Serological reactivity against *T. cruzi*-derived antigens: Evaluation of their suitability for the assessment of response to treatment in chronic Chagas disease.

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ABSTRACT

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, affects more than 6 million people worldwide. Following a mostly asymptomatic acute phase, the disease progresses to a long-lasting chronic phase throughout which life-threatening disorders to the heart and/or gastrointestinal tract will manifest in about 30% of those chronically infected. During the chronic phase, the parasitemia is low and intermittent, while a high level of anti-*T. cruzi* antibodies persist for years. These two features hamper post-chemotherapeutic follow-up of patients with the tools available. The lack of biomarkers for timely assessment of therapeutic response discourages a greater use of the two available anti-parasitic drugs, and complicates the evaluation of new drugs in clinical trials. Herein, we investigated in a blinded case-control study the serological reactivity over time of a group of parasite-derived antigens to potentially address follow up of *T. cruzi* chronically infected subjects after treatment. We tested PFR2, KMP11, HSP70, 3973, F29 and the InfYnity multiplexed antigenic array, by means of serological assays on a multi-national retrospective collection of samples. Some of the antigens exhibited promising results, underscoring the need for further studies to determine their potential role as treatment response biomarkers.

1. Introduction

Chagas disease, caused by infection with the protozoon parasite *Trypanosoma cruzi*, affects more than 6 million people, mainly in Latin America where vectors that transmit the infection are endemic (WHO Media Centre, 2018). The risk of infection is directly associated with poverty, and migratory movements have changed the epidemiological pattern of the disease in recent decades (Gascon et al., 2010).

Two phases can be observed in the progression of the disease: acute and chronic. The former is short-lived and characterized by detectable parasitemia levels, but since it is generally asymptomatic or has non-specific mild symptoms it goes unnoticed and untreated (Rassi et al., 2010). It is followed by a life-long chronic phase throughout which the parasite persists in tissues. About 70% of chronically infected individuals remain asymptomatic (indeterminate form), but the remaining 30% will ultimately develop cardiac and/or digestive associated pathology (determinate form) (Gascon and Pinazo, 2015; Rassi et al., 2010).

Diagnosis of the acute phase consists in detecting bloodstream circulating parasites by direct parasitological examination, or the

parasite DNA by highly sensitive molecular amplification techniques like quantitative PCR (qPCR) (Picado et al., 2018). In the chronic phase parasitemia is low and intermittent, which hinders direct detection of the parasite (Alonso-Padilla et al., 2017). Diagnosis is then achieved with serological methods, by means of the detection of specific anti-*T. cruzi* IgG class immunoglobulins. Due to genetic and antigenic diversity of the parasite (Zingales, 2018), two serological tests based on distinct antigenic sets are recommended for definitive diagnosis (Pan-American Health Organization, 2018).

Two anti-parasitic drugs are available to treat the infection: benznidazole (BNZ) and nifurtimox (NFX) (Gascon and Pinazo, 2015; Rassi et al., 2010). Lately, there has been a paradigm shift in Chagas disease treatment based on clinical and immunological evidence, agreeing in favor of providing anti-parasitic treatment for all chronically infected subjects who do not have advanced tissue damage (Viotti et al., 2014). BNZ and NFX are highly efficacious when administered early during the acute phase, and nearly 100% effective in the treatment of newborns that congenitally acquired (acute) *T. cruzi* infection (Carlier et al., 2015). Moreover, both are better tolerated by children compared to adults (Altcheh et al., 2011). Data from a randomized study with chronically infected Bolivian adults, using clearance of the parasite or decrease in parasitemia (evaluated by qPCR) as therapeutic response criterion, indicated a BNZ efficacy of 82% (37/45) at 12-month follow-up (Torrico et al., 2018). However, the "gold standard" to assess therapeutic response is conventional serology seroconversion (Pan-American Health Organization, 2018). But it can take decades for a *T. cruzi*-infected subject to become seronegative after treatment, at least when evaluated with current serological assays (Fabbro et al., 2007; Sguassero et al., 2018; Viotti et al., 2006). As a result, there are no tools available for a timely treatment-response assessment. Absence of early biomarkers of cure hinders patients follow-up in their daily clinical management or during the performance of clinical trials (Alonso-Padilla et al., 2020; Gomes et al., 2009). For the latter, protocols for the molecular detection of the parasite using serial qPCRs have been proposed (Parrado et al., 2019; Torrico et al., 2018). These can reveal treatment failure when a positive result is reported. However, a negative PCR result does not necessarily indicate that the parasite has been cleared, due to the low amount and fluctuation of circulating parasites during the chronic phase and the persistence of intracellular parasites in tissues (Sánchez-Valdéz et al., 2018; Zhang and Tarleton, 1999). Besides, qPCR is an expensive technique that requires of equipped molecular biology laboratories and highly trained personnel, scarce in endemic regions.

New potential markers of early therapeutic response have been tested in different clinical contexts, although none is commercially available (Pinazo et al., 2014; Floridia-Yapur et al., 2019; Nagarkatti et al., 2020). Some of the most promising are based on specific IgG responses against *T. cruzi* antigens. In chronically infected adults, IgG levels against kinetoplastid-specific proteins Paraflagellar Rod protein 2 (PFR2), Kinetoplastid Membrane Protein 11 (KMP11), and the Heat Shock Protein 70 (HSP70), were significantly reduced at 3 – 6 months after BNZ treatment (Fernández-Villegas et al., 2011). Two years post-treatment, the decrease was either sustained or further reduced in 67%, 50% and 34% of those evaluated for each antigen (Pinazo et al., 2015). Similarly, reactivity against the immune-dominant repetitive epitope 3973, contained in the parasite TcCA-2 protein (Buschiazzo et al., 1992), was higher in adult patients having cardiac or digestive alterations as compared to those in the indeterminate phase (Thomas et al., 2012). Reactivity against 3973 decreased between 9 and 24 months post-treatment in the 49% of BNZ-treated patients in the indeterminate stage and in the 34% of those in early cardiac stages (Fernandez-Villegas et al., 2016). Besides, an increase in reactivity against 3973 was observed in 24% of indeterminate and 36% of symptomatic patients following treatment, which could correspond to therapeutic failure (Fernandez-Villegas et al., 2016).

T. cruzi Ca^{2+}-binding flagellar protein F29 has also been assessed as an early marker of response to treatment with NFX or BNZ in children and in adults (Sosa Estani et al., 1998; Fabbro et al., 2013). Samples from treated and untreated chronically infected individuals were evaluated using an ELISA test based on recombinant F29 antigen and conventional serology. Since significant differences in the reactivity towards F29 were observed between treated and untreated patients, controlling for clinical and parasitological status, the F29-based ELISA might also be a useful method to monitor response to drug treatment.

Recently, a group of peptide antigens described by Zrein and co-workers has also been reported as part of a multiplexed assay for diagnosis (Zrein et al., 2018). It encompasses fifteen specific antigenic domains (epitopes) selected from *T. cruzi* proteins: CRA, surface antigen 2, MAP, TcD, SAPA, TcR39, TcR69, 60S ribosomal protein L19, trans-sialidase, cruzipain, KMP11, TSSA, mucin-like protein, mucin TcMUCII and Tc40 (Zrein et al., 2018), which were analyzed in an ELISA-type assay (Granjon et al., 2016). In it, all the aforementioned antigens are coated (printed) onto a single 96-well plate well at a specific position, including positive and IgG loading controls (Granjon et al., 2016; Zrein et al., 2018).

The aim of the present study was to evaluate the reactivity over time against aforementioned antigens of a retrospective collection of serum samples. These originated from different epidemiological contexts and geographical regions encompassing samples from untreated and treated chronic *T. cruzi*-infected adults. A group of sera from non-infected individuals from Bolivia was included as control. The potential applicability of the antigens as markers of treatment response was confronted to the criteria proposed in a recently published Target Product Profile (TPP).

2. Materials and methods

2.1. Ethics

The study complied with the principles of the Declaration of Helsinki. Its protocol and informed consent documents were reviewed and approved by the Ethical Committees of Fundación CEADES (ref.: NHEPACHA-16,032,013; Cochabamba, Bolivia); Hospital General de Agudos "Dr. I. Pirovano" (ref.: DI-2015–522-HGAIP) and Hospital Interzonal General de Agudos "Eva Perón" (refs.: 24/14 and 11/17) from Buenos Aires (Argentina); Consejo Superior de Investigaciones Científicas (ref.: 094/2016; Madrid, Spain); Instituto de Medicina Tropical (ref.: CEC-IMT 12/2016; Caracas, Venezuela); Nucleo de Estudos da doença de Chagas (ref.: 2.245.763; Goiania, Brazil); and Hospital Clínic of Barcelona (ref.: HCB/2013/8031; Barcelona, Spain). All patients included in the study signed an informed consent. For those under 18 years old, the informed consent was signed by the assigned tutor.

2.2. Study groups

2.2.1. Epidemiological classification

The study population included untreated and treated *T. cruzi* chronically infected subjects diagnosed at the country of origin, and non-infected individuals as control subjects. Three study groups were defined: (i) healthy subjects, seronegative to *T. cruzi* infection using two different serological techniques (group 1); (ii) untreated *T. cruzi* chronically infected subjects (group 2); and (iii) *T. cruzi* chronically infected subjects either in the indeterminate (asymptomatic) or symptomatic form that fulfill treatment criteria with BNZ or NFX (Bern et al., 2007). From the latter, those who had been followed up for at least two years post-treatment were included in group 3. Women were predominant in the three study groups and accounted for 59% of the participants. Average ages were 32.6, 44.4 and 35.8 years old, respectively in the "control" (group 1), "infected and untreated" (group 2), and "infected and treated" subjects (Supplementary Table 1).

Study collection was conformed retrospectively, and all control subjects within group 1 were from Bolivia due to unavailability of

negative control samples from elsewhere. Controls were from urban and peri-urban areas of Cochabamba (Bolivia), where there is a low incidence of other related infectious diseases like leishmaniasis. Subjects within the other groups were from six different countries in South America (Supplementary Table 1). Inclusion criteria were: participant had to be from a country and region where Chagas disease is endemic; signed informed consent given by the participant or by the participant's legal representative; and *T. cruzi* infection status diagnosed with two distinct serological tests (Pan-American Health Organization, 2018). Exclusion criteria were: pregnancy; present metabolic disorder and/or immune-suppression (accordingly to clinical history); previous treatment up to six months before BNZ or NFX administration, or throughout the follow-up period, with drugs of known trypanocidal capacity (i.e. azoles, amiodarone, dodrenarone); lack of compliance with sample quality criteria (i.e. repeatedly freeze-thawed sera); no evidence of any other concomitant infectious disease at the time of the original samples.

The serological status communicated by the laboratories of origin of the samples was confirmed using a commercially available recombinant ELISA (bioelisa Chagas, Biokit, Lliça d'Amunt, Spain). Agreement of the three diagnostics was needed for inclusion in the analysis. Subjects in group 1 ($n = 42$) had to be *T. cruzi* seronegative and asymptomatic or having chronic cardiomyopathy of non-infectious etiology. Samples from *T. cruzi*-infected individuals were from two types: 107 samples were from the same number of "infected and untreated" individuals (group 2), and 228 samples were from 114 "infected and treated" individuals (Fig. 1). It was with the 72 treated subjects from who two samples were timely available that study group 3 was conformed. All these subjects had received the standard dose of BNZ or NFX and completed 30 to 60 days of treatment. From these, the sample closest in time to treatment start (PRE) and that closest to two years from initiation of treatment were assayed (POST; between 12 and 36 months).

2.2.2. Clinical characterization of the study cohort

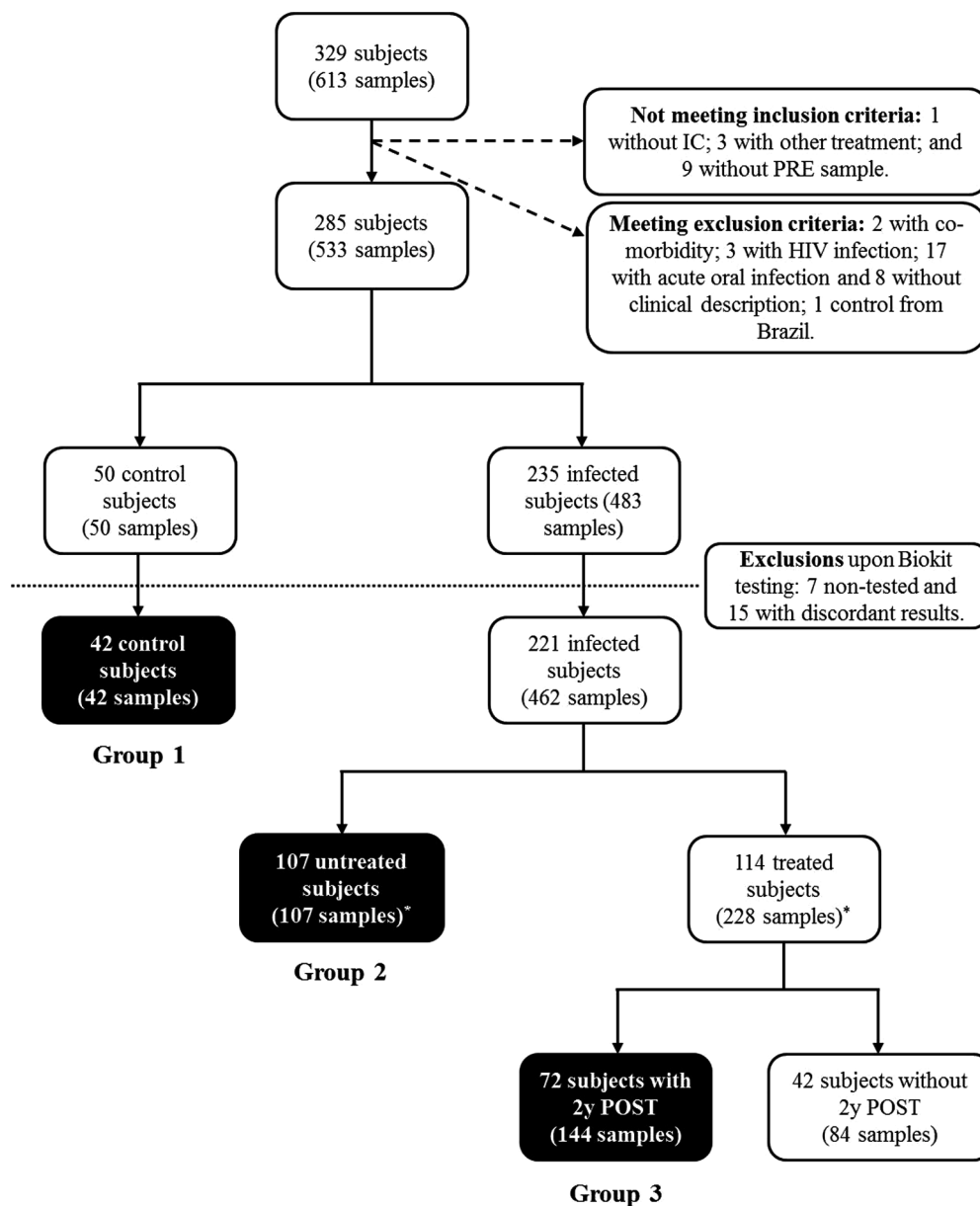*T. cruzi*-infected subjects were classified accordingly to the type and

Fig. 1. Quantitative flowchart description of the study groups consolidation. For untreated subjects we only had one sample, while from all treated subjects we used the first available sample to determine the antigens' sensitivity and specificity (*). From those treated subjects with two samples available we used the closest to treatment start (PRE) and the closest to 2 years from treatment initiation (POST; between 12 and 36 months) (group 3).

level of symptomatology associated to Chagas disease as established in the study protocol. Heart involvement and damage to the digestive tract were evaluated. Similar proportions of chronically infected subjects had demonstrable clinical manifestations in the two groups of *T. cruzi*-infected subjects (untreated or treated), and no overt symptomatology predominated in either of them (Supplementary Table 2). Cardiac involvement was most abundant and only 4/40 (10%) and 3/36 (8%) had digestive involvement, respectively in the groups of "infected and untreated" and "infected and treated" subjects. Using a modified Kuschnir̍s scale, there were similar proportions of subjects having only early heart lesions (Kuschnir̍s modified grades 0 – 1) in each of those untreated versus treated individuals. Only 15 out of 221 individuals (6.8%) were classified as Kuschnir 2 - 3, and four *T. cruzi*-infected subjects (two in each group) were not classified. Eight of those reported with Kuschnir 2 – 3 were in the untreated group. Among the remaining seven, four classified as Kuschnir 2 were treated accordingly to the clinical information recorded in the NHEPACHA database. The three Kuschnir 3 individuals included in the "infected and treated" group were at its border line. Although they did not strictly comply with treatment criteria, received treatment upon agreed decision with the patient. NHEPACHA working group decided to include these three samples into the analysis so that their information was not loss.

Co-morbidities were reported in 9% of *T. cruzi* infections (20/221; mainly immune-suppression related to HIV infection) while no such information was available for 2% (6/263) of the study population. No co-morbidities were reported for healthy subjects (group 1).

2.3. Antigens production and assaying

T. cruzi recombinant proteins KMP11, HSP70, and PFR2 were over-expressed and purified as described (Fernandez-Villegas et al., 2011), while peptide 3973 was synthesized using a simultaneous multiple peptide solid-phase method (Thomas et al., 2012). PFR2, KMP11, HSP70 and 3973 ELISAs were performed as described (Fernandez-Villegas et al., 2011; Thomas et al., 2012). F29 was produced and evaluated as previously determined (Fabbro et al., 2013). InfYnity̍s array of peptide antigens were obtained by solid-phase peptide synthesis and purified by HPLC method to obtain over 90% purity. Only cruzipain was expressed as *E. coli* recombinant protein. The multiplexed assay was performed as described (Granjon et al., 2016). On the other hand, soluble *T. cruzi* epimastigote antigens (STcA) were obtained and used as described (Fernandez-Villegas et al., 2011), while Biokit ELISA was performed following the manufacturer instructions. In all cases, samples were tested in triplicate, and positive and negative controls were included in all plates.

2.4. Data structure and study parameters

Protocols for sample selection, shipping, labeling, management and storage were established for a blinded assessment, and protocols for test the performance with each of the corresponding antigens and their pre-defined cut-off values were also defined. Source documents for the collected data were the patient̍s clinical history, laboratory analyses performed during the study, and results from cardiologic examinations. Digestive examination results were included if there had been suspicion of digestive tract involvement. The data collection and management was performed using the OpenClinica open source software version 3.13 (OpenClinica LLC and collaborators, Waltham, MA, USA).

All serum samples received a unique identifier which precluded information on their origin and any other identifier, thereby maintaining them blinded for the laboratories that performed the serological tests. Results were registered and all data procedures were centralized, managed and analyzed by ISGlobal Biostatistics and Data Management Unit.

The study parameters included: study group; diagnosis as *T. cruzi* infection positive or negative according to the serological status

provided by each participating site and confirmed by Biokit; sample time between the date of the start of treatment and date of sampling; and sample moment, which refers to whether the sample was collected before treatment (PRE) or after treatment (POST). More specifically, the latter takes into account those samples that were collected near to or after two years of follow up upon treatment.

2.5. Statistical analysis and methods

Data were described as frequencies and means (with standard deviation, SD) for discrete and continuous variables, respectively. Time and serological results for each antigen were described using median and interquartile range (IQR) values, and the Wilcoxon Rank Sum test was performed to statistically assess differences between values. Proportions were compared using Chi-squared test or Fisher's exact test if the application conditions of the former were not met. The analysis was carried out using Stata and the significance level was set at 0.05 (Stata Statistical Software: release 16, StataCorp LLC 2019, College Station, TX, USA).

In order to determine antigens' capacity to correctly classify cases (PRE samples) and controls, we calculated: (i) the proportion of correctly classified subjects or diagnostic efficacy [DE = ((TP + TN) / (TP + FP + TN + FN)) × 100]; (ii) sensitivity [Se = (TP / (TP + FN)) × 100]; (iii) specificity [Sp = (TN / (TN + FP)) × 100]; and the rates of (iv) false positives [FPR = (FP / (TN + FP)) × 100] and (v) false negatives [FNR = (FN / (TP + FN)) × 100]. Original results for each antigen under study were compared to results using the Biokit analysis. Receiver operating characteristic (ROC) curves were constructed for each of the antigens to summarize their diagnostic ability. The cut-off points in optical density readings (OD) that were used to discriminate between positive and negative readouts were provided by the laboratories of origin of the antigens (Supplementary Table 3). Alternative cut-off points were calculated as described by Frey and co-workers (Frey et al., 1998) based on the negative controls' standard deviation and sample size (N) (Supplementary Table 3).

The reactivity over time for each antigen was calculated using the median percentage change in reactivity of samples between PRE and 1 – 3 years POST treatment from group 3. Reactivity variation was calculated for each subject using the PRE time-point as reference: the difference between PRE and POST values was divided by the PRE value and multiplied by 100. When available, criteria to identify whether this variation could be associated to "therapeutic response" were provided by the laboratories developing each antigen. For instance, criteria for antigens PFR2, KMP11 and 3973 were a decrease $\geq$ 40% for POST versus PRE reactivity, while the decrease for HSP70 had to be $\geq$ 30%, and such decrease was required in at least two out of the four antigens (Egui et al., 2019). On the other hand, parasite persistence upon measuring InfYnity̍s IBAG37 (MAP) antigen reactivity is expected if its score remains $\geq$ 2, while parasite clearance would be recorded if < 2 (Zrein et al., 2018). For this specific antigen, a change (increase or decrease) is considered significant if it exceeds one score (e.g. 3 to 1 or 2 to 0.5) (Zrein et al., 2018). For F29 antigen no criteria for "therapeutic response" was provided, and changes in its response were categorized in ranges of 10% frames. In every case, a heat-map was built to summarize the evolution of the antigens' reactivity between PRE and 2-year POST treatment time-points. Less to more intense green was used for decreasing values, non-varying values are in white, increasing values were highlighted using less to more intense red, and missing values are in gray.

3. Results

3.1. Description of the study groups

Out of 533 samples retrieved upon applying inclusion and exclusion criteria, seven were not available for confirmation with Biokit, 15 gave a

discordant result between what was reported from the sites of origin and the latter, and those derived from acute cases were discarded (Fig. 1). From Biokit confirmed samples: 42 were from control subjects (group 1), and 462 samples were from a total of 221 *T. cruzi*-infected subjects (Fig. 1). Overall, Biokit results coincided with the reported original classification, indicating that the samples had been appropriately stored and shipped.

With respect to treated subjects, most were administered with BNZ for as long as 60 days (Supplementary Table 4). Concerning treatment compliance, 96% of subjects completed their treatment, and 4% of them reflected treatment interruption (Supplementary Table 4). These subjects received treatment for at least 30 days and therefore they were included in the study. Note that there were POST samples available from only 72 of those treated subjects, who ultimately encompassed group 3 (Fig. 1).

3.2. Analysis of the antigens' sensitivity and specificity parameters

Reactivity values based on OD were significantly different between infected and control populations (Fig. 2). For the qualitative analysis of the diagnostic performance of each antigen, we compared results from all *T. cruzi*-infected subjects without (group 2) or before ("infected and treated", group 3) treatment to those from subjects in the control group (group 1). "Positive" or "negative" result of any given sample was based on their antigen-specific OD and the corresponding cut-offs as defined by the laboratories of antigen origin (Supplementary Table 3). The capacity to correctly classify uninfected control subjects (group 1) and *T. cruzi*-infected subjects was calculated by comparing the "positive" or "negative" status for each corresponding antigen with that reported by the sample donor site (and confirmed using the Biokit test; Table 2).

InfYnity array had a 96% diagnostic efficacy (DE), while that of KMP11 was 92% (Table 2). These were the only that reached a Se rate > 90%. However, Sp for InfYnity array was 74%. In comparison, Sp rates for PFR2, KMP11 and 3973 were ≥ 93%. Regarding false positive (FPR) and false negative (FNR) rates, KMP11 kept both under 10% due to its very good Se and Sp registered parameters.

When ROC curves for each of the antigens used were plotted, the area under curve (AUC) values for KMP11 and 3973 were ≥ 0.960. InfYnity set of synthetic peptides yielded an AUC = 0.998. In contrast, AUC

Table 2
Summary of the classification power of each antigen evaluated.

| Parameter	PFR2	KMP11	HSP70	3973	F29	InfYnity
No. subjects	217/	211/	217/	219/	221/	221/
*	259	253	259	261	262	263
DE	74	92	55	85	72	96
Se	70	91	49	84	80	100
Sp	95	98	83	93	27	74
FPR	5	2	17	7	73	26
FNR	30	9	51	16	20	0

* Number of infected subjects over the total considered for the analysis of the performance parameters of each antigen. DE: diagnostic efficacy; Se: sensitivity; Sp: specificity; FPR: false positive rate; FNR: false negative rate. All the values are given in%. Values ≥ 85% are highlighted in bold. (IDs of all antigens used in the study can be checked at Supplementary Table 3).

values for PFR2, HSP70 and F29 were respectively 0.869, 0.698 and 0.627 (Figure S1).

HSP70 and F29 had poor performances using the cut-offs defined originally, but if these were calculated using independent methods (Frey et al., 1998) (Supplementary Table 3), their capacity to correctly identify samples within control group 1 improved. These antigens were respectively miss-classifying seven and 30 negative controls (Supplementary Table 5). Using the newly calculated thresholds, the number of miss-classified control samples was reduced to four and one, respectively (Supplementary Table 5).

3.3. Evolution of seroreactivity to antigens from pre to post treatment samples

The median time for samples taken POST treatment was 2.1 years (IQR = 0.7). The size of group 3 tested samples ranged from 65 to 72 depending on the antigen analyzed due to unavailability of missing samples. As a matter of reference, we calculated the reactivity trend of the same PRE and POST samples over the commercial ELISA test used for confirmation of the results informed by the laboratories of origin of the samples (i.e. Biokit), as well as the reactivity trend over an in-house developed ELISA [named STcA; (Fernández-Villegas et al., 2011)]
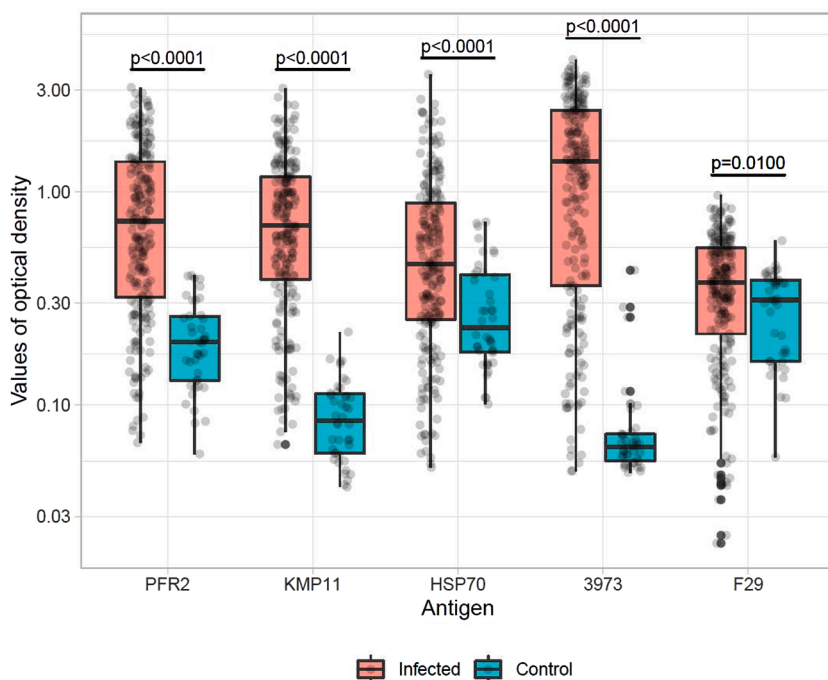
Fig. 2. Box-plots showing values of optical density (OD, presented in log scale) for each of the individual antigens tested with samples from *T. cruzi*-infected subjects and non-infected controls. Each dot represents a single sample. Medians between groups were compared using the Wilcoxon Rank Sum test. The number of control subjects' samples considered was 42 for all antigens, except for F29 that was 41. At least 211 samples from infected subjects were considered: 217, 211, 217 and 219 for PFR2, KMP11, HSP70 and 3973; and 221 for F29. Differences were due to samples availability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

based on whole *T. cruzi* lysate soluble antigens (Fig. 3A). This in-house ELISA test was developed at IPBLN laboratory (Granada, Spain) and it had also been applied over the whole collection of samples with the same purpose of the Biokit assay, i.e. to ensure the good quality status of the samples received. Direct comparison of Biokit and the in-house ELISA showed very similar results (Supplementary Table 6).

Overall, there was a tendency for a decrease in reactivity between PRE and POST for all tested antigens and control ELISAs (Fig. 3A). In general, all individual antigens and InfYnity's array showed a smaller% of samples with increased reactivity after treatment (POST to PRE) in comparison to Biokit (21%) or STcA (28%) (Fig. 3B).

Antigens with the lowest proportion of samples with increasing reactivity were KMP11 (6/65; 9%) and F29 (6/71; 8%). In fact, KMP11 (59/65; 91%), F29 (65/71; 92%) and IBAG37 (62/69; 90%) had the highest proportion of samples decreasing from PRE to POST (Fig. 3B). Interestingly, a substantial decrease (higher than 40%) in reactivity was observed in 48% of the subjects' samples evaluated on HSP70, while this reduction was 32% on PFR2, 30% on KMP11, 27% on 3973, 25% on F29 and 20% on IBAG37 (Fig. 3C). A decrease in reactivity higher than 40% was observed only in 6% and 8% of the subjects when Biokit and STcA were used (Fig. 3C). In fact, 71% of the patients showed a decrease in reactivity lower than 10% with Biokit (Fig. 3C).

Overall, antigens that reported a greater variation of median change between PRE and POST (treatment) time points, taking into the
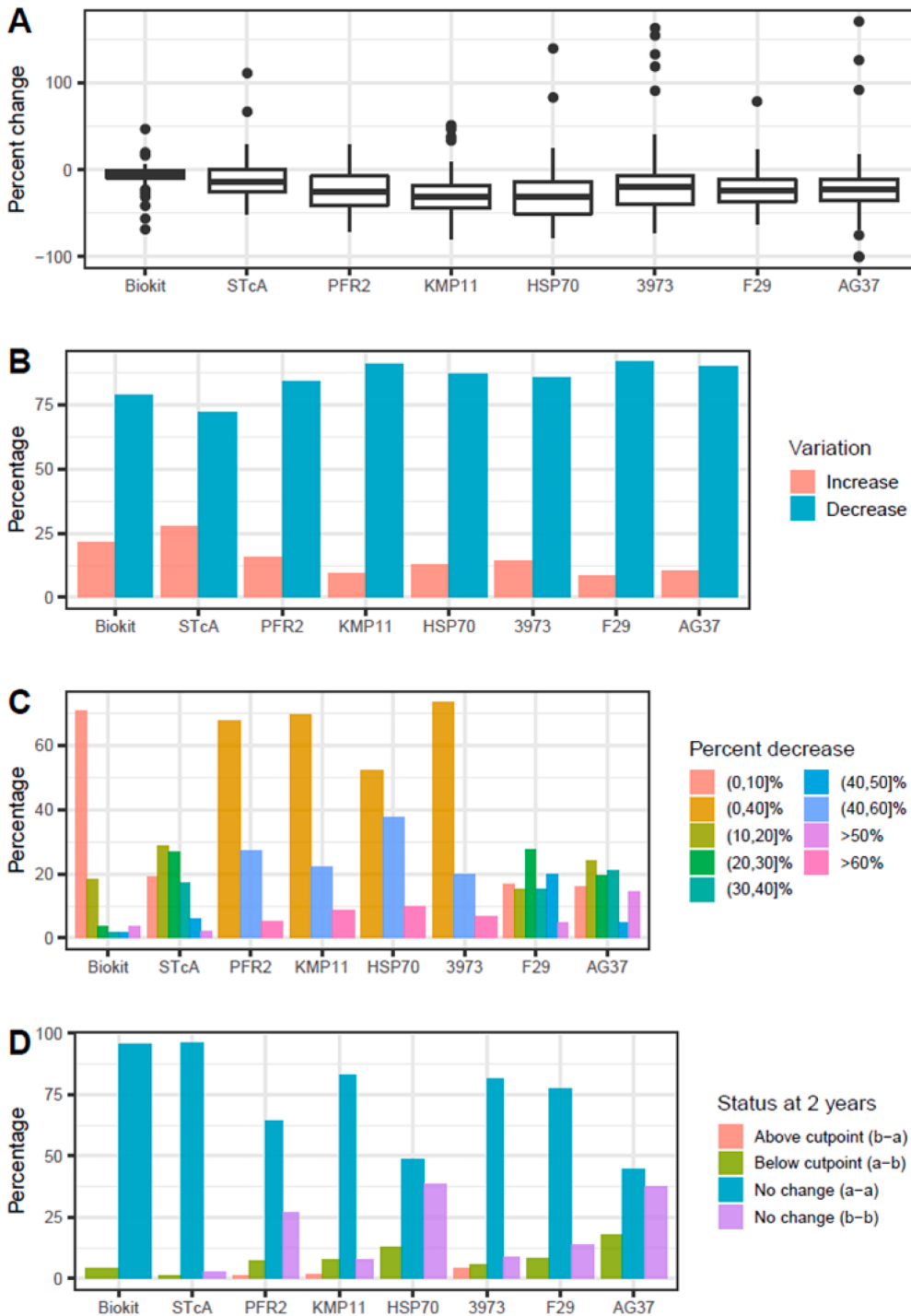
Fig. 3. Description of reactivity changes observed between PRE and POST samples of group 3 subjects. (A) Box-plot showing samples reactivity as median percent change with IQR for each antigen; (B) POST to PRE variation (increase or decrease) indicated as percentage of samples in each condition per antigen; (C) for those samples that showed a decrease of reactivity, changes in response were categorized in ranges of 10% frames; (D) status of samples at 2-years indicated as transitions above (a) or below (b) the corresponding antigens' acknowledged thresholds (cut-offs): b-a, transition from below to above cut-off (increase); a-b, transition from above to below cut-off (decrease crossing cut-off, i.e. seronegativization); a-a, serological status unchanged maintaining samples' reactivity always above cut-off; b-b, serological status unchanged maintaining samples' reactivity always below cut-off.

calculation also the samples that showed increased POST reactivity, were KMP11 (−30.9) and HSP70 (−31.1) (Fig. 3A). Regarding "therapeutic response" criteria provided by the laboratories of origin of the antigens (see "Statistical analysis and methods" section), 26 subjects complied with the decrease on at least two of the antigens PFR2, KMP11, HSP70 and 3973 (Table 3) (Egui et al., 2019). In relation to the other antigen with informed criteria of parasite clearance, IBAG37, it was found that seven subjects complied with the decrease of at least one score, of which five were coincident with those that responded to the antigens aforementioned.

Samples serological status was categorized as "above" ("a") or "below" ("b") the originally provided cut-off for each antigen in order to identify therapeutic response events. This is, going from "positive" to "negative" in each of them. Few of these transitions occurred in any of the antigens studied (Figure S2). In general, all antigens reported a higher number of negative POST samples than Biokit or STcA assays. HSP70 and IBAG37 had the highest number of positive to negative transitions (a-b), even though this was only 13% (9/70) and 18% (13/69), respectively (Fig. 3D). According to guidelines, a change from positive to negative status (threshold shifts from above to below cut-off) in both, Biokit and STcA, would indicate that a *T. cruzi*-infected individual did successfully respond to treatment (Pan-American Health Organization, 2018).

Fig. 4 summarizes serological reactivity trends observed for each of the antigens between PRE and POST samples. Overall, the fold change decrease over time of conventional tests was almost flat (Fig. 4). In contrast, KMP11 and 3973, the two best performing antigens in this study in terms of Se and Sp, generally had a decreasing fold change reactivity, as indicated by their predominantly green coloring (Fig. 4).

4. Discussion

Availability of reliable biomarkers for the early assessment of therapeutic response for Chagas disease is a major challenge towards its control. Herein, we investigated the serological reactivity over time of a series of parasite-derived antigens on a retrospective collection of samples, so that they might be considered for further evaluation as potential biomarkers for follow up of treated *T. cruzi*-infected subjects. Retrospective analysis and integration of cohorts is a difficult undertaking because of inherent potential bias and restrictive analysis for data. In order to avoid the use of complex regression analyses, the number of samples analyzed from the multiple countries and categories of samples was limited. This was in part due to incomplete clinical data for some registries, but also to the follow-up period (one to three years after treatment).

In terms of clinical evolution, there was no significant change in the subjects analyzed ($n = 49$) over post-treatment follow up, and just one patient did evolve from not having demonstrable clinical signs pretreatment to having cardiac involvement after it. Likely, this might be

Table 3
Number of subjects complying with "therapeutic response" criteria.*, "1″ means positive compliance with response criteria; $, "0″ means not complying with response criteria (Egui et al., 2019).

Antigen				Number of subjects
PFR2	HSP70	KMP11	3973	
1*	1	0	0	5
0$	1	1	0	5
1	1	1	1	4
1	1	0	1	3
1	1	1	0	2
0	0	1	1	2
1	0	1	0	1
0	1	1	1	1
0	1	0	1	1
1	0	0	1	1
1	0	1	1	1

due to the limited time of follow-up.

With respect to the antigens diagnostic capacities, HSP70 and F29 did not perform as reported or expected (Sosa Estani et al., 1998; Fabbro et al., 2013; Fernández-Villegas et al., 2011). This may have been due to variability in antigen batches, or to the prevalent origin of the samples tested and its link with infective strains circulating (Balouz et al., 2017; Guzman-Gomez et al., 2015). The retrospective collection of samples used in the study was heterogeneous, but certain origins prevailed over the rest. In subsequent prospective studies, a more balanced heterogeneity should be pursued.

A "positive" or "negative" reaction with any given antigen reveals the quality and quantity of the immune response, potentially indicating "over-expression" or lack of detection at a particular time point (e.g. PRE) (Pinazo et al., 2014). In order to ascertain whether a biomarker might be associated with infection, an updated expert consensus on a TPP for a test for the early assessment of Chagas disease treatment efficacy has determined that the Se for an acceptable biomarker should be ≥ 60% and its Sp ≥ 90% (Alonso-Padilla et al., 2020). Results from the present study indicate that KMP11, 3973 and PFR2 would obey those established criteria. For these antigens, a decrease ≥ 40% in reactivity in at least two of them has been proposed as treatment response criteria (Egui et al., 2019). In another study with the parasite-derived antigen TcTASV that threshold was determined at $a > 30\%$ decrease in the antibody levels to acknowledge treatment impact (Floridia-Yapur et al., 2019). It must be noted that TcTASV was evaluated with a pediatric cohort on that occasion (Floridia-Yapur et al., 2019). In a more recent work, Nagarkatti et al. evaluated the reactivity to antigen Tc_5171, a parasite hypothetical protein of unknown biological function, of a set of pre-clinical samples and a small collection of plasma samples from treated chronically *T. cruzi*-infected patients (Nagarkatti et al., 2020). Anti-Tc_5171 reactivity was shown to be infection specific and its levels decreased in a statistically significant manner after treatment (Nagarkatti et al., 2020). Nonetheless, further work comparing antigens performance with that of currently accepted tools (conventional serology) on a cohort of treated individuals that indeed responded to treatment (as evaluated with those conventional tools) will be needed to address their utility as treatment-response biomarkers.

In this study, KMP11 and 3973 were the two best-performing individual parasite-derived antigens to identify both negative and *T. cruzi*-infected subjects. Their sensitivity was 91% and 84%, and their specificity 98% and 93%, respectively, having a ROC AUC of 0.97 and 0.96. But it was HSP70 which had a higher percentage of subjects showing a higher rate of relative decrease (Fig. 3C), despite its diagnostic performance was poor (Table 2; Figure S1). Thus, a question might arise here on whether an antigen for timely evaluation of treatment response needs to have high diagnostic efficacy and ability to differentiate between a *T. cruzi*-infected or negative individual. It should not be disregarded that a certain level of change in a determined time frame could also provide the sought answer. Nevertheless, the major limitation of the study remains in lacking the clinical "gold standard" to ascertain whether a decline in antibody response overtime could be associated to cure.

Analyses conducted emphasize the need to continue the evaluation of antigens in order to eventually find potential biomarkers for the early assessment of treatment response in Chagas disease. In the search of an early response marker, serial samples collected pre- and after treatment over follow-up will have to be evaluated. Remarkably, such samples will necessarily have to be very well characterized epidemiologically, serologically and clinically. This type of research could be contemplated as part of well-funded clinical trials that evaluate new drugs against chronic *T. cruzi* infection, which include timely assessment of diagnostic efficacy and evaluation of clinical history. However, follow-up in these trials rarely goes beyond 24 months post-treatment. Moreover, expensive multinational trials to gain access to an epidemiologically diverse set of samples are complex to design and execute, thus costly and rare for Chagas disease. Per contra, setting up a large cohort, well-characterized epidemiologically, clinically and serologically, whence to have
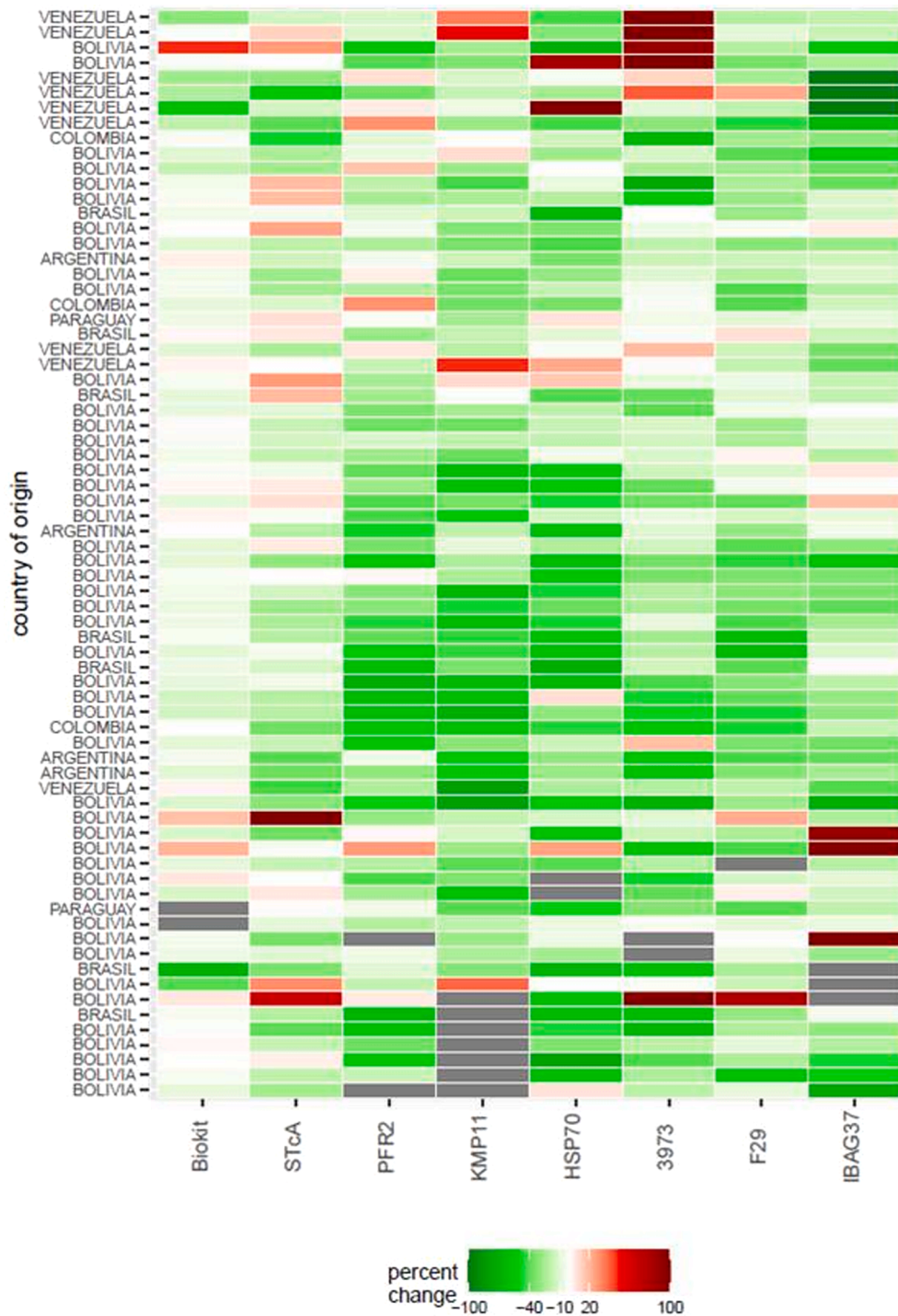
Fig. 4. Heat-map to illustrate in a color scale the PRE to POST reactivity changes of samples from group 3 subjects to each antigen under study, as well as over Biokit and STcA. Study subjects were grouped by reactivity pattern. Less to more intense green was used for decreasing reactivity values, non-varying values are in white, increasing values were highlighted using less to more intense red, and missing values are in gray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

longitudinal samples and evaluate fold change responses over time on several antigens should be feasible at a fraction of the cost of a clinical trial. Antigens' performance parameters such as sensitivity and specificity could then be validated against the "gold standard", i.e. serological reversion using two conventional tests, along the distinct time points. Despite seronegativization is a phenomenon that can take many years to occur, it is yet the only acknowledged tool to ascertain cure. Thus, long term prospective multi-national cohorts of *T. cruzi*-infected subjects will be crucial in the validation of any tests based on these or other biomarkers to come.

Declaration of Competing Interest

None.

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