progression was defined by the development of new electrocardiographic or echocardiographic alterations related to Chagas disease. [B] Patient with arterial hypertension prior to treatment. LAFB, Left Anterior Fascicular Block; LVH, Left Ventricular Hypertrophy; LVSD, Left Ventricular Systolic Diameter; MMI, Mild Mitral Insufficiency; MLAD, Mild Left Auricular Dilatation; RRBB, Complete Right Bundle Branch Block; VA, Ventricular Extrasystoles.

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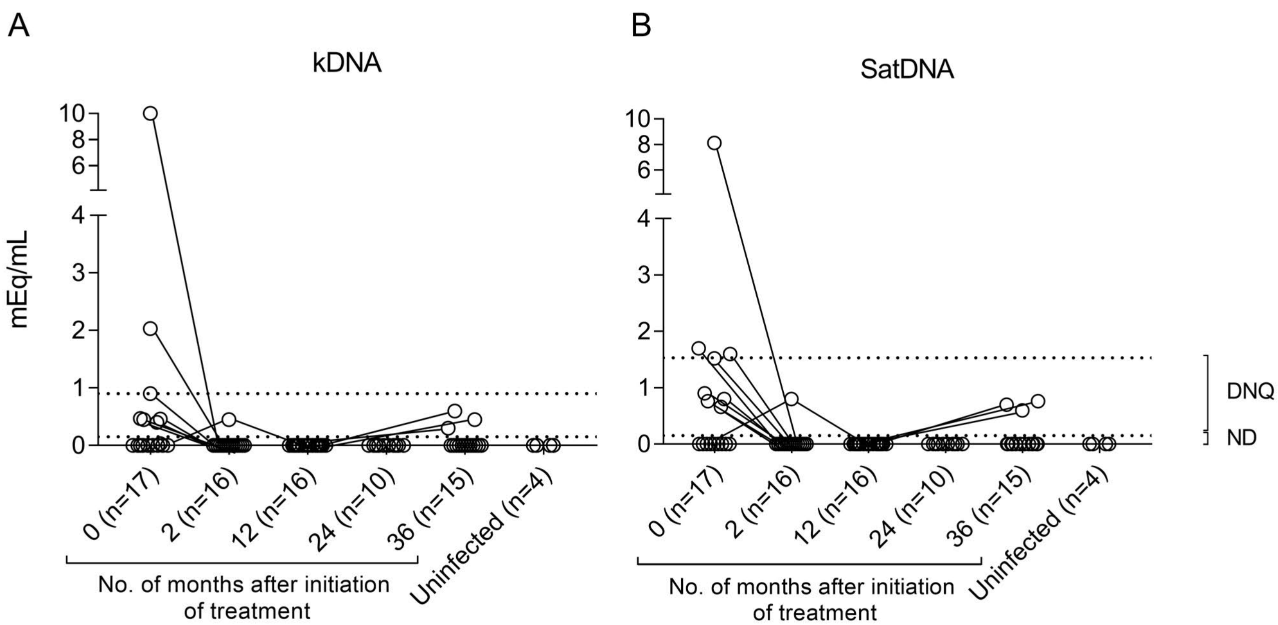
Fig. 1. Monitoring of parasitological response following intermittent administration of BZ. Blood samples collected prior to treatment and at several post-treatment time-points were analyzed by *T. cruzi* kDNA and SatDNA qPCR assays. Blood samples of uninfected subjects were tested as controls. Each circle represents the maximum qPCR value for each patient at each time-point. Dotted lines represent the limit of detection of each qPCR method, 0.90 and 1.53 par. eq./mL for kDNA and SatDNA qPCRs, respectively. ND, no detectable; DNQ, detectable but non-quantifiable.

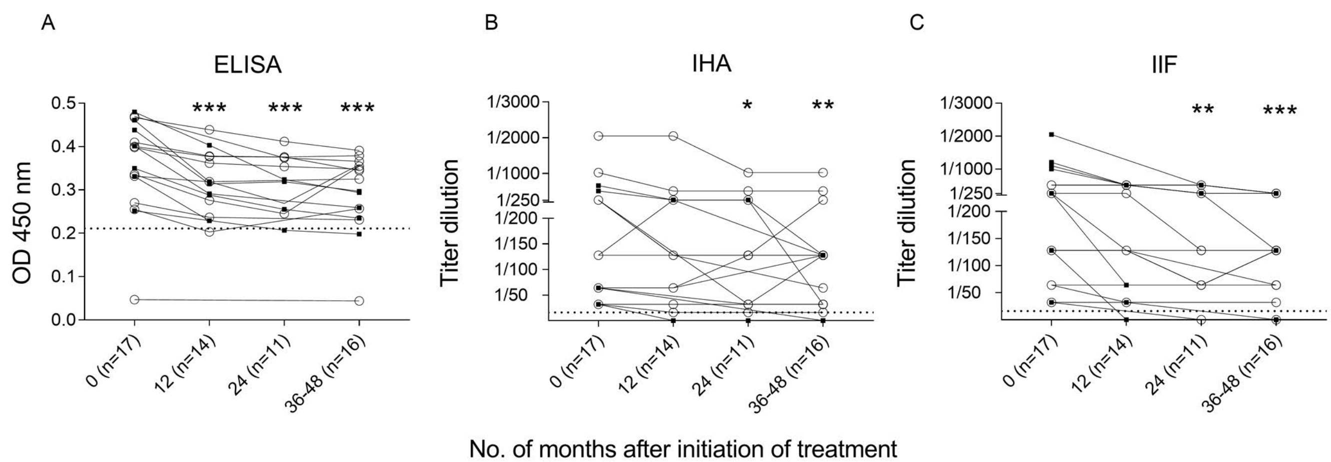
Fig. 2. Monitoring of *T. cruzi*-specific antibodies by conventional serological tests following intermittent treatment with BZ. *T. cruzi*-specific antibodies, as determined by ELISA, IHA and IIF, were measured prior to treatment and at different time-points after completion of BZ administration. Each open circle represents the data for single subjects. Broken horizontal lines show the reactivity threshold for each serological test. ***P< 0.001, **P< 0.01 and * P < 0.05 versus pretreatment levels (time 0) by ANOVA for repeated measures after log transformation of the IHA and IIF data. Black square symbols indicate decreased reactivity compared with baseline reactivity, as defined in the Materials and Methods.

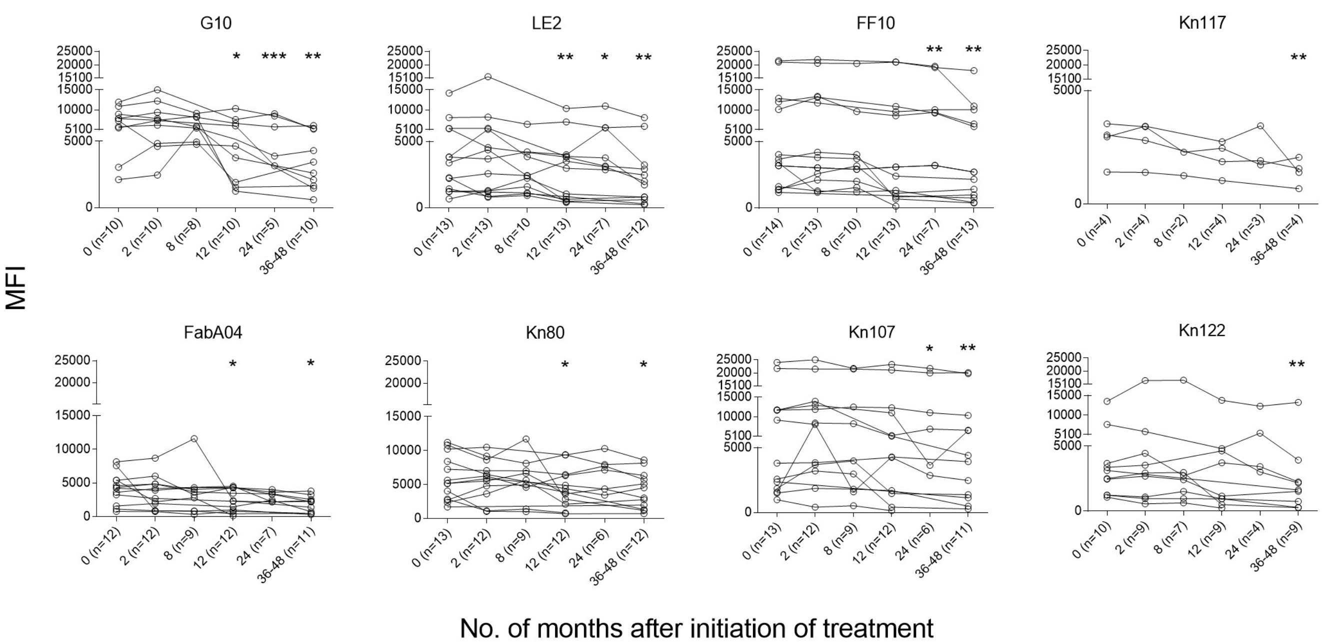
Fig. 3. *T. cruzi*-specific humoral response measured by multiplex assay in chronic Chagas disease patients after intermittent administration of BZ. Plots exhibit representative data for single subjects for the different proteins assessed. Each point represents the mean fluorescence intensity (MFI) for reactive proteins out of 10 assessed, analyzed both prior to treatment (time 0) and at several post-treatment time-points. *** P < 0.001, ** P < 0.01 and * P < 0.05 versus pretreatment levels (time 0) by ANOVA for repeated measures.

Fig. 4. Sera levels of MCP-1 and MIG in chronic Chagas disease patients treated with intermittent doses of BZ. A cytometric bead array was used to measure the concentrations of chemokines in the sera of subjects with chronic *T. cruzi* infection (circles) at different time points

following intermittent administration of BZ and in uninfected subjects (triangles). Changes from baseline (time 0) were assessed using ANOVA for repeated measures. ** and $^{\#\#}$ P < 0.01 versus baseline (time 0); * P < 0.05 versus baseline. The horizontal line in uninfected subjects shows the median values.


A
kDNA
10
8
6
4
3
2
1
0
mEq/mL
0 (n=17)
2 (n=16)
12 (n=16)
24 (n=10)
36 (n=15)
Uninfected (n=4)
No. of months after initiation of treatment
B
SatDNA
10
8
6
4
3
2
1
0
0 (n=17)
2 (n=16)
12 (n=16)
24 (n=10)
36 (n=15)
Uninfected (n=4)
No. of months after initiation of treatment
DNQ
ND


A
ELISA
OD 450 nm
0.0
0.1
0.2
0.3
0.4
0.5
***
***
***
0 (n=17)
12 (n=14)
24 (n=11)
36-48 (n=16)
B
IHA
Titer dilution
1/50
1/100
1/150
1/200
1/250
1/1000
1/2000
1/3000
*
**
0 (n=17)
12 (n=14)
24 (n=11)
36-48 (n=16)
C
IIF
Titer dilution
1/50
1/100
1/150
1/200
1/250
1/1000
1/2000
1/3000
**
***
0 (n=17)
12 (n=14)
24 (n=11)
36-48 (n=16)
No. of months after initiation of treatment


G10
LE2
FF10
Kn117
FabA04
Kn80
Kn107
Kn122
MFI
No. of months after initiation of treatment

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