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Parasitology

Use of an enzyme-linked immunosorbent assay that utilizes the *Tc*13Tul antigen of *Trypanosoma cruzi* to monitor patients after treatment with benznidazole

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ABSTRACT

*Tc*13Tul antigen is expressed in the mammalian stages of *Trypanosoma cruzi*, the etiological agent of Chagas' disease. Here, we designed and validated an enzyme-linked immunosorbent assay using the recombinant *Tc*13Tul (*Tc*13Tul-ELISA) and found that it had 82.5% sensitivity and 97.05% of specificity.
To evaluate whether the decrease in antibodies against *Tc*13Tul may be used as an early marker of the effect of chemotherapy with benznidazole, sera from 30 *T. cruzi*-infected children were evaluated by *Tc*13Tul-ELISA before and after benznidazole treatment. While in Group A (6 months-4 years old, n = 16) the decrease of more than 30% of *Tc*13Tul-ELISA values showed a sensitivity similar to that of conventional serology (CS); in Group B, (5–12 years old, n = 14) the decrease of *Tc*13Tul-ELISA values was a better parameter than negativization of CS to monitor the impact of treatment. Therefore, the dosage of anti-*Tc*13Tul antibodies may be useful as a methodology complementary to CS to evaluate chagasic patients undergoing chemotherapy with benznidazole.

1. Introduction

Chagas' disease is a chronic illness caused by the protozoan *Trypanosoma cruzi* that affects 11 million people in Latin America (Dias et al., 2002). *T. cruzi* is normally transmitted by triatomine bugs. However, transmission can also be congenital, or occur by blood transfusion or organ transplantation, even in countries where the disease is not endemic (Schmunis and Yadon, 2010). The course of infection includes an acute phase that lasts until 3 months after the infection, an indeterminate phase without symptoms, and a chronic phase in which approximately 30% of the patients present clinical evidence of heart disease or megavisceras. Specific chemotherapy with benznidazole or nifurtimox has been recommended for treatment of acute (Lugones et al., 1969), congenital (Altcheh et al., 2005), and reactivated infection (Altclas et al., 2005; Riarte et al., 1999) and for children up to 15 years of age with chronic infection (de Andrade et al., 1996; Sosa Estani et al., 1998) (OPS/MSF, 2005). In contrast, recommendations for anti-parasitic treatment in adults with chronic infection have remained controversial for many years; nevertheless, long-term follow-up by several observational studies published since the 1990s shows a trend in favor of the application of the treatment since it has been shown it reduces disease progression to cardiomyopathy independently of parasite elimination (Reviewed by Sosa-Estani et al., 2009 and Bern, 2011). In Argentina, by regulation of the Ministry of Health, the serology for *T. cruzi* is systematically carried out on all pregnant women and the treatment of *T. cruzi*-infection is indicated in: i) patients in the acute phase of the disease, regardless of mode of transmission, ii) in children and adolescents under 19 years in the chronic phase, iii) in living reactive donors for organ transplantation and iv) laboratory accident or surgical material contaminated with *T. cruzi* (Ministerio de Salud de la Nación Argentina, 2012).

The use of conventional serology (CS) tests (enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF) and indirect hemagglutination (IHA) is still the main method for Chagas' disease diagnosis in the chronic phase. The non-infective stage of *T. cruzi* (the epimastigote stage) is generally used as a source of antigen for CS tests. However, available test kits for Chagas' disease diagnosis have variable sensitivity and specificity and show discrepancies or inconclusive results, such as false-positive or false-negative reactions (Otani et al., 2009). For that reason, the World Health Organization recommends using at least two of those serological tests in parallel (WHO, 1991).

Several lines of evidence indicate that the earlier the specific treatment is initiated, the greater the chance of cure (Freilij et al.,

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2007). The primary criterion of cure is the negative seroconversion of CS (Cançado, 1999), whereas the secondary criterion to monitor the impact of treatment is the reduction of antibody titers in repeated serological tests (Fabbro et al., 2007; Viotti et al., 1994; Viotti et al., 2011). Since the decrease in antibody concentrations after chemotherapy is a slow process, CS sometimes is not able to determine which patients have been cured. For this reason, there is special interest in validating biomarkers of parasite load decrease with diagnostic purposes. In this regard, it has been documented that conventional immunoassays prepared with highly specific *T. cruzi* antigens, such as *trans*-sialidase (Pereira-Chioccola et al., 2003), F29 (Sosa Estani et al., 1998), or GP57/51 (Gazzinelli et al., 1993), can be useful in the assessment of cure after chemotherapy.

*Tc*13 is a trans-sialidase family protein bearing five amino-acid (EPKSA) repeats at its C-terminal region coded in several strains of the parasite (Campetella et al., 1992; García et al., 2003). *Tc*13 antigens are expressed on the surface of the amastigote and trypomastigote stages of *T. cruzi* but not detected on the epimastigote (Souto-Padrón et al., 1989). In previous studies, EPKSA repeats have shown high specificity and sensitivity for detecting antibodies in sera from acute and chronic Chagas' disease patients (Burns et al., 1992; Ferreira et al., 2001; Peralta et al., 1994; Reyes et al., 1990; Vergara et al., 1991). Recently, we have demonstrated that the use of the *Tc*13Tul antigen, a *Tc*13 antigen of the Tulahuén strain of *T. cruzi* which also contains part of the N-terminal domain, can detect specific IgM and IgG by ELISA in sera from *T. cruzi*-infected mice (García et al., 2008). Here, we describe the development of an ELISA using the recombinant antigen *Tc*13Tul, with the aim to detect specific antibodies in Chagas' disease patients. In addition, to define an early marker of therapeutic impact, we evaluated whether antibodies against *Tc*13Tul antigen decrease after anti-*T. cruzi* chemotherapy with benznidazole. For this purpose, we evaluated a population of immunocompetent, chronically-infected children (6 months to 12 years old) born to *T. cruzi*-infected women who were diagnosed by CS in our Institute and returned after trypanocidal treatment for the serological follow-up.

2. Materials and methods

2.1. Recombinant antigens

The *Tc*13Tul protein is encoded by a 2391-bp fragment (GenBank Accession no. AF092099), which codifies for 2 of 3 SXDXGXTW motifs of the N-terminal domain and 45 EPKSA repeats. *Tc*13Tul protein was expressed in the pMalp2 vector as a fusion to the maltose-binding protein (MBP). MBP-*Tc*13Tul and MBP, expressed by the wild type vector, were purified by amylose resin, as previously described (García et al. 2003).

2.2. Serological diagnosis and CS techniques

Diagnosis of *T. cruzi* infection was performed at the Diagnosis Dept. of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben" (Buenos Aires, Argentina) by three serological tests which detect specific IgG (ELISA, IIF and IHA). The diagnosis was based on positivity or negativity of two out of three tests. Serological techniques were developed in our Institute using "in-house" antigens obtained from epimastigote forms of *T. cruzi*, compliant with domestic and international rules. Whole epimastigote cells are used for IIF, while crude cell lysates are used for IHA and ELISA. The sensitivity of the tests varies between 99.0% and 99.8%. When two or three tests are simultaneously performed, the sensitivity ranges from 99.7% to 100% and the specificity from 97.4% to 97.9%. IHA and IIF assays were considered as reactive from sequential 1/2 serum dilutions between 1/32 and 1/256. ELISA test was considered positive when mean absorbance at 490 nm was higher than the cut-off value of 0.200 (Alvarez et al., 1968; Cura and Segura, 1998; Cura et al., 1993; De Rissio et al., 2010).

2.3. Serum samples

Serum samples used to design and validate the *Tc*13Tul-ELISA belonged to a reference panel prepared by the Diagnosis Dept. of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben" from patients who attended our institution and reside in Buenos Aires city and surroundings. Reactive and non-reactive sera from this panel were classified according to the results of serological and parasitological (xenodiagnosis) tests and epidemiological background.

Sera from patients with clinical and parasitological diagnosis of visceral leishmaniasis came from Posadas city (Misiones Province), an endemic area for visceral leishmaniasis in Argentina, and from Asunción city in Paraguay. Sera from patients with toxoplasmosis, HIV and hepatitis B were obtained from the Hospital Municipal Materno-Infantil de San Isidro "Dr. Carlos Gianantonio", Buenos Aires, Argentina. Cession of these sera was approved by the Ethical Committee of this institution. Sera from patients with autoimmune diseases were provided by Dr. Liliana Roquel. Sera from patients with other pathologies were obtained through the Instituto Nacional de Enfermedades Infecciosas – ANLIS "Dr. Carlos G. Malbran".

Serum samples from *T. cruzi*-infected children used to evaluate the performance of the *Tc*13Tul-ELISA before and after benznidazole treatment were obtained from children born to *T. cruzi*-infected women who were diagnosed by CS in the Diagnosis Dept. of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben". Children enrolled in this study have chronic Chagas' disease and were presumed to be congenitally infected. *T. cruzi*-infected children diagnosed at our institution were derived to pediatric hospitals for benznidazole treatment (5–7 mg/kg/day divided in 2 or 3 daily doses for 60 days). After the end of the treatment, patients returned to our institution to carry out the follow-up of the post-treatment CS for *T. cruzi* infection. It is important to note that post-treatment samples were collected with different schedule times.

2.4. Tc13Tul-ELISA

To standardize the *Tc*13Tul-ELISA, previous assays were carried out to determine the optimal concentration of recombinant proteins to be adsorbed to the solid phase and the best dilution of serum and conjugate to use. Best results were obtained with 0.25 μg per well of recombinant antigen and sera in a dilution of 1:400. To subtract non-specific reactivity directed to the MBP portion of the recombinant antigen, sera were simultaneously tested against MBP.

Flat-bottomed polystyrene plates (Nunc-Immuno MaxiSorp) were coated overnight at 4 °C with 50 μL of the recombinant antigen (MBP or MBP-*Tc*13Tul) diluted at a concentration of 5 μg/mL in phosphate-buffered saline (PBS), pH = 7.2. Afterwards, plates were blocked for 1 h with 5% skimmed milk in PBS and subsequently washed 3 times with PBS-0.05% $Tween_{20}$ (PBS-T). After washes, 50 μL per well of serum samples diluted 1:400 in 1% skimmed milk-PBS were incubated for 1 h at 37 °C. Each serum sample was incubated in duplicate simultaneously onto MBP- and MBP-*Tc*13Tul-coated wells. After 3 washes with PBS-T, plates were incubated for 1 h at 37 °C with horseradish peroxidase-labeled anti-human IgG (DAKO, Denmark) diluted 1:10,000 in 1% skimmed milk-PBS. After a new wash cycle with PBS-T, 50 μL of substrate solution was added to each well (0.1 mol/L citric acid, 0.2 M Na_2HPO_4, pH = 5, *o*-phenylenediamine dihydrochloride 4 mg/ml, 0.024% H_2O_2) and the plates were left to stand in the dark for approximately 15 min. Color development was stopped by adding 50 μL per well of 2 N H_2SO_4 and the absorbance was read at 490 nm (OD_{490nm}), using an ELISA microplate reader (Dynatech). For each serum sample, the OD_{490nm} obtained with MBP as antigen was subtracted from the OD_{490nm} obtained with MBP-

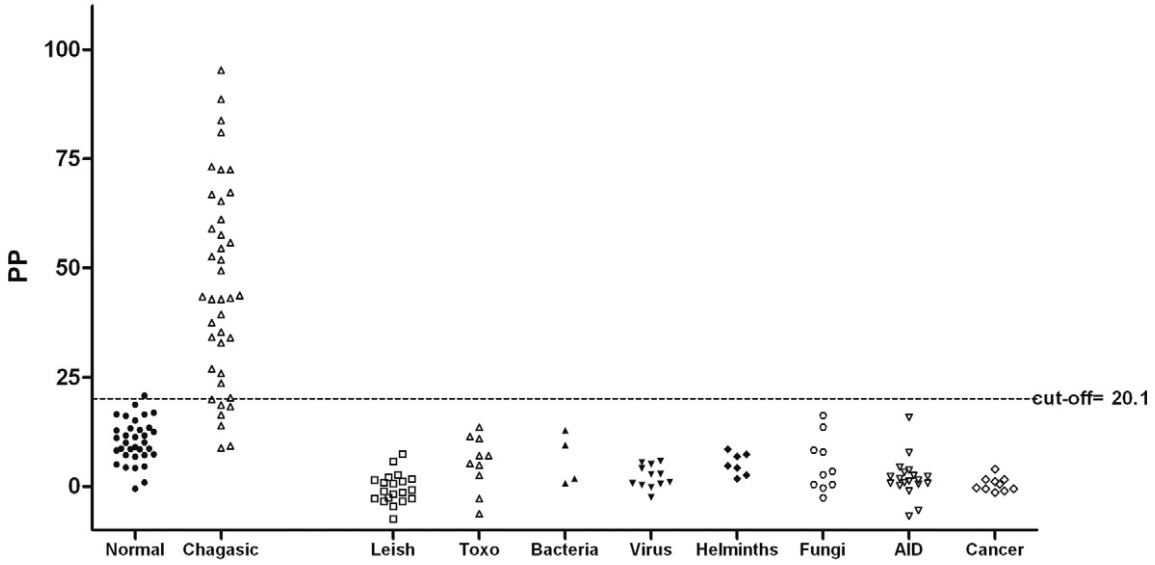

Fig. 1. Reactivities of sera from healthy individuals (Normal), patients infected with *Trypanosoma cruzi* (Chagasic) and patients with other pathologies analyzed by *Tc*13Tul ELISA. Results are expressed as the PP with respect to a strong-positive reference serum for *Tc*13Tul-ELISA. The dotted horizontal line indicates the cut-off value. Leish, patients with leishmaniasis (n = 20). Toxo, patients infected with *Toxoplasma gondii* (n = 10). Bacteria, patients with bacterial infections: mycoplasma (n = 2), chlamydia (n = 1) and syphilis (n = 1). Virus, patients with viral infections: HIV (n = 8), HBV (n = 1), measles virus (n = 1), VZV (n = 1) and CMV (n = 1). Helminthes, patients infected with *Toxocara* (n = 3), and *Echinoccocus* (n = 4). Fungi, patients with fungal infections: histoplasmosis (n = 3), coccidioidomycosis (n = 3), and aspergillosis (n = 4). AID, patients with autoimmune diseases positive for: anti-mitochondrial antibodies (AMA) (n = 8), anti-parietal cell antibodies (n = 1), smooth muscle antibodies (SMA) (n = 2), anti-centromere antibodies (n = 1), anti-nuclear antibodies (AMA) (n = 5), anti-cardiolipin (n = 1), and anti-endomysial antibodies (n = 1). Cancer, leukaemia paediatric patients underwent chemotherapy (n = 10).

*Tc*13Tul as antigen (*Tc*13Tul net OD_{490nm}), and results expressed as a percentage of a high positive control serum sample (see Section 2.5).

Antibody titration in sera from Group B patients by *Tc*13Tul ELISA was obtained from sequential 1/2 dilutions between 1/50 and 1/6400. The highest dilution at which the *Tc*13Tul ELISA percentage positivity (PP) value greater than or equal to the cut-off was considered the antibody titer (endpoint).

A first approach to estimate the precision of the *Tc*13Tul-ELISA was performed with 13 serum samples from Chagas' disease patients and 10 serum samples from healthy individuals tested in duplicate along three consecutive days. Bartlett's test showed that the variances of the groups were equal ($P = 0.518$ and $P = 0.4875$ for chagasic and healthy sera, respectively).

2.5. Quality assurance and data analysis

The percentage positivity (PP) method of data expression and quality assurance (Wright et al., 1993) was used. Three reference sera were selected by the *Tc*13Tul-ELISA to be used as internal quality controls: a strong positive (C++), a moderate positive (C+) and a negative (C−). Four replicates of C++, C+, C− and conjugate controls (CC, serum diluent buffer without serum) were included on every plate. For each sample tested, the *Tc*13Tul net OD_{490nm} was expressed as a percentage of the mean *Tc*13Tul net OD_{490nm} of the four C++ replicates. For acceptance of results from individual plates, the mean OD_{490nm} of the four C++ replicates, and the PPs of at least three of the four replicates of the internal quality control sera had to fall within prescribed ranges.

Normal distribution of data were verified by Kolmogorov-Smirnov ($P = 0.200$) and the absence of outliers by the graphical method (box-plot). The repeatability and intra-laboratory precision of the *Tc*13Tul ELISA were assessed over raw data of 20 consecutive runs using the reference control sera in four replicates each (CLSI/NCCLS, 2004). The repeatability and intra-laboratory precision CV% calculated for the C++ were 11.39% and 24.5%, respectively.

The values of sensitivity and specificity were calculated according to Altman and Bland (1994a, 1994b).

Kaplan-Meier curves of negativization or decrease of antibodies were compared by Log-rank test and differences were considered significant with a *P* value ≤0.05.

2.6. Ethical considerations

To comply with the Argentinean Law # 25,326 of protection of personal data, all the serological samples used in this study were anonymous and codified without the possibility to identify the donor. This project was reviewed and approved by the Ethics Committee of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben".

3. Results

3.1. Development of the Tc13Tul-ELISA

3.1.1. Sensitivity and specificity

*Tc*13Tul-ELISA was assayed with 34 sera from healthy individuals and 40 sera from adult *T. cruzi*-infected patients diagnosed by CS

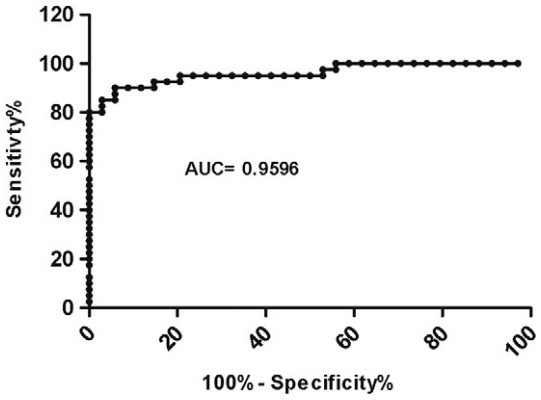

Fig. 2. ROC curve analysis of the *Tc*13Tul-ELISA performed with 40 sera from *T. cruzi*-infected patients and 34 sera from healthy individuals.

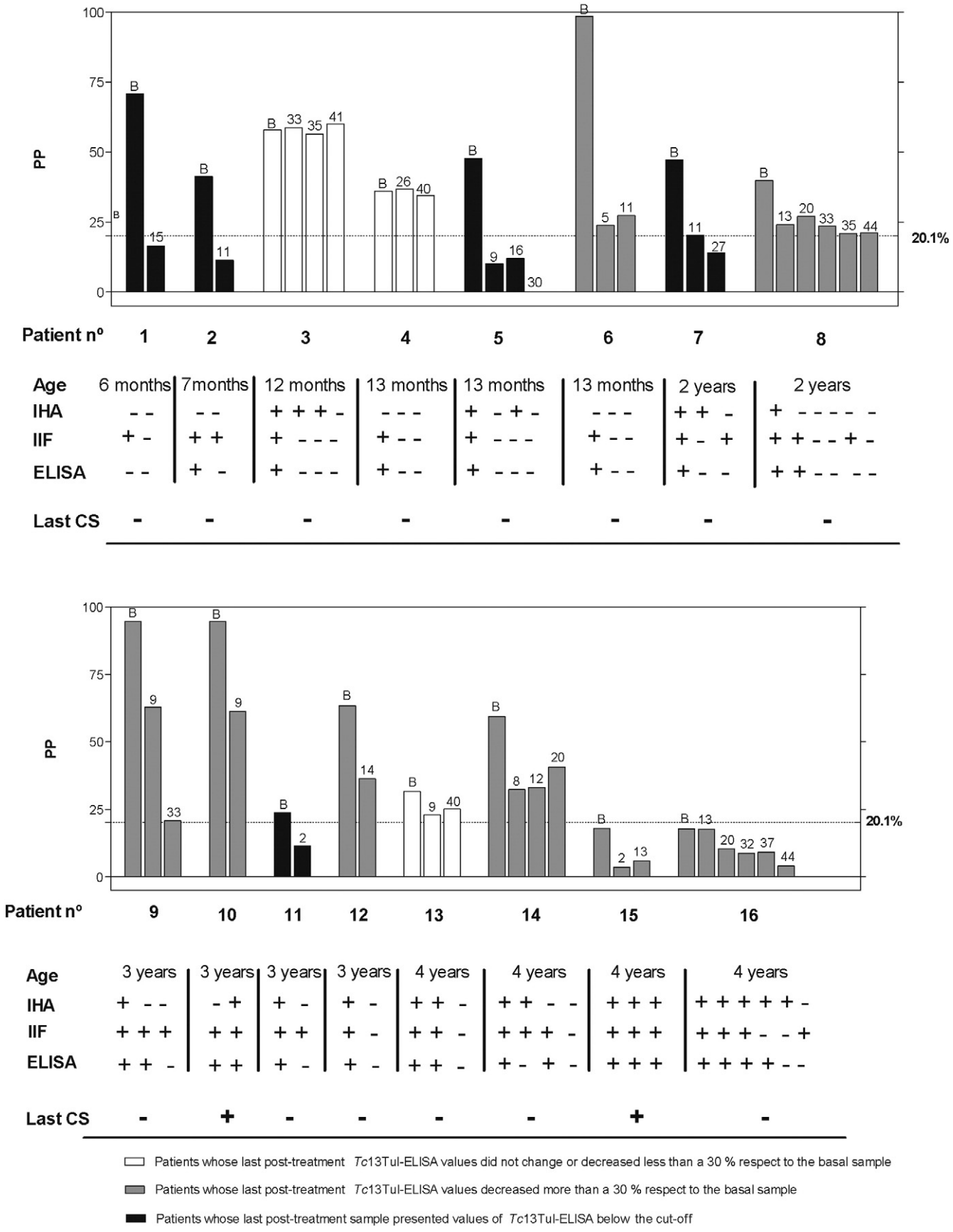

Fig. 3. *Tc*13Tul-ELISA values and CS data before and after benznidazole treatment of sera from children ranging from 6 months to 4 years old (Group A). *Tc*13TulELISA values expressed as the PP are shown in the bar graph, while the corresponding results obtained with the three CS techniques (IHA, IIF, and ELISA) are shown below. The B above the bars means that this bar corresponds to the pre-treatment sample (basal value). Numbers above the bars indicate the number of months elapsed after the pre-treatment sample. Last CS: −, when at least two of three CS techniques in the last post-treatment sample are −; +, when at least two of three CS techniques in the last post-treatment sample are +.

(Fig. 1). The cut-off value of negative sera estimated as two standard deviations above the mean was 20.1 PP. The sensitivity of *Tc*13Tul-ELISA was 82.5 PP (95% CI = 70.74–94.26) whereas its specificity was 97.05 PP (95% CI = 91.36–100). The specificity of recombinant *Tc*13Tul was also analyzed with serum samples from non-chagasic patients affected with other parasitic or unrelated diseases, including individuals with leishmaniasis (n = 20), toxoplasmosis (n = 10), bacterial infections (n = 4), viral infections (n = 12), helminthes infections (n = 7), systemic fungal infections (n = 10), autoimmune disease (n = 19) and cancer (n = 10). None of these sera showed values above the cut-off in our assay, even sera from patients infected with *Leishmania*, a parasite phylogenetically related to *T. cruzi* which usually shows serological cross-reaction (Umezawa et al., 1999), thus proving that *Tc*13Tul-ELISA is highly specific for the detection of anti-*T. cruzi* antibodies (Fig. 1). The receiver operating characteristic (ROC) curve analysis showed an area under the curve (AUC) of 0.9596 (95% CI = 0.917–1.00), indicating that the *Tc*13Tul-ELISA has a good diagnostic performance (Park et al., 2004) (Fig. 2).

3.2. Reactivity to Tc13Tul antigen in T. cruzi-infected children before and after benznidazole treatment

Sera from 30 *T. cruzi*-infected children were evaluated by *Tc*13Tul-ELISA before and after treatment with benznidazole with the aim to investigate whether this chemotherapy induces significant changes in reactivity towards the *Tc*13Tul antigen.

It has been documented that the earlier the treatment is initiated, the faster CS becomes negative (Bern, 2011; Sosa-Estani et al., 2009; Freilij et al., 2007). Therefore, patients were divided in 2 groups according to their age: Group A: children from 6 months to 4 years old (n = 16) and Group B: children from 5 to 12 years old (n = 14). Within each group, patients were classified on the basis of the *Tc*13Tul-ELISA results as: i) individuals whose last post-treatment sample presented values below the cut-off, ii) individuals whose last post-treatment sample presented values above the cut-off but decreased more than 30% with respect to the basal sample, and iii) individuals whose last post-treatment sample did not change or decreased less than 30% with respect to the basal sample. The 30% decrease in *Tc*13TulELISA post-treatment value respect to the basal sample was taken as indicative of the impact of treatment, since in a recent study Viotti and co-workers has established this cut-off for the conventional ELISA by comparing the serological evolution in benznidazole-treated vs. untreated groups (Viotti et al., 2011).

In Group A, 14 out of 16 children negativized CS in some of the post-treatment samples. Regarding *Tc*13Tul-ELISA results, 13 children showed more than 30% decrease in post-treatment samples respect to the basal value, while 5 of them presented post-treatment values below the cut-off (Fig. 3). Whereas negativization of the *Tc*13Tul-ELISA was not a good marker of therapeutic efficacy ($P = 0.016$), reduction of more than 30% in the post-treatment samples of the *Tc*13Tul-ELISA values demonstrated to be a criterion as valid as negativization of CS to evaluate the efficacy of benznidazole treatment ($P = 0.7228$) (Fig. 4).

In Group B, only 3 out of 14 patients negativized CS after treatment with benznidazole. Regarding *Tc*13Tul-ELISA, 7 children negativized their results, whereas 2 out of 14 children did not show values below the cut-off but reduced the *Tc*13Tul-ELISA post-treatment values more than 30% respect to the basal sample (Fig. 5). The analysis of the *Tc*13Tul-ELISA titers from Group B patients showed that patients who negativized the *Tc*13Tul-ELISA after treatment (Fig. 5 black bars) had at least a 2-fold titer reduction in the first post-treatment sample (Table 1 patients shown in bold letter). Regarding the patients who decreased more than 30%, the *Tc*13Tul-ELISA value after treatment (Fig. 5 gray bars and Table 1 patients shown in gray letter), only one of them showed a 2-fold reduction in titer. Remarkably, from 11 patients whose CS remained positive after chemotherapy, 5 of them negativized the *Tc*13Tul ELISA and 1 of them reduced its titer. The kinetics of negativization of CS versus negativization or titer reduction of the *Tc*13Tul-ELISA were significantly different ($P = 0.0439$) (Fig. 6). Thus, providing this novel ELISA can be very useful as a complementary technique to CS to monitor the impact of treatment in this age group.

4. Discussion

In a previous publication, we characterized the murine immune responses against *Tc*13 antigens and showed that the *Tc*13Tul recombinant protein is able to be used in ELISA to detect antibodies in *T. cruzi*-infected mice (García et al., 2008). In this study, we aimed to design and validate a similar assay to detect anti-*Tc*13 antibodies in sera from patients infected with *T. cruzi*, since it has been documented that they contain antibodies against the EPKSA repeats of *Tc*13

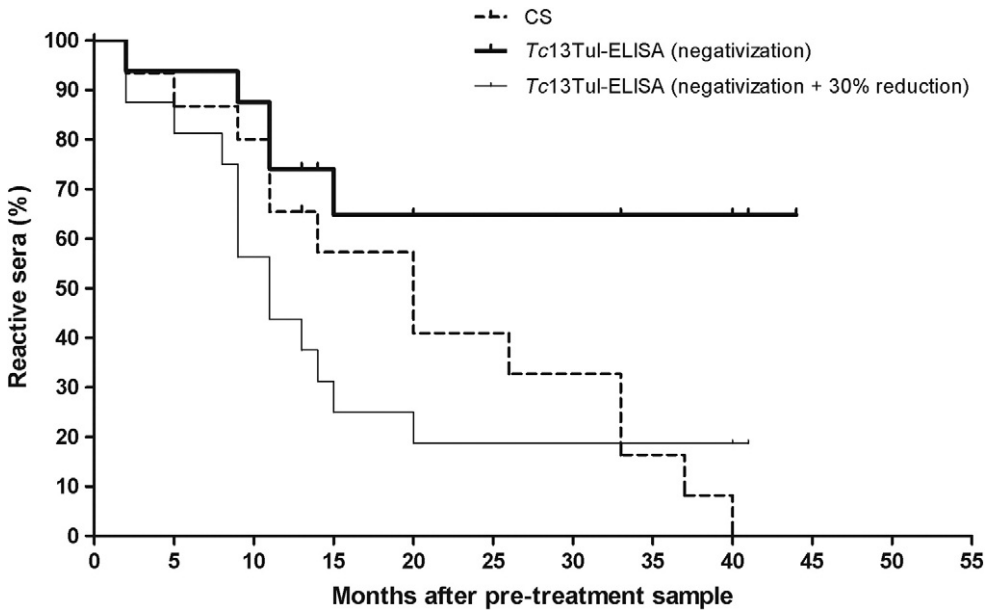

Fig. 4. Decrease in anti-*T. cruzi* antibodies by *Tc*13Tul-ELISA and negativization of CS after treatment with benznidazole in children ranging from 6 months to 4 years old (Group A).

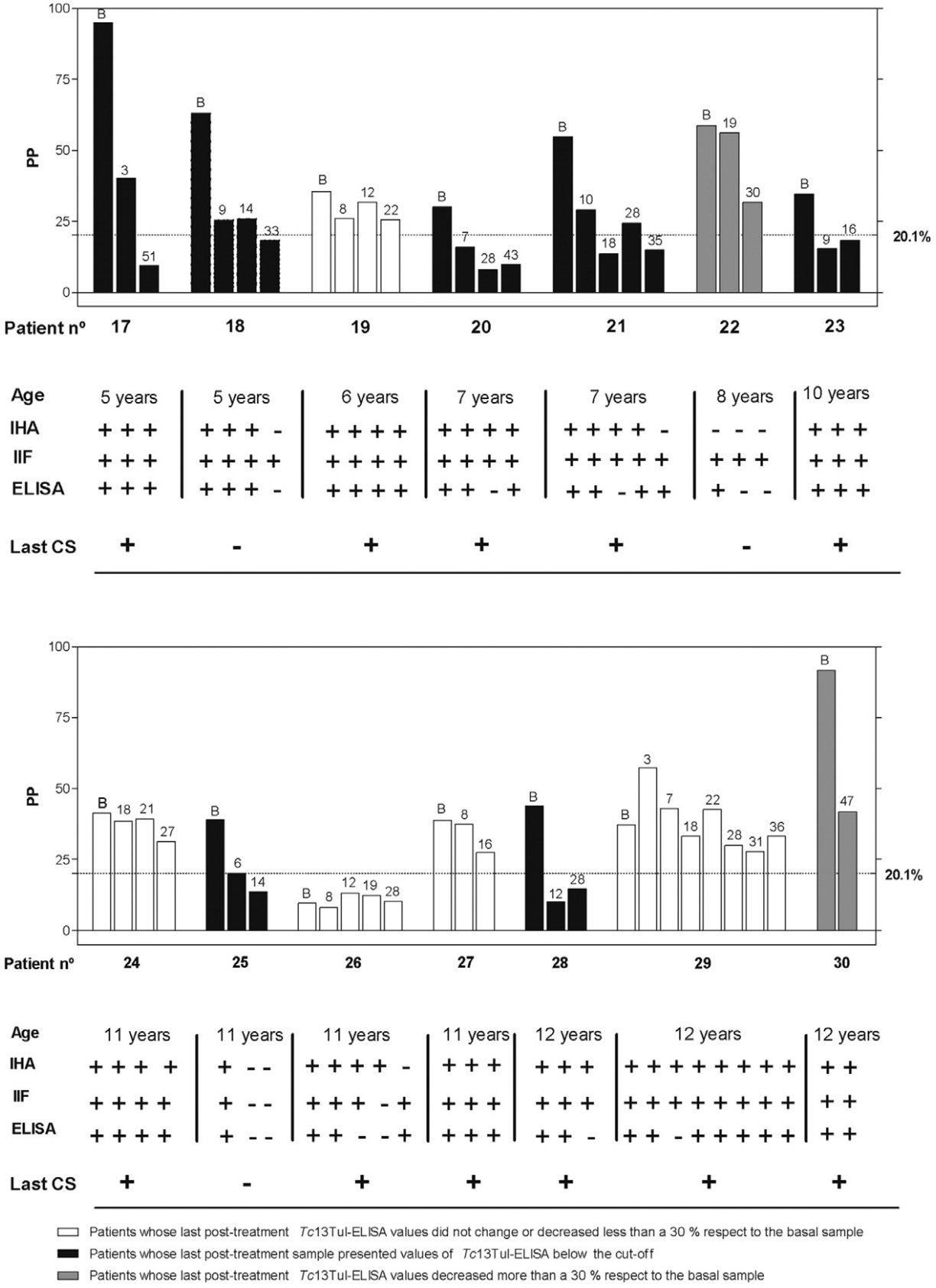

Fig. 5. *Tc*13Tul-ELISA values and CS data before and after benznidazole treatment of sera from children ranging from 5 to 12 years old (Group B). *Tc*13TulELISA values expressed as the PP are shown in the bar graph, while the corresponding results obtained with the three CS techniques (IHA, IIF, and ELISA) are shown below. The B above the bars means that this bar corresponds to the pre-treatment sample (basal value). Numbers above the bars indicate the number of months elapsed after the pre-treatment sample. Last CS: −, when at least two of three CS techniques in the last post-treatment sample are −; +, when at least 2 of 3 CS techniques in the last post-treatment sample are +.

Table 1
anti-*Tc*13Tul antibody titers before and after treatment of sera from patients ranging from 5 to 12 years old (Group B).

Patient n^0 [a]	Months after basal sample	Titer	Titer change[b]
17	**Basal**	**1/3200**	
	3	**1/400**	**−3**
	51	**1/200**	**−4**
18	**Basal**	**1/1600**	
	9	**1/400**	**−2**
	33	**1/100**	**−4**
19	Basal	1/400	
	8	1/400	0
	22	1/400	0
20	**Basal**	**1/400**	
	7	**1/50**	**−3**
	43	**1/200**	**−1**
21	**Basal**	**1/1600**	
	10	**1/400**	**−2**
	35	**1/100**	**−4**
22	Basal	1/800	
	19	1/1600	+1
	30	1/800	0
23	**Basal**	**1/400**	
	9	**1/50**	**−3**
	16	**1/100**	**−2**
24	Basal	ND	
	18	ND	
	27	ND	
25	**Basal**	**1/400**	
	6	**1/50**	**−3**
	14	**<1/50**	**−3**
26	Basal	ND[c]	
	8	ND	
	28	ND	
27	Basal	1/1600	
	8	1/800	−1
	16	1/400	−2
28	**Basal**	**1/800**	
	12	**1/50**	**−4**
	28	**1/100**	**−3**
29	Basal	ND	
	3	ND	
	36	ND	
30	Basal	1/3200	
	47	1/800	−2

[a] Patients whose last post-treatment sample diluted 1/400 presented values of *Tc*13Tul ELISA below the cut-off are shown in bold. Patients whose last post-treatment sample diluted 1/400 decreased their *Tc*13Tul ELISA values more than a 30% respect to the basal sample are shown in gray (patients n^0 22 and 30). Patients whose last post-treatment sample diluted 1/400 did not change their *Tc*13Tul ELISA values after treatment are shown in plain letters.
[b] Fold decrease (−) or increase (+) in titer dilution respect to the basal sample.
[c] ND, non-determined.

antigens (Vergara et al., 1991; Burns et al., 1992; Peralta et al., 1994; Houghton et al., 1999; Houghton et al., 2000; Ferreira et al., 2001).

The *Tc*13Tul-ELISA showed a high area under the receiver operating characteristic (ROC) curve (AUC = 0.9596) and a acceptable inter-assay variability (see Section 2.4, Material and Methods), 2 features that fit with a good overall performance of a diagnostic test (Bossuyt et al., 2003). The sensitivity and specificity of the *Tc*13Tul-ELISA were within the expected values by comparing with diagnostic assays performed with synthetic peptides and recombinant *T. cruzi* antigens which have tandem repeats, and taking into account that ELISAs carried out with either a peptide or a recombinant antigen show a high specificity but a limited sensitivity (Vergara et al., 1991; Brenière et al., 1997; Umezawa et al., 1999). It has been documented that individuals living in areas where Chagas' disease is endemic show higher levels of antibodies against repetitive antigens than those living in non-endemic areas (Peralta et al., 1994; Umezawa et al., 1999). In this regard, it is important to note that the sera used in this study belonged to individuals who reside in Buenos Aires city and its surroundings, a non-endemic area for *T. cruzi* infection. In addition, the lack of detection of anti-*Tc*13Tul antibodies in 17.5% of the Chagas' disease patients studied here is congruent with the loss of these antibodies in the chronic stage of the infection, as previously observed in *T. cruzi*-infected mice (García et al., 2008).

One of the problems affecting the control of Chagas' disease is the lack of reliable methods to evaluate the cure after treatment of the patient with trypanocidal drugs (Sosa-Estani et al., 2009). Conventional parasitological techniques, such as Strout method, haemoculture, or xenodiagnosis, lack sensitivity in the chronic phase of the infection (Fabbro et al., 2007; Sosa Estani et al., 1998; Viotti et al., 1994) and although detection of *T. cruzi* DNA by PCR have demonstrated promising data for the assessment of therapeutic effectiveness (Duarte et al. 2006, Murcia et al., 2010; Schijman et al. 2003), it has not yet been enough validated as a method to evaluate therapeutic cure. Therefore, persistent negative serological results after trypanocidal treatment is the indirect proof of parasite clearance and treatment success, so far. Although seronegative conversion after treatment in children is faster than in adults, it can take many years post-treatment indicating the need to identify early markers of therapeutic cure (de Andrade et al., 1996; Fabbro et al., 2007; Sosa Estani et al., 1998; Viotti et al., 2006). Alternatively, several reports have shown that a fall in serological titers can be a helpful tool to monitor the early impact of treatment, since it was associated with better clinical outcomes (Fabbro et al., 2007; Viotti et al., 1994; Viotti et al., 2011). On the other hand, it has been documented that IgG antibodies against some trypomastigote antigens decrease shortly after chemotherapy (Krettli, 2009; Pereira-Chioccola et al., 2003). In this context, we aimed to compare whether *Tc*13Tul-ELISA is more useful than CS to assess the efficacy of the treatment. To address this objective, we carried out a transversal study on 30 *T. cruzi*-infected children treated with benznidazole in which their pre- and post-treatment serum samples were simultaneously evaluated by *Tc*13Tul-ELISA and compared these results with those obtained by CS. Post-treatment samples were taken 2–44 months and 3–51 months after the treatment for Group A and B, respectively. Our study was performed on a paediatric population since nifurtimox and benznidazole have proven to be effective on these children (OPS/MSF, 2005) and, in Argentina, according to a regulation of the Ministry of Health, all *T. cruzi*-infected children must be treated.

We found that 87.5% of the patients of Group A and 21.4% of Group B underwent negativization of CS after benznidazole treatment (CS negativization A vs. B $P = 0.0015$). This observation was as expected since it is known that the fall in antibody titers after chemotherapy in young children is faster than in older ones (Freilij et al., 2007; Sosa-Estani et al., 2009). When we analyzed the negativization of *Tc*13Tul-ELISA, 31.2% and 50% of patients from Groups A and B, respectively, showed post-treatment values below the cut-off (*Tc*13Tul-ELISA negativization A versus B $P = 0.7407$), indicating that this parameter is not better than the CS negativization to evaluate the effect of the treatment in either group. If we take into account the reduction of anti-*Tc*13 antibodies after chemotherapy, we observed that 81.2% and 64.3% of children from Groups A and B, respectively, presented 30% decrease of their *Tc*13Tul-ELISA values after treatment respect to the basal sample (*Tc*13Tul-ELISA reduction A versus B $P = 0.2725$). In Group A, the evaluation of impact treatment by reduction of *Tc*13Tul-ELISA values in post-treatment samples was similar to that by CS negativization. On the other hand, in Group B, reduction of *Tc*13Tul-ELISA values after treatment showed to be a better parameter than CS to monitor the benznidazole effect, suggesting it could be considered a good predictor of the effect of chemotherapy. In this regard, the kinetics of the decrease in anti-*Tc*13Tul antibodies in Group B showed a pattern similar to that obtained by Sosa-Estani and co-workers by using an ELISA with the F29 *T. cruzi* protein to evaluate the efficacy of benznidazole treatment in children. In a double-blind, randomized clinical trial, 62% of the treated children negativized the F29 ELISA after a 4-year follow-up (Sosa Estani et al., 1998).

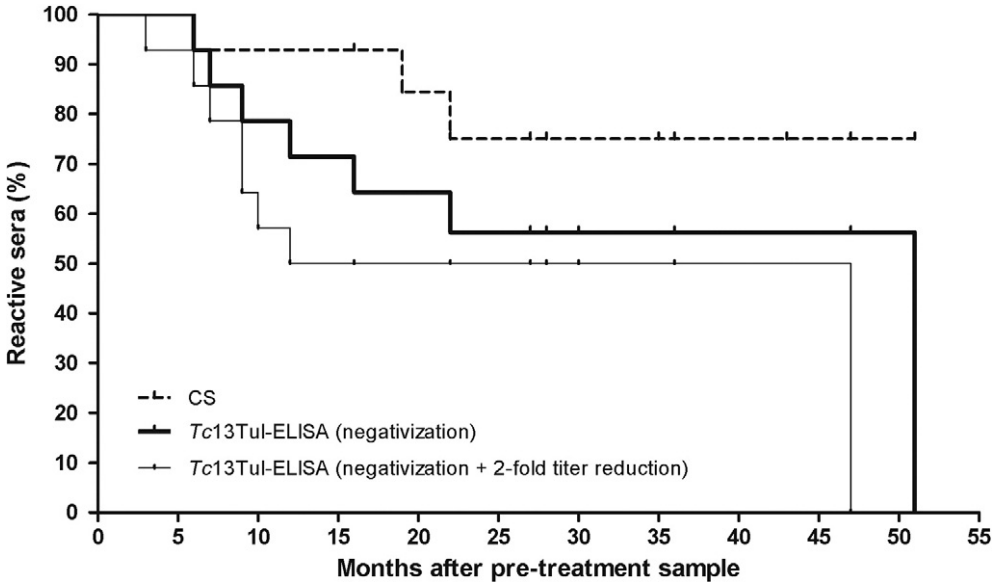

Fig. 6. Decrease in anti-*T. cruzi* antibodies by *Tc*13Tul-ELISA and negativization of CS after treatment with benznidazole in children ranging from 5 to 12 years old (Group B).

The etiological treatment of *T. cruzi* infection has proven to be beneficial to delay, reduce or prevent the progression to disease regardless of achieving parasite clearance (Fabbro et al., 2007; Sosa Estani et al., 1998; Sosa-Estani et al., 2009). Here we show that, in chronically infected children, when using the negativization or reduction of *Tc*13Tul-ELISA values as a complementary parameter to CS negativization to evaluate the impact of treatment a higher effect is observed. Moreover, in the group of age from 5 to 12 years old, *Tc*13Tul-ELISA was superior in detecting serological changes in shorter post-treatment timeline than CS. However, in order to determine whether unchanged CS with a decrease in *Tc*13Tul-ELISA values following treatment indicates that a curative response is ongoing, a longitudinal study to evaluate the *Tc*13Tul-ELISA values in function of time with a post-treatment sample collection on schedule will be designed.

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