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A Follow-Up Study of Chagasic Patients with Special Reference to *Trypanosoma cruzi* Persistence and Criteria of Chagas Disease Cure

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Abstract
A long-term follow-up study was carried out in acute chagasic patients evaluated from 1 to 23 year after receiving benznidazole treatment. Evaluation was performed combining clinical, serological, parasitological, molecular (Polymerase Chain Reaction, PCR) and immuno-histopathological methodologies in order to detect changes in the progression of the infection and to estimate the efficacy of the drug effect with the intention of establishing a criterion of cure for Chagas disease. Systematic observations at different time post primary infection revealed the degree of negative conversion recorded by each of the used tests and the tissular persistence of *T. cruzi* in 40% of the study patients. At the endpoint evaluation, four groups of individuals at different clinical conditions were recognized from the initially selected acute patients. These included typical chronic chagasic patients (35%), individuals with subclinical or inapparent infection (35%), symptomatic seronegative patients showing persistence of *T. cruzi*-tissue form (8%) and clinically cured patients (22%). Therefore, the here reached criterion of Chagas disease cure after a long-term evaluation with reliable methods involve those asymptomatic patients showing consistently negative sero-parasitological and molecular results for circulating parasites, as well as non-signal of *T. cruzi* tissue-form persistence. Statistical comparison between benznidazole-treated and untreated patients revealed no significant differences (P>0.05), neither in the recognized groups nor in the used methods. These results led us to conclude that therapeutic effect of benznidazole was unsatisfactory due to fails to eliminate *T.cruzi*-tissue form, which persisted for long periods in primary infected patients, raising doubts on its use as the drug of election for Chagas disease specific therapy.

1. Introduction

Chemotherapy against Chagas disease, the human manifestation of *Trypanosoma cruzi* infection, with nitroderivates drugs including benznidazole and/or nifurtimox may lead to

regression of clinical symptoms, thus preventing fatal outcomes during the acute phase as consequence of reduction in the parasite population circulating in the peripheral blood. The ability of the drug to keep the disease in check improving clinical condition of patients has been interpreted as parasitological cure (1, 2). However, although treatment focuses on killing the parasite, it is unable to eliminate the entire population mainly the amastigote tissue forms. In addition, drug effectiveness during the chronic phase of the disease remains unclear, and in some author's opinions, although treatment with these compounds may reduce risk for progression of Chagas heart disease, does not lead to the parasitological cure of *T.cruzi* infection (3, 4). Despite this, currently, there is general agreement in considering clinical, parasitological, molecular and serological characteristics as essential criteria to evaluate drugs used for treating Chagas disease (4, 5). However, limitations such as the long-term follow-up and the lack of reliable tests to ensure elimination of the parasite appear to conspire against these purposes (3).The clinical criterion itself for evaluation of nitroderivates drugs effect on Chagas disease has been considered of limited value, both in the acute and chronic phases of the infection: on one hand, due to the short time of abnormalities detected with paraclinical methods (or because many patients are symptomless during the course of infection) and, on the other hand, due to problems derived from the design and implementation of long-term follow-up studies of this long-lasting and slowly evolving disease (3, 5). Consequently, criterion of cure must be considered as the demonstration of the total elimination of *T.cruzi* in post-treated patient (including both tissue and circulating blood forms), showing consistently negative results when parasitological, molecular (PCR) and serological tests are reproducibly performed (4, 5, 6, 7, 8). Nonetheless, there is no consensus among authors regarding the criterion of cure in Chagas disease, which has stirred up controversy about the usefulness of the etiological treatment, transforming this issue in an interesting open field for investigation.

One more aspect to be taken into consideration during a Chagas disease follow-up study is the persistence of *T.cruzi* in tissues other than the heart, a fact relatively frequent that has been demonstrated by PCR assays in post-treated patients (9, 10). This finding is troublesome because it adds a new variable to the analysis on drug effects in chagasic patients, making more difficult to reach an agreement concerning the criteria of Chagas disease cure. However, considering that *T.cruzi* is a paninfective parasite able to invade any tissue of the mammal host, demonstration of parasite persistence related to the therapeutic protocols used for its control should be a matter of concern.

The present article deals with a longitudinal follow-up study on chagasic patients with 1 to 23 years post-acute infection. The study included clinical, sero-parasitological and molecular methods aimed at comparing the outcome observed in chronic patients at different times post primary infection, with the baseline characteristics showed during the acute phase. In addition, detection of *T.cruzi* persistence at oral tissue was recorded, combining immunohistopathological and molecular (PCR) techniques, trying to assess the effect of benznidazole on the parasite throughout the infection. All these observations were carried out hoping to shed some light on the controversial criterion of Chagas disease cure.

2. Patients and Methods

2.1. Patients Baseline Characteristics at the Acute Chagasic Infection

The here included chagasic patients had been previously diagnosed when suffering acute *T.cruzi*-infection between 1989 and 2011. Some of these patients were part of previous studies carried out in diverse localities from Barinas state of western Venezuela where Chagas disease is endemic (2, 9, 11).The 60 selected patients (30♀ and 30♂) showed, for the time they suffered the primary acute chagasic infection, an average age of 20±14 years old (range: 1-57 years old). The female group with 17±11 years old (range: 1-52 years old) and males being 23±15 years old (range: 3-57 years old).

The parasitological diagnosis confirmed by direct and indirect tests (blood smear or direct examination, xenodiagnosis or hemoculture) detected 49 of 57 (86%) patients with blood circulating parasites (BCP); in 14% (8/57 of patients) no parasites were seen (NPS).

The serological diagnosis showed 96% (50/52) seropositive to circulating *T.cruzi* antibodies when 3 serological methods, including direct agglutination test (DAT), indirect immunofluorescence antibody test (IFAT) and an enzyme-linked immunosorbent assay (ELISA), were used. In addition, all the seropositive acute patients showed antibody titers ≥ 512 of anti-*T.cruzi* IgM when tested by IFAT following procedures previously described (11). Conditions and procedures for serological and parasitological methods have been reported elsewhere (2, 9).

Clinical examination revealed 55 (92%) symptomatic and 5 (8%) asymptomatic patients. From the former, 22 (40%) showed severe symptoms and in the other 33 (60%) mild symptomatology was detected. In addition, 38 (63%) patients showed Romaña´s sign, in 4 of them (7%) chagoma was detected and 18 (30%) showed no signs at all. Cardiological examination showed 42% of patients with both abnormal electrocardiogram (EKG) and echocardiogram (ECO) being the pericardial effusion the major abnormality detected during the echocardiographic study, which was present in 12 of 23 infected individuals (52%). The baseline characteristics observed in the selected patients during the primary acute chagasic infection are detailed in Table 1.

The treatment procedures carried out in 52 of the acute initially diagnosed patients included oral administered benznidazole, receiving the adults a maximum dosage of 5 mg/kg/day and children 10 mg/kg/day during 60 consecutive days. The 5 asymptomatic patients (Nº: 8, 14, 45, 46, 57), and 3 more patients showing mild symptoms (Nº: 18, 20, 37), did

not receive treatment and were considered as the control group for statistical purposes.

Table 1. *Baseline characteristics of the study patients at the initial acute chagasic infection.*

SERO-PARASITOLOGICAL, CLINICAL AND PARACLINICAL DIAGNOSIS															
Age group (years)	Patients Nº / %	Gender		Parasit + ve	Serol. + ve	Symptoms			Clinical signs			EKG		ECO	
		Fem Nº%	Male Nº%	Nº/ %	Nº/%	Severe Nº / %	Mild Nº / %	Asymp Nº / %	RS Nº/%	Ch Nº/%	NS Nº/%	Ab Nº/%	N Nº/%	Ab Nº/%	N Nº/%
1-10	18 / 30	11 / 37	7 / 23	16 / 28	14 / 27	5 / 23	12 / 36	1 / 20	9 / 24	1/25	8/44	5 / 21	11/33	5/22	10/31
11-20	17 / 28	7 / 25	10 / 33	13 / 23	14 / 27	5 / 23	10 / 30	2 /40	13/34	0/0	4/22	7 /29	9/27	5/ 22	10/31
21-30	15 / 25	9 / 30	6 / 20	11 / 19	15 / 28	7/32	6 /18	2 /40	10/26	2/50	3/17	8 /33	7/21	8/ 35	7/22
31-40	4 / 7	2 / 7	2 / 7	4 / 7	3 / 6	3/14	1 /3	0 /0	4 /11	0/0	0/0	1 /4	3/9	1/ 4	3/9
41-50	2 / 3	0 / 0	2 / 7	2 / 4	1 / 2	1/4	1 /3	0 /0	0 /0	1/25	1/6	0 /0	2/6	1/ 4	1/3
51-60	4 / 7	1 / 3	3 / 10	3 / 5	3 / 6	1/4	3 /9	0 /0	2 /5	0/0	2/11	3 /13	1/3	3/ 13	1/3
Total	60 / 100	30 /50	30 / 50	49 / 86	50 / 96	22/37	33 / 55	5 / 8	38/63	4/ 7	18/30	24/42	33/58	23/42	32/58

Parasit. +ve: Detected BCP; Serol. +ve: Seropositive; Asymp. Asymptomatic; RS: Romaña´s sign; Ch: Chagoma; NS: No sign; EKG: Electrocardiogram; ECO: Echocardiogram; Ab: Abnormal; N: Normal.

2.2. Follow-up Protocol, and Sample Collection and Processing

All the selected patients who were diagnosed during the Chagas disease acute phase were regularly re-evaluated every 6 to 12 months at the out-patient cardiologic unit of the "Luis Razetti" General Hospital in Barinas, Venezuela. The clinical evaluation consisted of detecting symptoms attributable to Chagas disease or its sequels, following cardiac examination for detection of electrocardiographic and echocardiographic abnormalities. In addition, during the evaluation, including the endpoint sampling, a 10 ml blood sample was taken to be processed for hemoculture in NNN culture medium (3ml distributed into 3 tubes), part of the blood sample for PCR assays and the remaining amount centrifuged for serology, which included assessment for anti-*T.cruzi* antibodies (DAT, IFAT, ELISA) and specific anti-*T.cruzi* IgM and IgG using IFAT. Conditions and procedures for sero-parasitological and molecular techniques were performed as indicated above.

2.3. Detection of *Trypanosoma cruzi* Persistence at Oral Tissue

During the endpoint evaluation each patient was clinically examined at the dentistry unit of the above mentioned General Hospital. The condition of the oral cavity and its classification according to the degree of gingival inflammation was established by a dentist. Once confirmed the degree of inflammation, a small gingival biopsy ($3mm^3$) was taken and divided into two parts, one frozen to be processed for PCR assay and the other part preserved in 10% formaldehyde until used for immunohistopathological studies. To detect the presence of *T.cruzi*-DNA in the sample of inflamed gum a specific PCR was carried out using primers S35 (5´-AAA TAATGTACGGGT GAG ATG CAT GA-3´) and S36 (5´-GGGTTC GAT TGGGGTTGGTGT-3´) following protocols previously established (12). The immunohistopathological evaluation was carried out by Giemsa staining (GS) of histological sections looking for tissue parasites, and by two additional immunohistochemical methods including an indirect immunofluorescence test (IIFT) and the peroxidase anti-peroxidase (PAP) technique to specifically identify *T.cruzi* antigenic deposits in tissue. Details for these protocols have been previously reported (9, 10).

2.4. Diagnostic Criteria

The criteria taken into consideration to establish the clinical, parasitological, serological and molecular status of the here included chagasic patients, both in acute and chronic phase, have been previously reported (2, 9, 10, 11, 13). In addition, criteria of electrocardiographic and/or echocardiographic abnormalities were also considered. These included left and/or right bundle-branch block, atrial fibrillation, ventricular premature contractions and sinus bradycardia (<50 beats/min) for EKG, while for ECO left ventricular ejection fraction (LVEF) < 50%, increase of left ventricular diastolic (>55mm) and systolic (>35mm) diameter, aneurysm and pericardial effusion were the considered variables analyzed.

In relation to *T.cruzi* persistence at oral tissue of chronic chagasic patients (CCP), observation of signals including the presence of the parasite itself, positive reaction for antigenic deposits and/or detection of part of its genome, when GS, PAP-IIFT and PCR assay were respectively used, were considered criterion enough for parasite persistence at gingival inflammation foci.

2.5. Ethical Considerations

This study was approved by the ethical committee of the "Luis Razetti" General Hospital, Ministry of Health, Barinas, Venezuela and by the Research Council of University of Los Andes, Merida, Venezuela. A written consent was obtained from each of the patients, and their representative in case of children, in order to comply with the criteria established by the Biomedical Committee of the National Research Council of Venezuela.

2.6. Statistical Analysis

Statistical significance among baseline characteristics detected in acute chagasic patients and those observed in CCP during the long-term follow-up, including comparison between benznidazole-treated and non treated patients for each study parameter, was estimated using an X^2 contingency table and the adjustment with Yates' correction accordingly (14).

3. Results

3.1. Clinical, Sero-Parasitological and Molecular Follow-up in Chronic Chagasic Patients

A longitudinal follow-up of 60 unquestionable chronic chagasic patients (CCP), with 1 to 23 years post primary acute infection (PPI), was performed to describe the spectrum of Chagas disease related to time. To accomplish this task, clinical, sero-parasitological and molecular re-evaluations were carried out in order to compare the baseline characteristics showed by the time they were diagnosed with acute Chagas disease (1989 – 2011) with those detected in CCP at the endpoint sampling performed in 2012. At the time of the endpoint evaluation, the current age average was 35±14 years old (range: 8-73 years old) and the average follow-up time was 15±5 years (range 1-23 years).

The parasitemia, followed up by multiple hemoculture, revealed the presence of *T.cruzi* blood circulating trypomastigotes in only 2 (3%) patients with 12 and 14 year PPI. In the remaining 58 CCP (97%) no parasites were seen at any time, including samples of patients with 1 to 11 and 15 to 23 years PPI. Statistical comparison of parasitemia values between acute patients (Table1) and that detected during the follow-up in CCP (Table 2) revealed a highly significant difference among patients before and after treatment with benznidazole (X^2=71.6; P<0.001). The overall serological follow-up, revealed a decreasing number of seropositive patients during the study period, from 96% detected in the baseline acute cases to 70% (42/60) at the endpoint sample collection of CCP. In all cases seropositives fulfilled the established criteria for serological diagnosis (2 positive out of 3 tests) and maintained specific anti-*T.cruzi* IgG titers when IFAT was done. Similar to what occurred with parasitemia, an increase of seronegative patients (30%) was recorded by the time of the endpoint examination, which also resulted statiscally significant when compared with the baseline serological data (P<0.001). Analyzing the possible effect of treatment on sero-conversion, the results revealed that 16 out of 52 patients (31%) treated with benznidazole during the acute infection became sero-negative at different times PPI, being the major conversion recorded between 15 to 21 years and the maximum proportion (25%) detected at year 16 PPI. In addition, it is interesting to note that 2 of the 8 (25%) patients who did not receive benznidazole treatment became seronegative to anti-*T.cruzi* antibodies at 16 and 21 years PPI. Details on the serological results obtained each year of the follow-up study are shown in Table 2.

Table 2. *Clinical, sero-parasitological and molecular follow-up of chagasic patients.*

Year of acute infection	Time PPI (year)	Patients Nº (%)	Hemoculture		Serology		Blood PCR		Clinical symptoms		Paraclinical examination			
											EKG		ECO	
			+ve HC	-ve HC	+ve	-ve	+ve	-ve	Sympt	Asympt	Normal	Abnormal	Normal	Abnormal
2011	1	1 (2)	0	1	1	0	0	1	1	0	0	1	1	0
2010	2	3 (5)	0	3	2	1	1	2	1	2	2	1	2	1
2008	4	1 (2)	0	1	1	0	0	1	0	1	1	0	1	0
2007	5	2 (3)	0	2	2	0	1	1	1	1	1	1	1	1
2005	7	1 (2)	0	1	0	1	0	1	0	1	1	0	1	0
2004	8	1 (2)	0	1	1	0	0	1	1	0	0	1	0	1
2002	10	1 (2)	0	1	1	0	1	0	0	1	1	0	1	0
2001	11	2 (3)	0	2	0	2	0	2	0	2	2	0	2	0
2000	12	2 (3)	1	1	2	0	0	2	0	2	2	0	2	0
1998	14	7(12)	1	6	6	1	0	7	4	3	5	2	4	3
1997	15	3 (5)	0	3	2	1	0	3	1	2	3	0	2	1
1996	16	12 (20)	0	12	6	6	2	10	6	6	6	6	8	4
1995	17	2 (3)	0	2	1	1	0	2	1	1	1	1	1	1
1994	18	6 (10)	0	6	4	2	0	6	2	4	4	2	5	1
1992	20	3 (5)	0	3	3	0	2	1	0	3	3	0	3	0
1991	21	5 (8)	0	5	3	2	1	4	3	2	4	1	3	2
1990	22	3 (5)	0	3	3	0	1	2	1	2	2	1	2	1
1989	23	5 (8)	0	5	4	1	2	3	4	1	3	2	2	3
Total Nº		60	2	58	42	18	11	49	26	34	41	19	41	19
(%)		(100)	(3)	(97)	(70)	30	(18)	82	(43)	(57)	(68)	(32)	(68)	(32)

PPI: Time post primary acute infection; HC: Hemoculture; Sympt: Symptomatic; Asympt: Asymptomatic

The PCR assay detected 11(18%) patients positive to *T.cruzi*-DNA in blood samples, as confirmed by the amplification of a band of 330bp when the S35/S36 primers were used. The group of PCR-positive had a course of infection from the acute phase between 2 and 23 years. Among the 11 PCR-positive patients, 3 of them (27%) did not receive treatment during the acute phase, which occurred 5, 16 and 20 years prior to the endpoint sample collection. The remaining 8 (73%) PCR-positive patients, that received complete benznidazole treatment when they suffered the acute *T.cruzi*-infection during the period 1989-2011, showed a regular distribution in time.

Regarding the clinical symptoms detected in chagasic patients before (during acute phase) and after (CCP-follow-up) receiving benznidazole treatment, statistical comparison revealed significant differences (X^2= 40.2; P<0.001). However, comparison of the observed results on cardiological examination, including EKG and ECO in both groups of

treated and non-treated patients, revealed no significant differences (P>0.05). During the clinical evaluation of the 60 CCP with 1 to 23 years PPI, 34 (57%) asymptomatic were detected, resulting in two different groups: one group consisted of 13 (38%) patients seronegative to anti- *T.cruzi* antibodies showing, in addition, normal EKG and ECO, and negative results in both HC for BCP and blood PCR assay. In the other group of 21 (62%) asymptomatic patients, persistence of anti-*T.cruzi* antibodies (seropositive) was observed. All these patients also showed normal EKG and ECO when cardiac evaluation was carried out. However, in two of them (Nº 29 and 45) BCP were detected in the observed HC, and in 7 more positive PCR in blood were recorded. On the other hand, from the 26 detected symptomatic individuals 5 (19%) were seronegative and 21 (81%) seropositive for anti-*T.cruzi* antibodies. In the group of seronegatives all the patients presented abnormal EKG, while 3 abnormal and 2 normal ECO were confirmed at the cardiologic examination. Meanwhile, in the seropositives group, 7 and 5 individuals with normal and 14 and 16 with abnormal EKG and ECO were detected, respectively. Details on the variables considered during the follow-up study, and the relation serology-clinical findings, are presented in Table 2. From the 60 study CCP, one sudden death occurred 18 months after the endpoint evaluation was performed. By the time of the last examination the patient was 53 year old with 16 year PPI, maintaining anti-*T.cruzi* IgG titers (1:256) and presenting a symptomatology with abnormalities in both the EKG and ECO. In addition, one of the 52 patients receiving benznidazole treatment (Nº 6) showed a strong reaction to the drug similar to a pleomorphic rash or multiform erytheme, which judging by the presence of blisters and skin pain seems to be a moderate Stevens-Johnson syndrome-like, which disappeared shortly after treatment was suspended.

3.2. *Trypanosoma cruzi* Persistence at Oral Tissue of Chronic Chagasic Patients

Clinical evaluation carried out at the oral cavity of the study patient revealed mild, moderate and severe gingival inflammation in 57%, 37% and 6% of the individuals, respectively. In all patients, a degree of inflammation was detected at any time PPI. Observations of biopsies taken from gingival inflammatory foci processed by Giemsa staining (GS) showed 20% positive results, detecting disperse scanty amount of *T.cruzi*-amastigotes mainly located on gingival fiber (Fig.1A). When immunohistochemical techniques were used, antigenic deposits were detected using both IIFT and PAP in 23% and 28% of patients, respectively (Fig.1B-C). In addition, the use of the PCR assay allowed detecting in 12 of the 60 patients (20%) the presence of the expected specific bands matching with *T.cruzi*-DNA (330bp). The results obtained with different histological, immunohistochemical and molecular (PCR) methods satisfied the established criteria to consider positive the detection of *T.cruzi* persistence at the oral tissue of CCP at different times PPI. Taking together the above results, 24 of 60 (40%) patients showed signals indicating parasite persistence. Different signals detected with the immunohistochemical techniques allowed us to demonstrate parasite's persistence in patients as early as 1 year until 23 year PPI, which may suggest the long lasting capacity of the parasite's survival in the human host oral tissues (Table 3). In relation to coincidence among two or more tests indicating *T.cruzi* persistence in oral tissues of CCP the following frequency was observed: IIFT-PAP (18%); GS-PAP (12%); GS-IIFT (10%); GS-PCR (8%); PAP-PCR (12%) and IIFT-PCR (7%). These results support the above statement on the demonstration of *T.cruzi* persistence and its capacity for lasting for long periods at the oral tissues of CCP.

Table 3. *Detection of Trypanosoma cruzi persistence at oral tissue of chagasic patients at different time post primary acute infection.*

Time PPI (years)	Nº(%) Patients	Nº Patients with degree of gingival inflammation			Immunohistochemical and molecular findings								Persistence signal	
					GIEMSA STAIN		PAP		IIFT		PCR			
		Mild	Moderate	Severe	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
1	1 (2)	1	0	0		*	0	1	0	1	1	0	1	0
2	3(5)	1	2	0	1	2	1	2	0	3	1	2	1	2
4	1(2)	1	0	0	0	1	0	1	0	1	0	1	0	1
5	2(3)	0	2	0	1	0*	1	1	0	2	1	1	1	1
7	1(2)	1	0	0	0	1	0	1	0	1	0	1	0	1
8	1(2)	0	1	0	0	1	1	0	1	0	0	1	1	0
10	1(2)	0	1	0	0	1	1	0	1	0	1	0	1	0
11	2(3)	2	0	0	0	2	0	2	0	2	0	2	0	2
12	2(3)	2	0	0	0	2	1	1	0	2	1	1	1	1
14	7(12)	6	1	0	2	4*	2	5	3	4	0	7	3	4
15	3(5)	1	1	1	0	3	0	3	0	3	0	3	0	3
16	12(20)	8	3	1	2	9*	4	8	3	9	4	8	6	6
17	2(3)	1	0	1	2	0	2	0	1	1	2	0	2	0
18	6(10)	2	3	1	1	4*	1	5	2	4	0	6	2	4
20	3(5)	3	0	0	1	2	2	1	1	2	0	3	2	1
21	5(8)	1	4	0	0	5	0	5	0	5	0	5	0	5
22	3(5)	1	2	0	0	3	1	2	2	1	1	2	2	1
23	5(8)	3	2	0	1	3*	0	5	0	5	0	5	1	4
Total Nº	60	34	22	4	11	43	17	43	14	46	12	48	24	36
%	100	57	37	6	20	80	28	72	23	77	20	80	40	60

PAP: Peroxidase-anti-peroxidase; IIFT: Indirect immuno-fluorescent test;
*: 1 of the samples not done

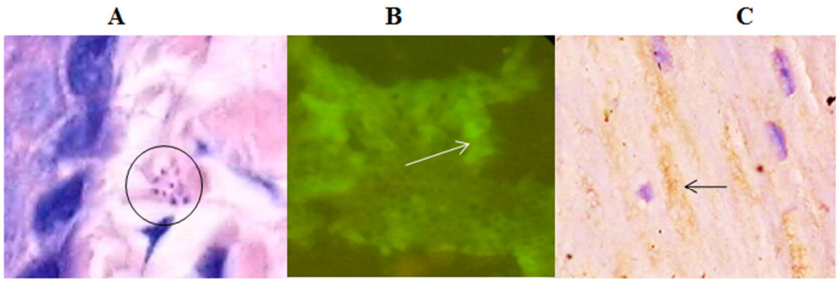

Fig. 1. *Detection of Trypanosoma cruzi tissular form and persistent antigenic deposits at oral samples from chronic chagasic patients at different times post primary infection. A. Giemsa stain showing tissular amastigotes at gingival fiber (Circled). B. Antigenic deposits detected by indirect immunofluorescence test (Arrowed). C. Antigenic deposits in gingival section detected by peroxidase anti-peroxidase technique (Arrowed). (1000X).*

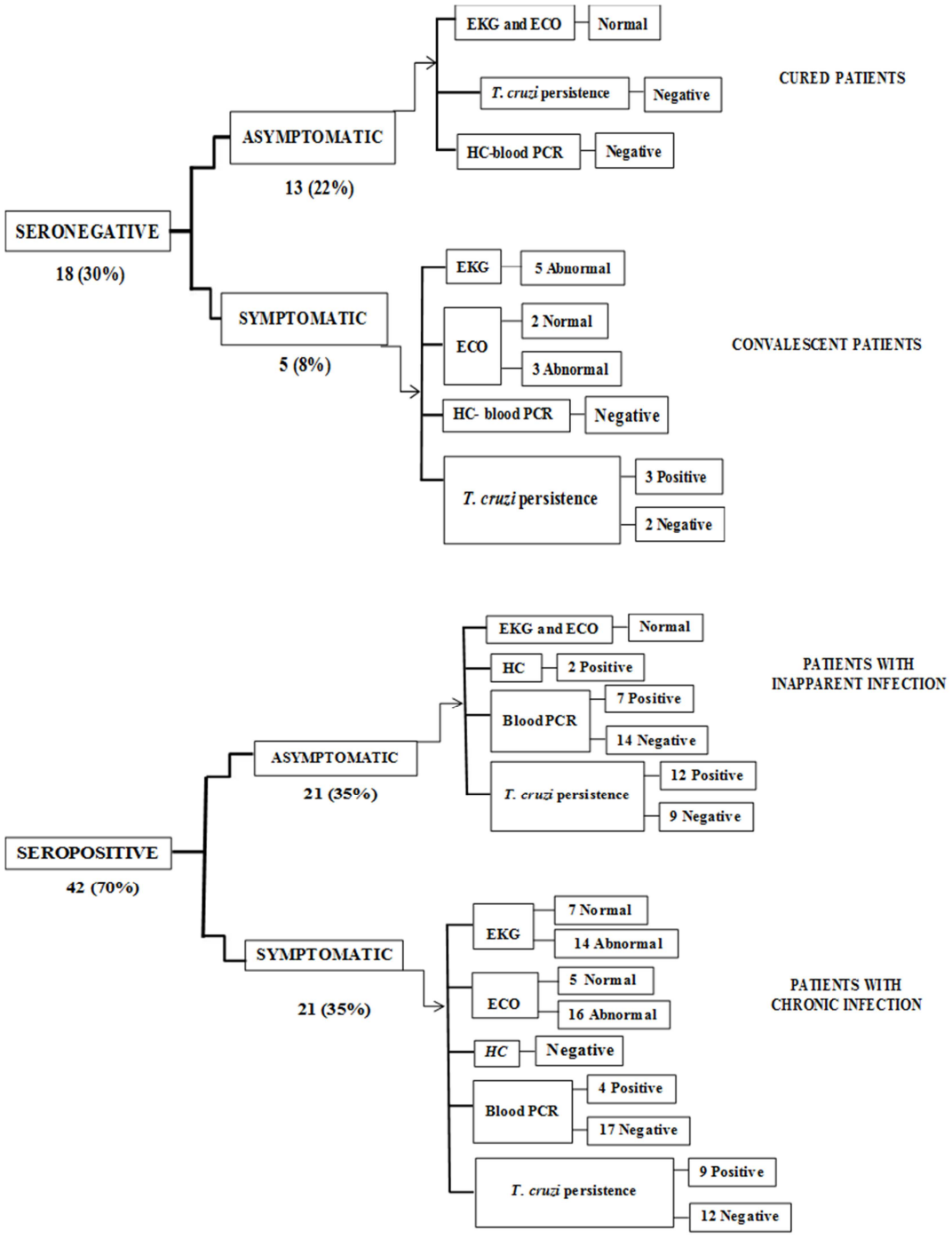

Fig. 2. *Classification of clinical condition at the endpoint of a follow-up study on chronic chagasic patients treated with benznidazole.*

3.3. Criteria of Cure in Chagasic Patients

Taken together all the above results of clinical, sero-parasitological and molecular (PCR) evaluation and detection of *T.cruzi*-persistence in a long-term follow-up, four different groups in the studied cohort of 60 CCP were identified. This includes a first group made up of 21 patients (35%) that were seropositive, symptomatic, with electrocardiographic and echocardiographic abnormalities, absence of BCP but with blood PCR positive and *T.cruzi* persistence at oral tissue, demonstrated by different techniques. This group with these characteristics is proposed to be recognized as the typical chronic chagasic patient. A second group was also made up of 21 *T.cruzi*-seropositive patients, although they were asymptomatic, with normal EKG and ECO, showed parasite persistence including BCP detected by HC, positive blood PCR and tissue parasites, which was identified as patients with inapparent, occult or subclinical infection. The third group, identified as convalescent patients, included those *T.cruzi*-seronegative symptomatic patients (N=5), which showed abnormal EKG and ECO and *T.cruzi*-persistence in tissue. Finally, the fourth group consisted of 13 (22%) *T.cruzi*-seronegative asymptomatic patients, characterized by showing normal EKG and ECO and negative *T.cruzi*-persistence with all parameters and techniques considered here, and it was recognized as the group of cured patients. Details on the elements considered as criteria of cure in chagasic patients, including its quantification are summarized in Table 4. In addition, the proposed division using the considered criteria and the identification of each patient are shown in Fig. 2 and 3, respectively.

Table 4. *Summary results of clinical, sero-parasitological and molecular (PCR) evaluation and detection of T.cruzi-persistence in a long-term follow-up of chagasic patients.*

		OBSERVED RESULTS IN: N°/ %						CLINICAL CONDITION						*Trypanosoma cruzi* PERSISTENCE			
Clinical	**Pat.**	**HC**		**SEROLOGY**		**Blood PCR**		**SYMPTOMS**		**EKG**		**ECO**		**AT ORAL TISSUE**			
Criterion*	**N° (%)**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**Sympt.**	**Asympt.**	**Abn.**	**Normal**	**Abn.**	**Normal**	**G.S.**	**PAP**	**IIFT**	**PCR**
Cured	13(22)	0	13	0	13	0	13	0	13	0	13	0	13	0	0	0	0
Convalescent	5 (8)	0	5	0	5	0	5	5	0	5	0	3	2	3	3	2	3
Inapparent	21 (35)	2	19	21	0	7	14	0	21	0	21	0	21	6	10	7	6
Chronic	21 (35)	0	21	21	0	4	17	21	0	14	7	16	5	2	4	5	3
Total	60	2	58	42	18	11	49	26	34	19	41	19	41	11	17	14	12
%		3	97	70	30	18	82	43	57	32	68	32	68	18	28	23	20

HC: Hemoculture; G.S.: Giemsa stain; PAP: Peroxidase-anti-peroxidase; IIFT: Indirect immuno-fluorescent test; Abn: Abnormal; *: For definition see the text

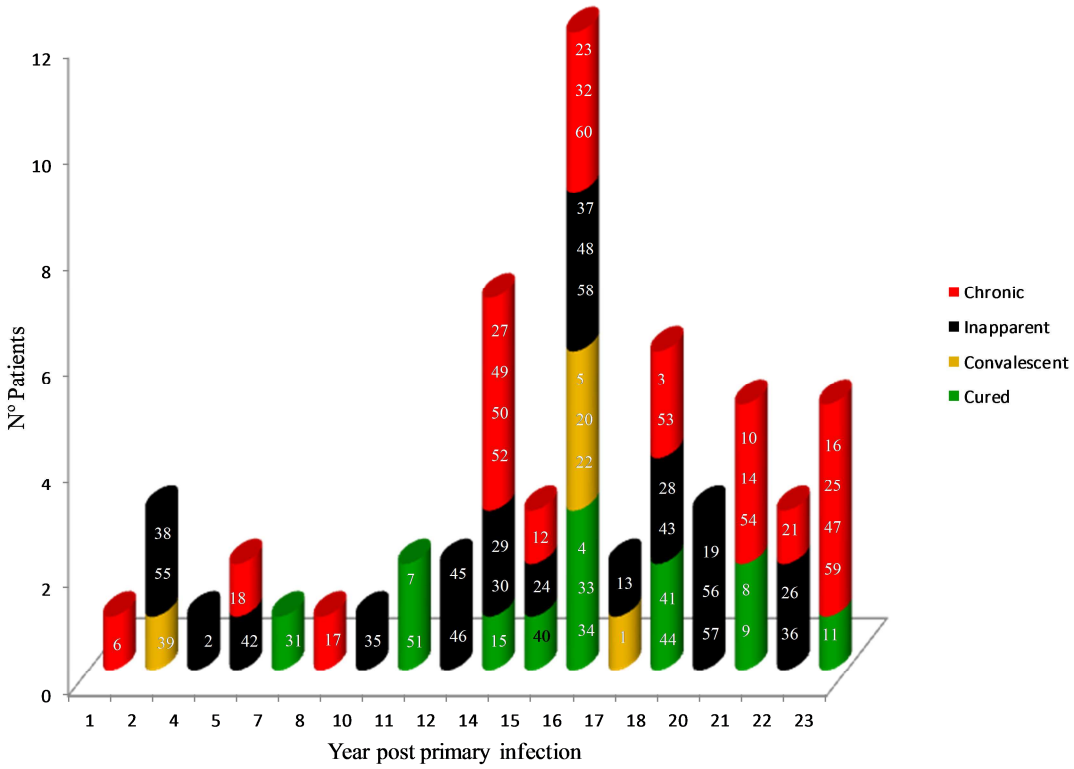

Fig. 3. *Identification of patient clinical condition after a long-term follow-up from 1 to 23 year post primary acute Chagas disease infection. Number inside bar identify patients. Color indicates clinical condition.*

4. Discussion

The lack of consensus concerning criteria of cure of Chagas disease following patients treatment with nitroderivates drugs i.e. benznidazole and/or nifurtimox, has generated an interesting controversy among researchers who have provided different proofs and arguments on the subject. While some authors reject clinical criterion or gives it a limited value arguing either slow or asymptomatic evolution of the infection, others have considered as cured those patients showing consistent negative conventional serological tests (6, 15). In addition, some authors have considered essential for the evaluation of anti-chagasic drugs to follow different criteria derived from clinical, parasitological and serological methods, which must be reliable, applicable in long-term follow-up and able to confirm elimination of both blood circulating trypomastigotes and amastigote tissue forms of *T.cruzi* (3, 15). More recently, the use of *T.cruzi*-specific PCR assays has proven to be a highly sensitive technique suitable for the direct detection of any form of the parasite, resulting thus useful for the follow-up of chagasic patients after specific chemotherapy which, at the same time, is a reliable method to establish criteria of cure for this long-lasting disease (12, 16).

In the present work, we have chosen and applied 5 different methods to carry out a long-term follow-up in 60 selected unquestionable acute chagasic patients, 52 (86.7%) treated with benznidazole and 8 (13.3%) untreated patients, the latter considered as the infected control group. All of them came from, and were diagnosed, in western Venezuela where Chagas disease is endemic. The methodology here used to monitor the progression of the infection included: i.parasitological techniques to detect BCP directly in fresh sample or indirectly by HC; ii.serological methods (DAT, IFAT and ELISA) including detection of IgM and IgG levels in each patient; iii. Periodical clinical evaluation to detect symptoms attributable to Chagas disease complemented with electro and echocardiographic examination; iv.molecular assessment of part of the *T.cruzi* genome by a specific PCR assay and immunohistopathological techniques for the detection of the parasite itself or its antigenic deposits in oral tissue samples taken at the endpoint evaluation of patients. This complex multifarious, painstaking and time consuming methodology was applied in order to estimate the success of treatment with benznidazole in the selected cohort of chagasic patients, avoiding inefficient criteria previously reported using single methods for Chagas disease chemotherapy evaluation. The study cohort was made up selecting patients at different times post primary acute infection, including individuals bearing infection from 1 to 23 years. This group consisted of this particular set of patients since the study was aimed at, on one hand, complying with a long-term follow-up study as previously recommended (3) and, on the other hand, having the opportunity to evaluate different patients at distinct periods of infection evolution at the same time. This way we had the possibility to comparatively analyze the complex spectrum of the Chagas disease progression as it naturally appears in endemic areas. Although this kind of designs may introduce a major variability for a comprehensive evaluation of the course of *T.cruzi* infection as compared to considering the same cohort of patients throughout the years, it also makes it possible the continuous observation or testing of anti-chagasic drugs in a more realistic way. This monitoring system may provide more accurate early information for a better understanding on Chagas disease progression and its clinical prognostic, as well as it may serve to detect the usefulness of benznidazole to eliminate parasite persistence in infected patients.

The comparison of total baseline values recorded in the entire cohort of acute chagasic patients with those detected during the follow-up evaluation, revealed statistically highly significant differences ($P<0.001$) when parasitemia, serology and symptoms were considered before and after treatment with benznidazole, which should suggest a beneficial effect of the used drug. However, in no cases paraclinical examination including EKG and ECO showed differences between benznidazole-treated and untreated-control patients. On the other hand, when the untreated patients were compared among themselves only the parasitemia level showed differences between patients in acute phase and those followed up at different times PPI ($P<0.01$). This fact suggests that *T.cruzi* is consistently found in the blood stream during the first weeks of the infection and that, despite the suppressive effect of benznidazole on the BCP causing a remission of symptoms, there is part of the infected population showing a similar benign prognosis without drug intervention, as previously noted (17). Considering the here observed results regarding the evaluation of benznidazole as a therapeutic tool in Chagas disease management, it is possible to detect limitations in the used criteria due to peculiarities of the natural course of *T.cruzi* in the infected individuals. These limitations are evident in the clinical aspects taking into consideration that even in the absence of benznidazole treatment symptoms subsides within 1-2 months in the acute phase, and EKG as well as ECO tend to normalize in most cases, increasing the number of symptomless infected individuals as previously reported (15). Similar explanation may be used to interpret other characteristics considered in the progression of the infection including parasitologic, serologic and molecular (PCR) aspects, in which despite an increase of negative results of 97%, 30% and 82% respectively recorded, no statistical significant difference was detected when benznidazole treated and untreated-control patients were compared ($P>0.05$). This may indicate that the efficacy of benznidazole against *T.cruzi* infection showed to be limited in our cohort of treated patients and similar to the observed in non-treated ones, supporting experimental observations (18).

Regarding *T.cruzi* persistence at oral tissues of chagasic patients evaluated at different times PPI, the immunohistopathological and molecular findings revealed consistent results pointing to the presence of tissular forms of the parasite in 40% of the samples. This was further

demonstrated by the similar proportional figures recorded in the diagnostic tests, which were able to detect in the patient's tissue the presence of the parasite itself (20%), *T.cruzi* antigenic deposits (28-23%) and specific *T.cruzi* DNA (20%) when GS, PAP-IIFT and PCR techniques, were respectively used. These results also give evidence on the use of a reliable methodology to have confidence enough for the interpretation of this kind of long-term follow-up study. In addition, the detection of 22.7% average positive results with the here used different diagnostic methods, support a similar finding (22.5%) previously reported using PCR assay as unique technique, which confirm that *T.cruzi* tissue form persistence in chronic chagasic patients seems to be a common characteristic in the behavior of this parasite (10). The present findings together with those reported in gingival inflammatory foci of chronic chagasic patients, as well as the high persistence recorded in the heart following endomyocardial biopsies and the results showed in experimental models, provide strong evidence for parasite persistence as a primary cause to explain pathogenesis of Chagas disease (9, 10, 18, 19). These results also allowed us to argue against benznidazole as anti-*T.cruzi* drug of election, claiming for a more efficient treatment to eliminate this parasite from the host tissue. Supporting the latter, when the group of benznidazole treated patients was compared with the untreated-control, no significant difference was observed ($P>0.05$). In fact, although *T.cruzi* persistence was detected at all times of the progression of the infection between 1 to 23 years PPI, the benznidazole treated patients and the untreated-control showed 40% and 37.5% persistence respectively, indicating the limited effect of this drug against the parasite tissue forms.

The present long-term follow-up study of a cohort of unquestionable *T.cruzi*-infected Venezuelan patients evaluated from 1 to 23 year after receiving benznidazole treatment, allowed us to distinguish groups of individuals at different clinical conditions, which may be useful to evaluate the efficacy of the drug effect and to establish a criterion of cure for Chagas disease. Overall, we were able to distinguish typical chronic patients (35%), individuals with subclinical, asymptomatic or inapparent infections (35%), symptomatic seronegative people bearing *T.cruzi*-tissue form persistence (8%) and cured individuals (22%) characterized by showing negative sero-parasitological and molecular results, being asymptomatic and without tissue parasite persistence. The use of a variety of reliable tests such as parasitological, serological, molecular (PCR), clinical, paraclinical (EKG, ECO) and immunohistopathological for a long-term evaluation of chagasic patients give us confidence in the obtained results when benznidazole treated and untreated-control patients were compared in order to establish the efficacy of this anti-*T.cruzi* nitroderivate on the long lasting Chagas disease. Finally, considering that the main purpose of a specific anti-*T.cruzi* therapy is the elimination of the parasite, the criterion of cure should be the demonstration of the eradication of both circulating and tissue forms of the parasite, and confirmed with reliable tests by post-treatment consistent negative results.

5. Conclusion

The combination of reliable methods performed during the present work allowed us: i. To distinguish four different clinical conditions among chagasic patients after a long-term follow up. These included typical chronic patients, individuals bearing subclinical or inapparent infections, sero-negative symptomatic patients and cured patients. ii. To corroborate the *T.cruzi* tissue forms persistence, and its capacity for lasting during long periods in chagasic patients as a common behaviour of the parasite, adding evidence to explain pathogenesis of Chagas disease. iii. To recognize as criteria of Chagas disease cure those asymptomatic individuals, showing negative serology for specific *T.cruzi* antibodies, and negative *T.cruzi* persistence both as blood circulating and tissue forms. iv. To demonstrate the unsatisfactory therapeutic effect of benznidazole due to fails to eliminate *T.cruzi*-tissue form, which raise doubt on its use as the drug of election for Chagas disease specific therapy.

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