Prediction of parasitological cure in children infected with *Trypanosoma cruzi* using a novel multiplex serological approach: an observational, retrospective cohort study

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Summary

Background Assessment of therapeutic response with standard serological diagnostic assays in patients with chronic Chagas disease is a major challenge due to the long persistence of parasite-specific antibodies. The current consensus for parasitological cure is to monitor conversion from positive to negative *Trypanosoma cruzi* serology (seroreversion). However, because of robust humoral immune response, seroreversion by standard serological tests can take years to decades. Developing novel tests of parasitological cure or surrogates is thus a priority in the Chagas disease field. We aimed to evaluate the MultiCruzi assay as a predictive tool for parasitological cure in a cohort of treated infants and children with acute and chronic Chagas disease enrolled in a long-term retrospective longitudinal study with clinical, serological, and parasitological follow-up, and to explore whether MultiCruzi could predict parasitological cure more quickly than the current reference method.

Methods Patients from two retrospective paediatric Chagas disease cohort studies with clinical, serological, and parasitological follow-up, diagnosed and treated at the parasitology service, Hospital de Niños Ricardo Gutierrez (Buenos Aires, Argentina) were included in this retrospective cohort study. Serum samples were collected every 6 months to 12 months between Oct 22, 1990, and June 3, 2019, for cohort 1 and 1 month after birth for cohort 2 and then every 3 months for a year between July 23, 2012, and April 19, 2016. We evaluated serological follow-up with the Chagatest ELISA (Wiener Lab, Rosario, Argentina) and used this as a clinical reference method for the evaluation of seroreversion. We compared Chagatest ELISA results with results of MultiCruzi (InfYnity Biomarkers, Lyon, France), a novel antibody profiling multiplex assay, investigating seroreversion events with both of the assays and prediction of seroreversion with MultiCruzi using an interpretation formula.

Findings Combining experimental data from discrete analysis of 15 *T cruzi* antigens efficiently predicted seroreversion at an early stage, which was later confirmed by conventional *T cruzi* serology. In cohort 1 (n=69), which included children of three different age groups, we observed differences 2 years after therapy. In the 27 individuals from cohort 1 who were treated within the first 12 months of age, MultiCruzi predicted early seroreversion in 21 (78%) patients whereas nine (33%) patients showed seroreversion with Chagatest ELISA (seroreversion difference 0·44, 95% CI 0·26–0·63; p=0·0005). In the 12 patients from cohort 1 treated between 1 year and 2 years of age, MultiCruzi predicted early seroreversion in six (50%) patients, whereas only one (8%) patient was confirmed to be seronegative with Chagatest ELISA (seroreversion difference 0·42, 95% CI 0·14–0·70; p=0·0253). In the 30 patients from cohort 1 who were treated between 2 years and 19 years of age, MultiCruzi predicted early seroreversion in five (6%) patients, whereas no patients were found to be seronegative with Chagatest ELISA (seroreversion difference 0·17, 0·03–0·30; p=0·0253). In cohort 2 (n=27), which included only children younger than 1 year of age and had a shorter follow up (between 5 months and 32 months), the proportion of reported events was significantly different 180 days after treatment for the *T cruzi*-positive group (early seroreversion predicted in nine [90%] of ten patients with MultiCruzi and confirmed seroreversion in four [40%] of ten patients with Chagatest ELISA; seroreversion difference 0·50, 95% CI 0·19–0·81; p=0·0253) and for the *T cruzi*-negative group 90 days (early seroreversion predicted in five [29%] of 17 patients with MultiCruzi and confirmed seroreversion in one [6%] of 17 patients with Chagatest ELISA; seroreversion difference 0·24, 0·03–0·44; p=0·0455) and 180 days (early seroreversion predicted in 17 [100%] of 17 patients with MultiCruzi and confirmed seroreversion only in seven [41%] of 17 patients with Chagatest ELISA; seroreversion difference 0·59, 0·35–0·82; p=0·0016) after treatment.

Interpretation The MultiCruzi assay can be used as a predictive monitoring tool to assess parasitological cure in children. This approach might be a solution to forecast forthcoming seroreversion in treated adults infected with *T cruzi*, but this requires further investigation.

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For the Spanish translation of the abstract see Online for appendix 1

For the Portuguese translation of the abstract see Online for appendix 2

For the French translation of the abstract see Online for appendix 3

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Research in context

Evidence before this study

We searched PubMed and ClinicalTrials.gov for studies published between Jan 1, 1980, and June 1, 2020, with the keywords "Chagas disease", "monitoring", "parasite clearance", "treatment efficacy", "clinical trials", "parasitological cure", "*Trypanosoma cruzi*", and "Chagas disease diagnostics", with no language restrictions. Our search indicated that the major obstacle to treating Chagas disease is the difficulty in assessing whether patients infected with *Trypanosoma cruzi* have successfully eliminated the parasite. Although progress in Chagas disease research has been made during the past decade, clinicians still face the same challenge—ie, the absence of a standardised and validated test of parasitological cure to guide further treatment decisions, or to determine the risk of maternal–fetal transmission during pregnancy. Although there is no experimental proof in patients that full disappearance of anti-*T cruzi* antibodies is synonymous with absence of the parasite, there is a consensus that parasitological cure can be monitored via the conversion of patient serological status from positive to negative using conventional serological tests. However, seroreversion might take several years to decades in adults, depending on the time between infection and treatment administration, making evaluation of treatment efficacy difficult within standard clinical trial settings.

Added value of this study

Our study population included neonates, infants, and children infected with *T cruzi* and monitored up to 20 years of age. The advantage of observing the serology of this population is that children have been shown to become seronegative more rapidly than adults. Therefore, we were able to observe the kinetics of antibody decline and potential treatment effect on serology. We showed, for the first time to our knowledge, that the MultiCruzi ELISA assay can robustly measure antibody signals, thanks to its multiparametric nature. Furthermore, when used in combination with an interpretation formula, the assay might be able to predict the disappearance of antibodies and give a potential insight into parasitological cure in children infected with *T cruzi*. The test highlights the concept of so-called serological signatures, and could enrich studies of serological profiles in different populations. This study adds value because of its original and interdisciplinary approach and might represent a framework shift for predicting serological outcomes after treatment in individuals infected with *T cruzi*.

Implications of all the available evidence

Mathematically driven approaches and the use of selected clinically proven biomarkers are important strategies for evaluation of parasitological cure in patients with Chagas disease and for speeding up efficacy evaluation of new anti-parasitic drugs. In the future, such approaches might permit appropriate patient counselling following treatment, promote acceptance of treatments for Chagas disease, and facilitate clinical trial evaluation. A model built around the paediatric population could serve as a basis for the construction of predictive models in adults.

Introduction

Chagas disease—caused by *Trypanosoma cruzi*, a protozoan parasite—accounts for 7000 deaths per year and is currently estimated to have infected over 6 million people, imposing a massive economic burden on endemic countries throughout Latin America, and increasingly in the USA and Europe due to globalisation and human migration.[1,2] The major transmission route in endemic areas, such as Gran Chaco in Latin America, is via a haematophagous triatomine vector. The Pan American Health Organization estimates that throughout Latin America, 65 million people are at risk of Chagas disease because of potential exposure to infected vectors.[1] In areas under vector control and in non-endemic areas, vertical transmission is increasingly reported due to migration of infected women of childbearing age. Vertical transmission of *T cruzi* occurs in 5–8% of newborns from mothers infected with *T cruzi*.[3] An estimated 40 000 infected women of childbearing age live in the USA, where several hundred congenital Chagas disease cases are expected to occur annually.[4] Fortunately, anti-trypanocidal treatment is effective in neonates and children if administered shortly after infection.[5] Thus, early diagnosis and treatment of congenital Chagas disease is of the utmost importance to eliminate the parasite and prevent progress towards chronicity and severe clinical symptoms.

Direct parasite detection techniques in neonates (micromethods) are cumbersome and technically difficult as they require expert microscopic examination or *T cruzi* PCR that requires a high volume of sample. Although useful, PCR can fail to detect parasites because parasitaemia is extremely low and fluctuates over time in patients with chronic Chagas disease, and parasites can hide in tissues and organs.[6,7] In routine settings, parasitological micromethods applied to infants fail to detect more than 50% of infections.[3] Another obstacle clinicians face is that, during pregnancy, maternal IgG antibodies are passively transferred to the fetus, making them seropositive even though they might not necessarily be infected with the parasite. At birth, it is virtually impossible to know whether the neonate is infected based solely on *T cruzi* serological tests. Only monitoring antibody dynamics during the first year of life would allow for such a diagnosis. Therefore, an optimal diagnostic interpretation model for congenital Chagas disease requires a multistep procedure starting from maternal screening with longitudinal follow-up of infants by PCR and serology testing.[3] If serology remains steadily positive after 8 months of age, an infant is infected and needs to

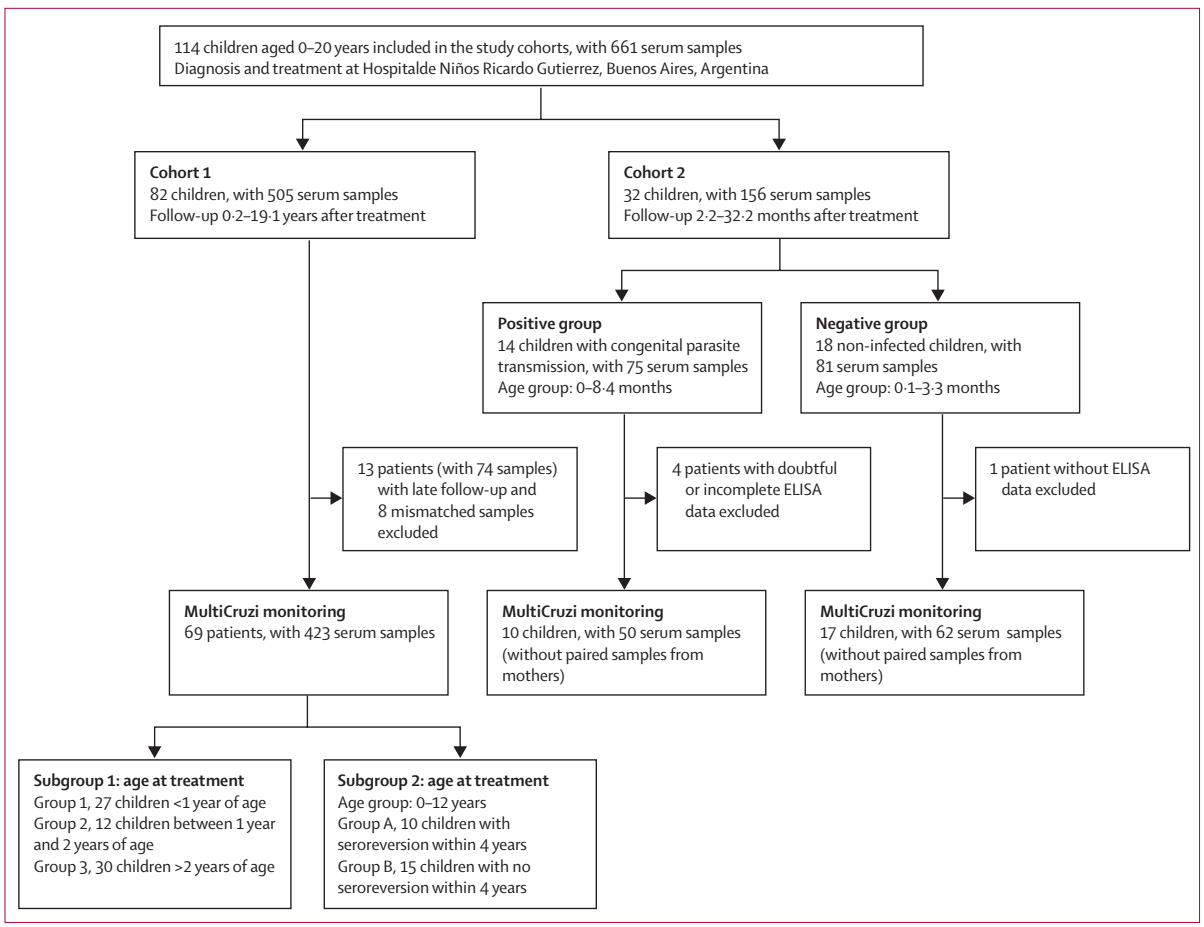

Figure 1: **Flow diagram of participants enrolled in the study**

be treated.[8] The main difficulty in monitoring parasitological cure stems from the need for long-term follow-up to observe the complete disappearance of anti-*T cruzi* antibodies. Indeed, seroreversion by standard serological tests can take years to decades.[6,9,10] This factor has an impact, not only for counselling of patients with Chagas disease, but also for evaluation of the results of clinical trials and the development of new drugs. Thus, there is an urgent need for qualified tests to assess treatment efficacy in children and adults infected with *T cruzi* as soon as possible after therapy.[11] A previously developed antibody multiplex assay, called MultiCruzi (InfYnity Biomarkers, Lyon, France), has shown good performance for serological confirmation of Chagas disease.[12,13] This assay allows monitoring of the reactivity of 15 highly specific antigens and provides the opportunity to study the diversity of serological profiles. Given the robust evaluation of signal intensity, this technique could be an interesting tool to monitor evolution of the combination of multiple antibody reactions over time.

We aimed to evaluate the MultiCruzi assay as a predictive tool for parasitological cure in a cohort of treated infants and children with acute and chronic Chagas disease enrolled in a long-term retrospective longitudinal study with clinical, serological, and parasitological follow-up, and to explore whether MultiCruzi could predict parasitological cure more quickly than the current reference method.

Methods

Study design, population, and origin of the samples

Patients from two retrospective paediatric Chagas disease cohort studies[5] with clinical, serological, and parasitological follow-up, diagnosed and treated at the parasitology service, Hospital de Niños Ricardo Gutierrez (Buenos Aires, Argentina) were included in the study (figure 1). The main advantage of studying samples from infant and child populations is that they show faster seroreversion of *T cruzi* antibodies by conventional serological tests upon effective treatment compared with adults.[5,14]

The inclusion criteria for cohort 1 were as follows: children with Chagas disease treated with benznidazole or nifurtimox, with at least 6 years follow-up after treatment. Patients with chronic diseases (renal, hepatic, or neurological) and congenital heart diseases were not

eligible for the study. Serum samples were collected every 6 months to 12 months between Oct 22, 1990, and June 3, 2019. This cohort comprised 82 infants and children with Chagas disease who were treated with the anti-parasitic drugs benznidazole or nifurtimox at standard doses. The median age at diagnosis was 2·0 years (range 0–20). The median serological follow-up time after treatment was 8·2 years (IQR 5·3–11·0; figure 1). Chagas disease was diagnosed in patients younger than 8 months by direct observation of *T cruzi* using microhaematocrit test or quantitative (q)PCR; patients older than 8 months were diagnosed by *T cruzi* by conventional serology, positivity being confirmed through reactivity of two of the three following techniques: indirect haemagglutination, ELISA, or particle agglutination. During follow-up, a decrease in *T cruzi* antibodies was observed by conventional serology, and seroreversion occurred in 32 (39%) of 82 patients. 13 individuals were excluded from the analysis because their respective follow-up started too late after the first treatment and therefore could not produce early enough post-therapy information. 69 children (33 boys and 36 girls) were therefore eligible for the analysis.

The inclusion criteria for cohort 2 were as follows: children younger than 1 year of age, born to a mother with a positive serology test for Chagas disease. Patients who had previously received treatment for Chagas disease who were unable to complete the scheduled visits, or with another disease that could complicate interpretation of results, were excluded. Serum samples were collected 1 month after birth and then every 3 months for 1 year between July 23, 2012, and April 19, 2016. This cohort comprises 32 infants and children with shorter follow-up (figure 1). 23 (72%) of 32 infants and children were matched with their mothers' samples. The diagnostic criteria for congenital *T cruzi* infection were as follows: in infants younger than 8 months, parasite presence confirmed by direct parasitological microhaematocrit test, in infants older than 8 months, positive *T cruzi* conventional serology by two of three of indirect haemagglutination, ELISA, or particle agglutination, mother with Chagas disease, or did not receive blood transfusions and had not resided in an area endemic for the Chagas disease vector (ie, could not have been exposed to an alternative source of Chagas disease).

The cohort was divided into two groups. The first group comprised 14 infants infected with *T cruzi*, with a median age of 0·8 months (IQR 0·2–3·1) treated with benznidazole 5–8 mg/kg/day in two to three doses for 60 days as soon as parasitaemia was confirmed. Patients were checked every 3 months by Chagatest ELISA (Wiener Lab, Rosario, Argentina), haemagglutination, and qPCR until both parasitaemia and serology results were negative after treatment. The median serological follow-up time after treatment was 8·3 months (IQR 6·8–9·7). Four patients were excluded from the analysis because of inconsistent or incomplete results in sample collection. Thus, ten infected infants (four boys and six girls) were eligible for analysis. The second group comprised 18 non-infected infants with positive serology (maternal transfer of antibodies) with a median age of 1·1 months (IQR 0·4–1·7). Serological follow-up every 3 months by conventional serology showed the disappearance of antibodies at 8 months. The median follow-up time was 8·4 months (IQR 7·4–9·4). One patient was excluded from the analysis because of incomplete serological test results. 17 infants (seven boys and ten girls) were eligible for analysis. Samples were stored at –20°C with the prior authorisation of the patients' parents and were sent to InfYnity Biomarkers for MultiCruzi analysis.

Cohort 1 was subdivided into three groups based on patient age at the start of treatment and to avoid any bias related to residual maternal antibodies in infant bloodstream. Group 1 included individuals treated within the first 12 months of age (n=27), group 2 included individuals treated between 1 and 2 years of age (n=12), and group 3 included all children treated after the age of 2 years (n=30).

To explore whether prediction of seroreversion, upon drug treatment, can determine whether a patient with Chagas disease has successfully responded to treatment in a timely manner, cohort 1 was further investigated by restricting patients to a follow-up shorter than 4 years, with an age group limited to 0–12 years, of which group A (n=10) showed seroreversion with conventional *T cruzi* ELISA within 4 years and group B (n=15) showed no seroreversion within 4 years. A similar timespan and age group were previously used to monitor treatment efficacy in children.[15,16]

Study protocols were reviewed by the Research & Teaching Committee and the Bioethics Committee of the Ricardo Gutierrez Children's Hospital (Buenos Aires, Argentina) and are compliant with the national and international standards for Chagas disease. The studies were done with the consent of parents and assent of children, to whom the study was explained orally, and a consent form was signed by parents. From the age of 7 years, consent was requested from children and signed by parents. From the age of 16 years, children signed the consent form themselves. The study was done in accordance with the harmonised tripartite standards for good clinical practice and the convention of children rights included in the national constitution and the legislation of the government of the city of Buenos Aires. Anonymity of the patients included in the studies is ensured. Patient identity was kept confidential in compliance with the personal data protection law no 25326 (Argentina), and was encoded in the results of the studies (identification through a code of numbers and letters).

Serological tests

We used the conventional serological method to detect circulating anti-*T cruzi* antibodies and results were

interpreted according to the manufacturer's instructions (Chagatest ELISA) and used as comparator for the MultiCruzi test. For every patient in both cohorts, serology was evaluated at the initial visit and repeated at each visit during follow-up after treatment. During the 29-year period of patient recruitment and sample collection, two versions of Chagatest were used (lysate-based or recombinant-based). The recombinant version uses a mixture of six recombinant antigens (SAPA, antigen 1, antigen 2, antigen 13, antigen 30, and antigen 36). These highly conserved antigens are from specific proteins of the epimastigote and trypomastigote stages of *T cruzi*. The lisado version uses parasite lysates of different *T cruzi* strains. There is good correlation between the two methods.[17,18] The Chagatest assay was validated by internal controls with a required minimum optical density for positive controls and a maximum optical density for negative controls. The mean difference between positive and negative controls should not be below 1·2. All steps were validated following the manufacturer's instructions. As per the manufacturer's guidelines, the cutoff was determined as the mean optical density on the negative control plus 0·2. Samples were considered reactive when the absorbance was greater or equal to the determined cutoff value. Obtained reference results were shared by the Hospital de Niños Ricardo Gutierrez.

We used the MultiCruzi immunoassay, developed by InfYnity Biomarkers, to monitor serological signatures and evaluate its usefulness as a predictive tool for parasitological cure.[12,13] The sciFLEXARRAYER bioprinting system (SCIENION, Dortmund, Germany) was used to print 15 *T cruzi* antigens, selected for their proven immunogenic properties, in duplicates into each well of a 96-well plate, under controlled humidity and temperature conditions (appendix 4 p 1). Relevant antigens were designed and obtained synthetically according to reviewed and published non-redundant sequences from UniProt. Of the 15 antigens, three were discrete typing unit (DTU)-specific derived from the sequences of TcI, TcII, and TcVI of *T cruzi* DTU. The other 12 antigens were not specific to any *T cruzi* strain and are conserved across DTUs. Additionally, positive control spots were printed in triplicate to define a precise spatial orientation pattern and validate the correct sequential distribution of all biological and chemical materials (human serum samples, enzyme conjugate, and substrate). To facilitate the visual interpretation of the test, cutoff control and medium control spots were embedded.

The MultiCruzi test was done as described previously.[12] Each plate was read and analysed using the sciREADER CL2 (SCIENION). The software calculates the median pixel intensity for each spot with the background noise subtracted. To establish the net intensity for each antigen, the mean value of duplicated spots was considered. Each batch of printed plates was validated with eight *T cruzi*-positive samples and eight *T cruzi*-negative sera or plasma samples from blood donors targeting reference intensities for the 15 antigens. Obtained results were made available to the assessors of the Chagatest assays.

Both Chagatest and MultiCruzi detect specific IgG antibodies against *T cruzi*. In Chagatest the measured general signal reflects the immunoreactivity of a mixture of antigens, whereas the MultiCruzi test can record informative antibody profiles of 15 antigens tested under identical experimental conditions. Both tests share the same enzymatic reaction—a peroxydase reacting with hydrogen peroxide and revealed by tetramethylbenzidine. The substrate used for the Chagatest is soluble, whereas a precipitation substrate is used for the MultiCruzi test.

Database

The raw data obtained as the output from the image analysis reader were submitted to validation steps using the internal controls embedded in each test. The mean pixel intensity of the reaction was corrected for background noise, expressed as a value ranging between 0 and 130, and used without preprocessing in the analysis. Inconsistent or aberrant signal measures or antigen patterns were retested. Patients with incomplete follow-up, insufficient ELISA data, absence of pre-treatment visits, or unmatched serological profiles on MultiCruzi were eliminated from the analysis (table). Validated data for both tests were incorporated into a Microsoft Excel 2016 database for further analysis.

Interpretation formula

We developed an interpretation formula to predict early seroreversion. As a reference, we used the ultimate result of conventional serology testing by ELISA, the current standard method to monitor parasitological response after treatment. By defining events for each technique (MultiCruzi assay and ELISA Chagatest), we calculated the time needed from pre-treatment to reach a sustainable event. For Chagatest, an event was defined as a change from positive to negative status (seroreversion), according to the manufacturer's instructions. Using data input from all antigens of the MultiCruzi assay, sustainable seroreduction was considered an event by the following method: at baseline, before any therapy, each patient was assessed for the count of initially reactive antigens reflecting the antibody diversity of each individual. At follow-up sampling, the signal decline was measured for each antigen. If at least half of the initially reactive antigens showed a 30% or more sustainable signal reduction compared with baseline, or at birth (in case of no treatment), then a patient was predicted to become seronegative at a later stage. The cutoff for signal reduction was set to 30% to exceed the maximum technical variability that might occur in immunoassay measurements. If an antigen reacted below the cutoff intensity at baseline, the antigen was no longer considered for the rest of the follow-up. This low intensity

See Online for appendix 4

	n (%)
Cohort 1	
Total	69 (100%)
Sustainable decline	45 (65%)
Non-sustainable decline	3 (4%)
Decline only at last follow-up visit	15 (22%)
No decline	6 (9%)
Cohort 2	
Total	27 (100%)
Sustainable decline	23 (85%)
Non-sustainable decline	1 (4%)
Decline only at last follow-up visit	2 (7%)
No decline	1 (4%)

Decline was considered sustainable if it occurred during the remaining follow-up visits and not sustainable if it occurred only during isolated visits in the middle of the follow-up period.

Table: **Proportion of patients with a decline in antigen reactivities in the MultiCruzi assay**

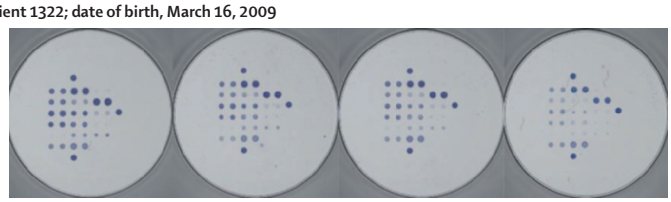

Figure 2: **Serological profiles of two patients followed up after treatment**
Antigen reactivities progressively declined after treatment. In both patients, the serological pattern was consistent from one visit to another. Around 4·5 years (patient 1322, cohort 1; A) and 8·5 months (patient 1489, cohort 2; B) after treatment, the overall antigen reactivities were markedly lower.

cutoff was based on the minimum measurable signal that provides acceptable reproducibility. Briefly, a patient was predicted to become seronegative at a later stage if at least half of the initially reactive antigens showed a 30% or more sustainable signal reduction compared with baseline. In case the event did not occur, the participant was censored at the last available timepoint (or visit). An advantage of this multiparametric approach is that the signal is composed of reactivities from 15 separate antigens (ie, the event is empowered by the possible signal reduction of every immune reaction).

Statistical analysis

We used graphical representations similar to univariate survival curves (Kaplan-Meier analysis) to investigate seroreduction as a prediction for seroreversion. Although the percentage curves presented in this study were calculated for the same patients, two different definitions were used to define an event. Thus, the curves are not independent and the event definition differs. Consequently, it would be incorrect to apply survival statistics (eg, log-rank test) for comparison. The graphical representation serves only to visually compare the decline rate of both test results combined with the event definition.

However, at specific timepoints, the proportion of events was compared between both tests with McNemar's test for dependent proportions. $p<0\cdot05$ was considered to indicate a statistically significant difference, but these results are informative only, as no correction for multiple testing was done. The seroreversion rate difference calculated between both tests was referred to as the seroreversion difference. Association of age and sex with the events was investigated using Fisher's exact test.

The original studies were registered with ClinicalTrials.gov, NCT04090489 (cohort 1) and NCT04084379 (cohort 2). Analyses were done with R version 3.0.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Details of the recruitment of the participants to the study can be seen in figure 1. We analysed the results of the samples from both cohorts and the immunoreactivity of each individual antigen using a digital image analyser. For each patient, different serological profiles were obtained reflecting the large antibody diversity in patients infected with *T cruzi*. Negative and positive results could be clearly separated (appendix 4 p 2).

We explored whether the evolution of serological patterns could be a strategy to predict early *T cruzi* antibody seroreversion by conventional serology in treated patients. Using the MultiCruzi assay, a progressive and sustainable decline in antigen reactivity was found after treatment in 68 (71%) of 96 patients infected with *T cruzi* (table). Besides this general decrease in signals, the serological patterns were consistent between different timepoints (see two examples in figure 2), supporting the robustness of the signal measured for each reactivity and highlighting the importance of studying full serological profiles rather than general serological signals.

After establishing an interpretation formula to predict early seroreversion, the performance of MultiCruzi in both cohorts of children was tested. Of the 15 antigens that are included in the test, at least seven showed reactivity in each patient at the initial examination. We

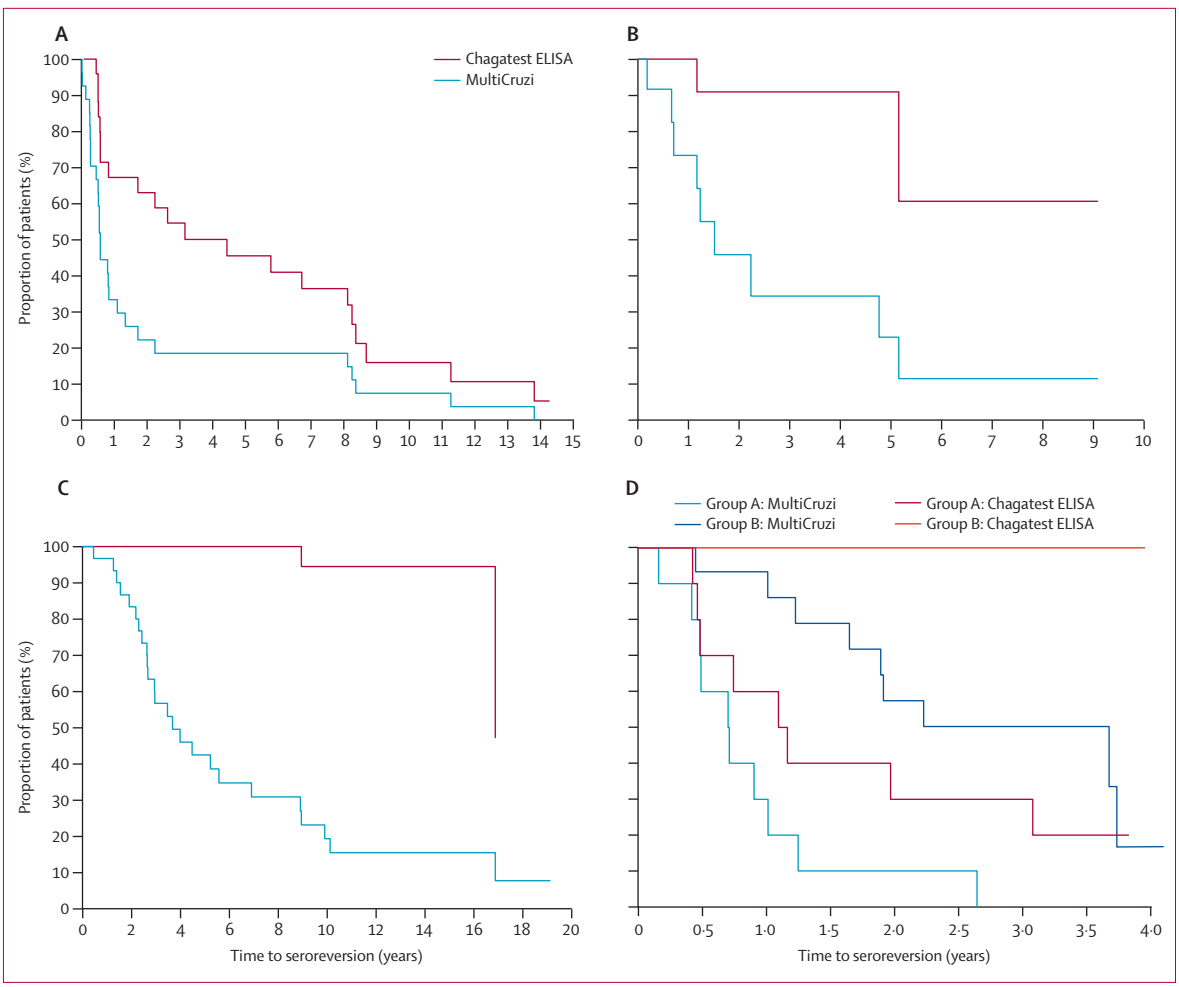

Figure 3: **Kaplan-Meier plots of time to seroreversion and prediction to seroreversion in treated individuals infected with *Trypanosoma cruzi* from cohort 1**
The proportion of treated individuals seroreverting according to conventional serology (Chagatest) and MultiCruzi during follow-up is shown. (A) Infants treated in their first year of life (n=27). (B) Children treated between 1 year and 2 years of age (n=12). (C) Children treated after the age of 2 years (n=30). (D) Patient samples are grouped into two classes: group A, seroreversion according to conventional serology visible within 4 years after treatment (n=10) and group B, no seroreversion according to conventional serology within 4 years after treatment (n=15).

observed discriminatory power among survival curves using conventional Chagatest ELISA and MultiCruzi (figure 3). MultiCruzi predicted early seroreversion more rapidly than conventional ELISA assay. In the 27 individuals from cohort 1 (group 1) who were treated within the first 12 months of age, MultiCruzi predicted early seroreversion in 21 (78%) patients, whereas nine (33%) patients showed seroreversion with Chagatest ELISA (seroreversion difference 0·44, 95% CI 0·26–0·63; p=0·0005). MultiCruzi could predict early seroreversion in 50% of patients within 6 months, whereas seroreversion using conventional serology was reached in 50% of patients after 3 years (figure 3A). In the 12 patients from cohort 1 treated between 1 year and 2 years of age (group 2), MultiCruzi predicted early seroreversion in six (50%) patients, whereas only one (8%) patient was confirmed to be seronegative with Chagatest ELISA (seroreversion difference 0·42, 0·14–0·70; p=0·0253). MultiCruzi could predict early seroreversion in 50% of patients within 1·5 years, whereas seroreversion using conventional serology was only reached in 40% of patients after 9 years (figure 3B). In the 30 patients from cohort 1 who were treated between 2 years and 19 years of age (group 3), MultiCruzi predicted early seroreversion in five (6%) patients, whereas no patients were found to be seronegative with Chagatest ELISA (seroreversion difference 0·17, 0·03–0·30; p=0·0253). MultiCruzi could predict early seroreversion in 50% of patients within 4 years, whereas seroreversion using conventional serology was reached in 50% of patients after 17 years of follow-up (figure 3C). When comparing the proportion of events at a specific time of appearance between both methods using McNemar's test, MultiCruzi and Chagatest were different at multiple timepoints, after 6 months for group 1, and after 2 years and 5 years for group 2 and group 3 (appendix 4 p 3). These data show a clear

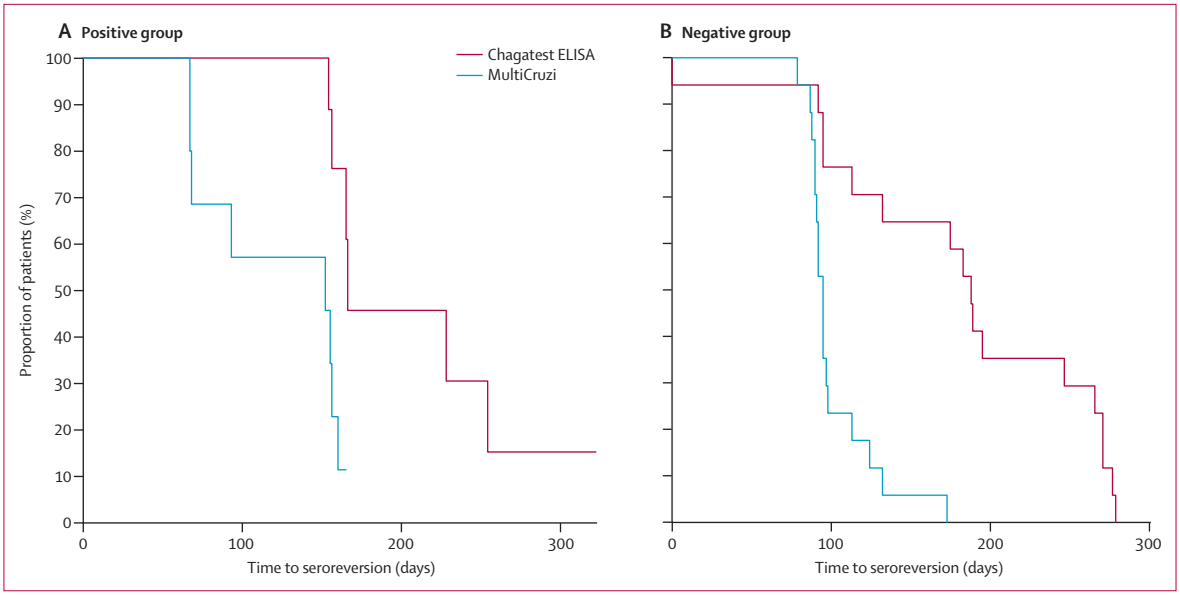

Figure 4: **Kaplan-Meier plots of time to seroreversion and prediction to seroreversion in cohort 2**
The proportion of treated individuals seroreverting according to conventional serology (Chagatest) and prediction of time to seroreversion with MultiCruzi during follow-up of children infected with *Trypanosoma cruzi* treated with benznidazole (n=10; A) and non-infected children (n=17; B).

correlation between the age of patients and the time needed to declare seroreversion—ie, the time required to predict seroreversion increases as the age of patients with *T cruzi* at the start of treatment increases. All patients confirmed as seronegative by conventional serology were predicted to serorevert earlier when using MultiCruzi. An association between age groups (< 1 year, 1–2 years, and >2 years) and test event was found for Chagatest (p<0·0001) but not for MultiCruzi (p=0·1359). We found no association between sex and test event (Chagatest p=0·43; MultiCruzi p=0·79).

We further analysed cohort 1 by restricting our analysis to patients with a follow-up shorter than 4 years and an age range limited to 0–12 years (figure 3D). In patients who showed seroreversion with conventional *T cruzi* ELISA within 4 years (group A), using the MultiCruzi assay, 80% were predicted to become seronegative after 1 year, whereas with Chagatest seroreversion was observed only after 3 years. In patients who showed no seroreversion within 4 years (group B), the MultiCruzi assay predicted seroreversion of 50% of patients less than 2·5 years after treatment, whereas with Chagatest no seroreversion was observed. We found no difference between the two tests in group A. However, in group B, we observed a difference between the methods 2 years after treatment (appendix 4 p 3). This finding indicates that the MultiCruzi might have the capacity to efficiently predict seroreversion in patients who do not serorevert in a set time period.

We applied the predictive interpretation formula to cohort 2. The serology signals in the samples reflect the passively transferred maternal antibodies and the antibodies generated by the childrens' immune systems. Using MultiCruzi data, 90% of the patient group was predicted to become seronegative after 165 days versus more than 320 days using the Chagatest ELISA assay (figure 4A). Similar results were obtained with infants and children theoretically cleared of the parasite. In this group, the signals measured reflect the progressive clearance of maternal antibodies. 90% of this patient group was predicted to become seronegative after 130 days with MultiCruzi versus 275 days with the Chagatest ELISA assay (figure 4B). Between 80 days and 100 days after treatment, the proportion of patients predicted to become seronegative increased sharply from 5% to 75%, possibly as a result of considerable clearance of maternal antibodies. McNemar's test revealed a difference in the proportion of events registered 180 days after treatment for the positive group (early seroreversion predicted in nine [90%] of ten patients with MultiCruzi and confirmed seroreversion in four [40%] of ten patients with Chagatest ELISA; seroreversion difference 0·50, 95% CI 0·19–0·81; p=0·0253). For the negative group, we observed differences after treatment at 90 days (early seroreversion predicted in five [29%] of 17 patients with MultiCruzi and confirmed seroreversion in one [6%] of 17 patients with Chagatest ELISA; seroreversion difference 0·24, 0·03–0·44; p=0·0455) and at 180 days (early seroreversion predicted in 17 [100%] of 17 patients with MultiCruzi and confirmed seroreversion in seven [41%] of 17 patients with Chagatest ELISA; seroreversion difference 0·59, 0·35–0·82; p=0·0016; appendix 4 p 3).

Discussion

Full disappearance of *T cruzi* antibodies, measured by conventional serology tests, is the current standard for

monitoring parasitological cure.[9,19] Yet this approach is not suitable for routine testing, as it can take decades for full seroreversion to occur in treated adults.[6,9,10] In this study, we used a multiplex assay to explore the correlation between antigen reactivities at given timepoints and prediction of seroreversion. We analysed the serological follow-up of two cohorts of children, treated with either benznidazole or nifurtimox, with 15 selected *T cruzi* antigens, and documented changes in their serological profiles subsequent to therapy. Using an interdisciplinary approach, experimental data from the MultiCruzi assay and reference data from a conventional serology assay were incorporated into a predictive formula and were able to show that predictions based on the experimental MultiCruzi results were sufficient to forecast future seroreversion.

This study assessed therapeutic response, which is an unmet medical need in patients with Chagas disease.[20,21] Over the past two decades, efforts have been made to identify adequate surrogate biomarkers of parasite clearance.[22–24] However, to date, only full and sustainable seroreversion is considered to be adequate—such markers require decades of follow-up. Using multiplex screening technology, a single specific antibody (Ab3) has been shown to be a potentially valuable biomarker to monitor parasite persistence, since it is found in a high percentage of PCR-positive adult patients with Chagas disease and is substantially reduced in patients after therapy or PCR-negative samples.[13] The findings presented in this report suggest that if all 15 IgG antigen reactivities integrated in the MultiCruzi protein array are measured simultaneously and interpreted with a predictive interpretation formula, the assay could predict early seroreversion in children. We observed good correlation between seroreversion predicted by the MultiCruzi assay and seroreversion effectively detected by standard Chagatest ELISA—all children that showed seroreversion could be reliably predicted using the MultiCruzi prediction model. The multiparametric aspect of MultiCruzi gives it an advantage over standard Chagatest ELISA because it records the information for different antigens individually. Each antigen provides information about the development of reactivity and the possible antibody load of the patient. This combination of independent variables enhances analysis and provides a reliable and robust indication of signal extinction associated with decreasing antibody counts. This sustained reduction throughout consecutive follow-up is an encouragement to further investigate the predictive value of MultiCruzi in adult patients. In conventional serology, the general signal measured reflects the immunoreactivity of a mixture of antigens and can be indirectly mitigated by non-reactive antigens, thus minimising the possible change in patient serology. The reactivity measured for cohort 1 and cohort 2 before treatment showed a minimum of seven reactive antigens, which constitutes enough information to predict seroreversion.

The MultiCruzi assay is a useful tool for determination of serological signatures compared with a collection of well-defined *T cruzi*- specific antigens. Determination of serological signatures is not possible in conventional screening assays, which can only measure signals of antibody dynamics. It is important to have a reliable method to identify neonates in need of trypanocidal therapy, thus avoiding unnecessary treatments and associated side-effects.[25] Furthermore, monitoring serological profiles allows an improved understanding of the dynamics of the immune response for each individual. For instance, the evolution of serological signatures might inform about tiny changes after therapy more rapidly and reliably than conventional serology techniques and might ensure optimal medical care with minimal anxiety for patients. Finally, our findings are not only relevant for Chagas disease, but also other infectious diseases, such as syphilis, where conventional serology testing remains the recommended approach to monitor treatment efficacy.[26]

Detailed validation of the MultiCruzi assay and adjustment of the prediction interpretation formula for adults could pave the way for this strategy to monitor clearance of parasite antibodies. Specifically, it is essential to evaluate and fine tune the prediction model for other study cohorts, including children and adults from different regions. This is particularly important as the serological course after treatment varies depending on time and duration of infection. The antibody dynamics in adults are known to be slow (decades); therefore, only reliable seroreduction based on multiple antigens can provide rapid information. This result could be achieved consistently by a serial dilution approach that helps with titrating antibodies. This approach to addressing seroreduction in adults is currently under investigation in our laboratories.

The limitations of this study concern its retrospective nature and the serological approach, which considered negative serology as a surrogate marker of treatment efficacy, as proposed by a wide consensus.

In conclusion, we propose a framework shift in the prediction of serological outcomes after treatment in individuals infected with *T cruzi*, by using a specific and sensitive multiplex assay combined with mathematical models. Our findings suggest that the MultiCruzi assay provides reliable information to detect and monitor patients infected with *T cruzi*. Future mathematically driven approaches could radically transform how parasitological cure in patients with Chagas disease is evaluated and speed up the assessment of efficacy of new anti-parasitic drugs in clinical trials.

Contributors

LJM, EC, PL, MZ, EmC, and JA wrote the report. LJM, EmC, GB, NG, LM, HP, JdB, MZ, ErC, and JA were responsible for the acquisition, analysis, or interpretation of data. JA, ErC, and MZ were responsible for study concept and design. All authors provided critical feedback on the manuscript. All authors had full access to all data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests
EC, LM, PL, and MZ are employed by InfYnity Biomarkers. All other authors declare no competing interests.

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