Chagas Disease Treatment Efficacy Biomarkers: Myths and Realities

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Abstract Chagas disease (CD), caused by *Trypanosoma cruzi*, affects millions of people worldwide. Although CD R&D has made progress during the last decade, clinicians and general practitioners are still facing the same challenge, i.e., the lack of adequate markers of clinical cure, hindering assessment of new drug efficacy in clinical trials and counseling of patients about treatment outcome. To date, no new markers have been validated as surrogates of seroreversion – the only marker of parasitological cure which is itself considered to be a surrogate of clinical benefit. *T. cruzi* DNA detected using PCR cannot currently be considered as a surrogate of seroconversion. Much emphasis has been placed on different *T. cruzi* antigens but no definite proof of correlation between titers, as determined by serology at a given timepoint, and seroreversion has been shown. Thanks to the improvement of analytical methods and the application of new methodologies, the identification of potential new markers is being facilitated, and some of these are progressing. However, there is a long journey from the identification of a potential biomarker to its clinical validation and acceptance by the regulatory authorities that requires a common effort from the entire Chagas community.

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1 Introduction

The last decade has seen an increase in the number of clinical trials assessing the potential of new drugs for Chagas disease (CD), focusing specifically on repurposed azoles. One of the main issues facing clinical researchers in the field, however, is the absence of clearly defined markers of clinical cure, due to the complexity and long development time of the disease. This fact, among others, has hampered efforts toward the development of new drugs for CD. The scope of this review is not to present an extensive overview of all the potential markers for assessment of treatment efficacy described so far, as this has already been done [1–3]. Instead, we focus on the current needs and challenges in this specific area, describe new technologies that have been applied to the identification of potential markers of interest and propose the steps that we consider should be taken in order to tackle this important issue.

Indeed, we believe that a concerted joint effort by the CD community is essential in order to gain a better understanding of how to define a biomarker for CD and how then to further develop and validate it, in order to answer this very complex and demanding research question. This is not only necessary to be able to run clinical trials for the registration of new drugs but also so that general practitioners will be able to inform patients about the outcome of their treatment.

2 Chagas Disease Overview

CD, also known as American trypanosomiasis, and its etiological factor, *Trypanosoma cruzi*, were discovered more than a century ago by Carlos Chagas [4]. Since then, CD epidemiology has changed; although still endemic in Latin America, the disease has spread into non-endemic countries due to population migration and has become a global public health issue [5–8]. CD is the most common cause of infectious cardiomyopathy worldwide [9]. Around 6–7 million people are infected worldwide and 10,000 die annually [10, 11]. The disease presents in two main phases: the acute phase, which is asymptomatic and typically undetected and lasts for a couple of months during which the parasite is readily identified through blood examination, and the chronic phase, which can last for decades while the infection is controlled by the immune system and the parasite is hardly detectable. While most infected patients in the chronic phase will remain asymptomatic, a certain proportion—between 10% and 40%—eventually develop symptoms, mainly cardiomyopathies and in certain cases digestive tract megasyndromes or both [12]. The major causes of mortality in these patients are progressive heart failure and sudden death [13].

There are two treatments currently available, benznidazole (Abarax/ELEA and Rochagan/LAFEPE) and nifurtimox (Lampit/Bayer), which are old nitroheterocyclic trypanocidal drugs. Although these drugs have been shown to be efficacious in both phases of the disease, particularly in children, their use is limited due to side effects occurring during treatment and impeded access to medication [14, 15]. There is an urgent need for new and safer drugs for CD.

3 Disease Progression and Treatment Efficacy Assessment: Current Challenges and Future Needs

A major hurdle for the clinical development of new drugs for CD is the absence of an adequate test that can assess successful treatment or show clinical benefit in a timely manner. The definition of cure criteria for CD has been subject to debate; the complicated development and pathology of the disease, coupled with the complexities of the parasite life cycle and its interactions with the host, make it a very difficult task to determine such criteria. Clinical cure criteria are very often discarded as they are considered to be too difficult to achieve and possibly because of a lack of understanding of the slow evolution of the disease from asymptomatic stage to cardiomyopathy and/or megacolon [16]. Another issue is the lack of consensus on the assessment of treatment efficacy and inadequate tools to address it [17].

Although there is no absolute proof in patients that parasitological cure is synonymous with clinical cure, i.e., halting the progression of the disease toward cardiac or gastrointestinal symptoms, there is a consensus that parasite persistence is needed for the development of CD. All current CD drug development efforts are therefore focused on strategies to eliminate *T. cruzi* from the human body. The only way to assess drug treatment efficacy is to use serological tests showing the disappearance of *T. cruzi* antibodies (seroreversion, synonymous with parasitological cure). This is clearly a major challenge, since seroreversion can take decades to occur in treated adults, if it occurs at all. This makes assessment of parasitological cure with the currently available tools in this category of patients complicated, if not impossible, and thus seroreversion is not useful as a clinical endpoint in clinical trials. There is, therefore, a need to identify surrogate markers for the absence of parasites that are quicker and more sensitive than seroreversion. A surrogate endpoint of a clinical trial is a laboratory measurement or a physical sign that is used as a substitute for a clinically meaningful endpoint that measures directly how a patient feels, functions, or survives. Changes induced by a therapy on a surrogate endpoint are expected to reflect changes in a clinically meaningful endpoint [18].

The need for surrogate markers of parasitological cure is further highlighted by the recent FDA approval of benznidazole (BZN) monotherapy exclusively for the treatment of chagasic children between 2 and 12 years of age [19, 20]. In *T. cruzi*-infected children, seroreversion can be observed fairly quickly, within months to a few years following treatment. The FDA approval of BZN for children was based on seroreversion observed in around 50% of children [21, 22].

Another important feature of CD is the fact that not all *T. cruzi*-infected patients will develop the disease—in the literature it is typically stated that around 10–40% of infected patients will develop symptoms of the disease [12]—and that possible host factors for susceptibility are not well understood [23]. It would be useful to identify markers of disease progression that could be used to predict which *T. cruzi*-infected people are likely to develop the disease, as treatment could then be focused on people most at risk.

Efforts to understand this phenomenon and to identify patterns or indicators that could be used to categorize patients at risk of developing the disease have so far not

been successful. Preliminary attempts have been made to identify candidate genes associated with the progression of the disease. Results from a genome-wide association study (GWAS) using the well-established REDS-II cohort of Chagas patients suggested that both cardiovascular- and immune-related polymorphism in some genes of interest could be associated with a genetic predisposition to chronic Chagas cardiomyopathy [24]. Another study found an association between HLA haplotype and resistance to chronic Chagas disease [25]. A recent study showed a potential association between variations in the inflammasome, particularly in NLRP1 and CARD11, and chronic Chagas cardiomyopathy [26]. Although all these studies suffer from the low number of patients used in the analysis, this is certainly an area of research that merits further investigation.

4 CD Biomarker Identification for Treatment Efficacy Assessment and Next Steps: The What(s) and How(s)

There is a need to identify a surrogate marker for the absence of parasites that is quicker and possibly more sensitive than seroreversion. Efforts to identify new potential biomarkers, comparing, for example, samples from healthy people and Chagas patients, can lead to a substantial amount of data that is not always easy to interpret and analyze. Even when potential markers of interest are identified, there is still a long way to go to ensure that these putative markers will be useful in practice [27]. This will include analytic (validation of an assay for the marker of choice) and clinical validation of the marker as well as regulatory acceptance.

It becomes very important therefore to define the attributes that should be required for a biomarker, in particular those related to the methodology used for the analysis of a chosen marker and its suitability in the field, its level of sensitivity and selectivity, and the current level of validation according to the types and number of samples tested, to name but a few criteria. The definition of a target product profile (TPP) for a biomarker and its associated test should clarify these points early on in order to avoid focusing work and testing on a marker of interest for which no suitable test could be available for routine analysis. A tentative TPP for Chagas disease assessment of treatment response has been described but seems to be biased toward the use of PCR [28]. A more general biomarker TPP was highlighted by Pinazo et al. in their biomarker systematic review [2].

Another point for consideration is related to the quality and type of samples used to identify and validate biomarkers. Specimens from patients at different stages of the disease and from healthy people are critical tools for this and for the development of better tests. The appropriate detailed information and handling procedure to be followed are defined in local or international guidelines, and standardization of specimen collection methodologies is critical [29]. Indeed, technical aspects such as the anticoagulant used, sample processing time, processing and storage temperatures, and thaw/freeze cycles are all variables that can impact the quality of specimens and their stability over time, thereby having an impact on analysis results [30].

Finally, the design of clinical trials from which the specimens originate and which are used for the identification and validation of potential biomarkers should be considered very carefully.

5 Pros and Cons of Currently Proposed Biomarkers of Cure for CD

Several CD biomarkers have been suggested and used to discriminate between CD-infected and CD-non-infected individuals when assessing chemotherapy in cohort studies with adults or children and to establish disease progression in CD patients. However, very few of them show high sensitivity, have been systematically studied, or could be used to determine treatment efficacy. In addition, not many would pass the quality criteria mentioned above (see Sect. 4). All the CD biomarkers suggested so far have been reviewed by different authors (see [1, 3]). In 2014, a systematic review by Pinazo et al. proposed 25 potential biomarkers for the evaluation of therapeutic efficacy [2]. Requena-Méndez et al. also reviewed some blood-derived biomarkers useful for disease progression and cure [31]. Here we describe the pros and cons of some of these biomarkers and also some promising new markers with the potential to be surrogate endpoints. A summary of candidates is presented in Table 1.

5.1 Parasite DNA Amplification and Antigens for Serological Tests

Parasite detection in blood by PCR and the evaluation of antibodies by serology are the main techniques used to monitor CD treatment response in patients and in CD clinical trials. The evaluation of treatment efficacy is affected by their limitations, which have been recognized as Achilles' heel of clinical trial outcomes. PCR has shown promising results for the assessment of therapy failure; a positive result clearly evidences failure to clear the parasite and thus ineffective treatment [57]. However, a negative PCR does not guarantee the absence of parasite and cannot confirm parasite clearance. False negatives occur due to fluctuations in parasitemia, the isolation of parasite in tissue or organs, and the intrinsic limit of detection of PCR and qPCR techniques [58]. Other distinct factors may contribute to the overall performance of PCR assays: the size of the serum sample for parasite DNA extraction, the sample collection tubes, the different PCR assay conditions, and the algorithm used to classify results can affect the evaluation of the samples, as was demonstrated by Wei et al. [59] for the STOP CHAGAS clinical trial that evaluated posaconazole for the treatment of CD [60]. Nevertheless, PCR is a promising tool that can be easily performed in clinical settings and used for clinical trials; thus, the investment in improving PCR methodologies is worthwhile. The CD community must focus on suitable strategies for parasite DNA extraction in lower sample volumes, the equivalence between blood and tissue parasitemia; the reduction of false

Table 1 Candidate Chagas disease surrogate biomarkers

Biomarker type	Biomarker	Results in Chagas disease	Potential as a test of cure	References
Parasite proteins	Trypomastigotes F2/3 antigenic fraction	Anti F2/3 decreases after BZN treatment and disappears after 4–21 months in children	Negative results earlier than conventional serology but need to be evaluated in adults	[32]
	Immunofluorescence assay of fixed trypomastigotes (ISIFA)	High titers in infected patients and low titers 6 years after treatment when patients were considered cured. High sensitivity and no cross-reactivity with other diseases	ISIFA can differentiate treated from untreated and those with treatment failure, but the assay requires fixed parasites	[33, 34]
	Trypomastigote mucin antigen A&T CL-ELISA	Measure anti-Gal Abs. Titers decrease after BZN treatment in adults and children	The gradual and consistent decrease of Abs after 3–6 years of treatment. Correlation with seroconversion only in adolescents.	[35–37]

Parasite recombinant proteins	Ag13 85 kDa protein with repeats of 5 amino acids	Anti-Ag13 is suitable for CD diagnosis in different populations, and titers decrease and disappear after 3 years posttreatment	Negative conversion occurs quicker compared to other antigens	[38]
	T. cruzi ribosomal acid protein P2β	Levels of Anti-P2β decrease in asymptomatic treated CD patients	Seroconversion only in half of the population 20 years posttreatment	[39]
	Immunodominant antigens KMP11, HSP70, PFR2, Tgp63	A significant drop in reactivity against antigens between 6 and 9 months in BZN-treated CD adults at different stages of the disease. Titers continue to drop after 24 months	Antigens are recognized by complement-dependent IgG1 which could be an advantage to observe rapid seroreversion	[40, 41]
	24 kDa calcium-binding protein (rTc24)	Anti-rTC24 Abs decreases within 6–36 months posttreatment	Good correlation with the CoML test and seroconversion by other antibodies	[42, 43]
	Flagellar calcium-binding protein (F29)	Seroreversion for the F29 antigen occurs between 6 and 48 months after BZN treatment in children	ELISA-F29 establishes seroreversion earlier than conventional serology in adults. But the time to convert could limit its use in clinical trials	[44, 45]
	Multiplex 16 r *T. cruzi* proteins	Decreased response of the panel 36 months after BZN treatment in adults	Strong correlation with conventional serology, but seroreversion was observed only in a subset of treated patients	[46, 47]
	Recombinant complement regulatory protein (rCRP)	Detect Abs complement-dependent as the CoML test. Positive reactions decrease 1–2 years after BZN treatment	Results correlate well with CoML test and do not require live parasites	[48]
	Putative microtubule-associated protein (MAP) antigen3	Selected antigen from a multiplex array of 15 antigens. Results correlate with PCR-positive and PCR-negative results in a cohort study 5 years after BZN treatment	Abs efficiently detects parasite persistence in infected individuals and PCR-negative treated individuals	[49]

(continued)

Table 1 (continued)

Biomarker type	Biomarker	Results in Chagas disease	Potential as a test of cure	References
Host biochemical markers	ApoA1	Downregulated in CD and normal levels after BZN or NFX treatment	Level return to normal after treatment and 3-year follow-up	[50, 51]
	ApoA1 and FBN fragments	Upregulated in CD and downregulated after BZN or NFX treatments	Correlation with seroreversion, early decrease after BZN treatment	
	Lytic antibody complement-mediated lysis (CoML) test	Abs decreases until becoming negative after parasite elimination in BZN and BFX treatments	Negative results can be obtained 1 year after treatment when serology is still positive but requires live trypomastigotes	[42]
Host prothrombotic markers	Prothrombin fragment 1 + 2 (F_{1+2})	A marker of thrombin generation in vivo increases early in CD and decreases after BZN treatment	Consistent decrease to normal levels after treatment and for 3-year follow-up in 96% study population	[36, 52]
	Endogenous thrombin potential (ETP)	Quantifies the ability to generate thrombin when activated through tissue factor addition upregulated in CD, decreases after BZN treatment		
	Soluble platelet selectin (sP-selectin)	Biomarker of in vivo platelet activation decrease during BZN therapy in adults and children	Consistent decrease after treatment, but upregulation does not occur in all CD cases	[21, 36]
Immunological markers	IFN-γ T cells	Three-fold decrease compared with pre-treatment between 1 and 3 years posttreatment	IFN-γ levels correlate with the severity of disease and can be used to monitor disease progression	[53]
	$CD3^+$ T cells	CD3+ T-cell proportion differs between treated and untreated patients and normalizes in cured patients	Despite values normalizing in cured patients, the number of cells cannot be used to predict parasitological cure	[54]
	$IL12^+$ $CD14^+$ cells	BZN-treated children show low levels of $IL12^+$ $CD14^+$ cells and high levels of IL-10 modulated type 1 cytokines profile	Potential to see immunological effects of treatment but needs further study	[55]
	$CD4^+$ LIR^+ T cells	Decrease of $CD4^+$ LIR^+ T cells after treatment between 2 and 6 months and for at least 2 years		[56]

negatives, as well as the validation and standardization of PCR assays; and the correlation of PCR readouts with seroreversion.

Conventional serology with different parasite antigens is commonly used for CD diagnosis and to evaluate antibody titers against *T. cruzi* after chemotherapy. Due to the long-term persistence of specific antibodies that are detected by serological tests, chronically infected patients must be followed up for several years after treatment until they can be considered cured using seroreversion as a measurement of parasite clearance [33]. The current criteria of cure consist of two nonreactive conventional serological assays with parasite antigens that are commercially available as diagnostic kits in endemic countries. The probability of cure might also be predicted by a decrease in antibody titers for *T. cruzi* over time, but this will depend on the specific antibody and the status of the disease before treatment [57]. However, serodiscordance remains a challenge in Chagas disease diagnosis and raises the question of the reliability of serology tests relying on one specific antigen depending on the region and patient stage [61–63].

Of all the *T. cruzi* antigens published in the literature, only a few have been evaluated in the long term and used to predict treatment efficacy. Antigens obtained directly from *T. cruzi* preparations as the F2/3 antigenic fraction (isolated from trypomastigotes) have been used to assess cure in children with congenital transmission [32]. Anti-F2/3 antibodies become negative 4–21 months after BZN treatment and earlier than conventional serology, suggesting that they may provide an earlier marker of cure. Likewise, Andrade et al. have shown that a chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) with a trypomastigote mucin antigen (A&T) successfully assesses treatment efficacy in BZN-treated adolescents [35]. When measured as negative A&T CL-ELISA seroconversion, 88.7% of the treated group were cured after 6-year follow-up. Using this assay, the BZN efficacy in children and adolescents by per-protocol analysis and by intention-to-treat approach was 84.7% and 64.7%, respectively. F2/3 and A&T antigens obtained from parasites seem to be candidate surrogate biomarkers, but their use in adult studies needs to be further evaluated since the reduction in titers and seroreversion can take longer. Pinazo et al. have shown that A&T CL-ELISA remains positive for 3 years after treatment in an adult population [36].

Since obtaining pure proteins from the parasite can be laborious, several recombinant proteins have been produced and tested for detecting anti-*T. cruzi* antibodies in ELISAs and immunoblots. Most of the specific antibodies against recombinant proteins are good at discriminating CD individuals from healthy controls and useful for monitoring patients after treatment. Recombinant proteins such as F29 (flagellar calcium-binding protein); P2β (ribosomal acid protein); KMP11, HSP70, PFR2, and Tgp63 (immunodominant antigens); Ag13 (85 kDa protein with repeats of 5 aa); and a multiplex of 16 *T. cruzi* proteins have been used to assess treatment efficacy and to attempt to predict cure (see Table 1).

Anti-F29 decreases quickly after BZN treatment in children and seroconverts in 62.1% of cases after 48 months [44]. Fabbro et al. have shown that Anti-F29 may take up to 14.5 ± 5.7 years to seroconvert after BZN or nifurtimox (NFX) treatment in adults but predicts cure earlier than conventional serology (22 ± 4.9 years) [45].

By contrast, anti-P2β can take more than two decades to seroconvert in treated asymptomatic patients despite a reduction in titers compared to their initial values [39]. Anti-KMP11, HSP70, and PFR2 decrease rapidly a few months (6–9 months) after BZN treatment in more than 70% of CD patients and continue to decrease during the 24 months posttreatment follow-up period [40]. This is perhaps because KMP11 and HSP70 are mainly recognized by IgG1 complement-dependent antibodies [41]. Anti-Ag13 also has shown a constant decrease in titers compared with other antigens and seroconverts in 6/9 patients after 3 years of treatment [38]. Finally, a panel of 16 proteins in a multiplex bead assay has shown a strong correlation with conventional serology tests in a short-term follow-up of 53 BZN-treated patients [46, 47]. Despite the evidence gathered so far, longer follow-up and tests in larger populations are needed to select the best Ag/Abs pairs that can be used to evaluate treatment efficacy in clinical trials, regardless of the type of treatment or the patient's disease status.

In the search for the ideal antigens and antibodies, Zrein et al. used an innovative multiparametric screening technology to identify antibodies that could be used as surrogate biomarkers [49]. After evaluating 15 antigens in a multiplex serology assay, Antibody 3 (Ab3), which recognizes *T. cruzi* putative microtubule-associated protein (MAP) (Antigen 3), showed a strong correlation (92%) with PCR-positive results in treated and untreated CD patients from the SaMi-Trop cohort study [64]. More importantly, Ab3 could discriminate PCR-positive patients from PCR-negative treated patients (AUC 0.74). Ab3 efficiently detected parasite persistence in most of the *T. cruzi*-infected individuals and detected a large number of parasite persistent cases within the PCR-negative group, which shows this assay to be even more informative than PCR [49]. These results suggest that Ab3 could be a good surrogate biomarker; however, Ab3 titers should be evaluated before treatment and in a non-infected population. Validation of other cohort studies, quantitative evaluation of serology, and following titers until seroconversion will determine if parasitological endpoints can be predicted with this antibody. Its usefulness for evaluating treatment efficacy in clinical trials will also depend on a quick change in Ab3 titers after treatment.

Detection of complement-dependent lytic antibodies seems to be an alternative to the detection of specific anti-*T. cruzi* antibodies. These lytic antibodies can be detected either by complement-mediated lysis (CoML) test or by indirect immunofluorescence (IIF). The antibodies appear as soon as 20 days post-infection and disappear as early as 1-year post-chemotherapy [42]. In the 10-year follow-up of a study of CD patients treated with BZN or NFX, patients showed consistently negative CoML test results at 6 to 33 months posttreatment despite positive IIF and conventional serology and thus were considered cured [65]. The disadvantage of this approach is the need for living infective trypomastigotes, which is not practical for clinical trials. One epitope of the lytic antibodies contains a high molecular mass (160 kDa) protein, *T. cruzi* complementary regulatory protein (CRP). Meira et al. found a good correlation between the CoML test and an ELISA using a recombinant CRP [48]. In a cohort study with 31 CD patients, both tests showed the same significant reduction in the number of positive samples over a period of 4 years after treat-

ment [48]. However, evaluation of a bigger population is needed to confirm if this ELISA could replace the CoML test and be used to establish parasite clearance [42]. A recombinant calcium-binding protein (rTc24) has shown a good correlation with CoML test in ELISA and immunoblot, but its potential as a surrogate endpoint has to be further investigated [43].

5.2 Host Biochemical Molecules

Since *T. cruzi* is an intracellular parasite that produces a chronic infection, biochemical molecules from the host such as metabolites, proteins, immunomodulators, and cell surface proteins can be affected due to infection and thus may be potential CD biomarkers. In addition, host biochemical molecules can be better surrogate biomarkers than antibodies against *T. cruzi* antigens or DNA amplification techniques, since their evaluation will not depend on the persistence of antibodies or the direct detection of parasites.

In the search for indicators of parasite signature, our group performed the first serum protein analysis of CD patients using mass spectrometry [50]. We used serum fractionation to evaluate both high- and low-abundant serum proteins and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) for intact protein analysis [66, 67]. In a panel of 435 sera from Venezuelan asymptomatic CD patients and healthy controls (HC), we identified 18 host proteins that were statistically different between the CD and control populations. To select biomarkers with the greatest discriminatory power, we used a biomarker pattern software to generate candidate decision trees. Five host markers showed high sensitivity (89%) and specificity (100%) and could distinguish asymptomatic CD adult patients from HC. Biomarkers were identified by MS/MS analysis as full-length and fragments of the apolipoprotein-A1 (ApoA1) as well as a fragment of fibronectin (FBN) [50]. It is worth mentioning that our success in detecting and identifying these biomarkers was due to our innovative intact protein approach, also known as "top-down proteomics."

We used the same strategy to search for biomarkers in a Bolivian CD population and to predict cure after treatment with NFX [51]. After comparing the serum proteins of CD vs HC, the same candidate biomarkers were identified, demonstrating the reproducibility of the approach across the South American population, an important factor considering the variety of infective *T. cruzi* strains in patients in this region. In addition, we observed that ApoA1 and FBN fragments were significantly upregulated in chronic or asymptomatic CD subjects compared to HC and 3 years after NFX treatment returned to levels similar to those seen in HC. In contrast, full-length ApoA1 was downregulated in CD individuals compared to HC and returned to normal levels during the follow-up period. All patients were seropositive 3 years after treatment, but using these biomarkers, we were able to predict an overall cure rate of 43.2%. These results suggest that these biomarkers might be useful in assessing treatment efficacy in CD patients and could lead to the development of a test of cure [51]. Following this lead, we have developed a proteomics-based immunoblot

that detects ApoA1 and FBN fragments in CD patients. This is a successful translation of proteomics-based studies into accessible tools for bench diagnosis. Results from these studies confirm these fragments as signatures of the parasite and their great potential as surrogate biomarkers.

5.3 Host Prothrombotic and Immune Markers

Host prothrombotic markers have been used to evaluate disease progression in the chronic phase of CD, but they could also be useful for treatment evaluation. In infection, immunothrombosis is activated after the recognition of a pathogen in order to inhibit its dissemination and survival; different infectious agents may cause responses to different degrees. Thromboembolic events are observed in cases of chagasis cardiomyopathy, and an increased risk of peripheral thrombotic phenomena and thrombosis in CD patients without heart failure or structural cardiopathy has been observed. These events can be attributed to the host immune system and to the parasite itself. Of all the biomarkers that can identify a prothrombotic state, markers for clotting activation that have shown the most consistent results are prothrombin fragment 1 + 2 (F_{1+2}) and the endogenous thrombin potential (ETP). These markers are elevated in the early stage of the chronic phase and decrease after therapy with BZN [52]. In a more recent study with 99 individuals, Pinazo et al. observed that F_{1+2} and ETP were abnormally expressed in 77% and 50% of infected patients before treatment but returned to, and remained at, normal levels 6–9 months after treatment in 76% and 96% of cases, respectively [36]. This data suggests these markers could assess short-term response to treatment; however, normal values can be observed in infected patients, and some patients show qPCR-positive results even when ETP values reach baseline after treatment.

Lastly, different cytokines and cell surface markers have been evaluated in CD patients and proposed as immune markers for disease progression. Within them, IFN_{Υ} T cells, $CD3^+$ T cells, $IL12^+$ $CD14^-$ cells, and $CD4^+$ LIR^+ T cells have been studied in CD-treated or CD-untreated populations. IFN_{Υ} is one of the main cytokines that regulate Th1 immune responses, and it is critical for innate and adaptive immunity against virus and intracellular parasites. High levels of IFN_{Υ} in peripheral blood mononuclear cell (PBMC) cultures correlate with severity of CD cardiomyopathy and are probably responsible for the strong Th1 response in CD patients with cardiac disease [68]. Laucella et al. have observed that IFN_{Υ} T-cell levels decrease after BZN treatment between 1 and 3 years posttreatment and become undetectable in almost 50% of treated patients [53]. Likewise, the proportion of $CD3^+$ T cells differs between treated and untreated patients and normalizes in cured patients without changes in the PBMC phenotype [54]. In patients treated with BZN during the early indeterminate stage, the number of $IL\text{-}12^+CD14^+$ cells decreases, and treatment induces an IL-10-modulated type 1 cytokine profile [55]. On the other hand, chronic CD patients have shown increased numbers of $CD4^+LIR\text{-}1^+$ among total PBMCs, relative to non-infected individuals, and these numbers decreased after BZN treatment [56]. Although these findings suggest that cell types and their mark-

ers can be used to assess the influence of treatment, their potential as surrogate biomarkers needs to be further studied.

Nonetheless, immunomarkers can be useful in phase I clinical trials of new drugs. In a mouse immunosuppressed model, the presence of MHC-peptide tetramers, which are specific for $CD8^+$ T cells recognizing a transialidase peptide, was monitored as a biomarker for treatment success [69]. Cured mice show an increased number of T cells displaying a central memory phenotype (T_{CM}) with surface markers CD62L and CD127, these markers are not present in T_{CM} and T effector memory cells (T_{EM}) in non-treated mice, and the phenotype could be used to determine treatment efficacy and cure [70].

6 New Developments: A Hopeful Way Forward?

During the last 15 years, new developments have changed the Chagas biomarker landscape. These developments have included both the emergence of new tools and technologies for the assessment of known/"established" markers (essentially *T. cruzi* antigens or antibodies) and the identification of potential new markers, in particular markers in the host, for the diagnosis of CD and the assessment of drug treatment efficacy. These include, among others, high-throughput technologies to identify RNA aptamers; new analytical devices such as biosensors; new mass spectrometry and NMR technologies allowing comparative analysis of proteins, metabolites, lipids, and mRNA in serum samples from healthy and infected patients (X-omics); as well as FACS and MRI.

6.1 Aptamers

RNA aptamers are short nucleotides that can bind specifically, and with high affinity, to targets in complex protein mixtures, membrane preparations, or whole cells. Their specificity depends on their hydrophobic and ionic interactions with the target as well as on their tertiary structure. Aptamers can be developed in vitro using an iterative procedure known as systematic evolution of ligands by exponential enrichment (SELEX). Without a priori knowing a specific target, this process can select RNA sequences with affinities similar to or lower than those seen with monoclonal antibodies [71–73]. This approach was first explored for CD by Nagarkatti et al. in order to concentrate *T. cruzi* parasites and facilitate their detection by PCR [74]. Using a whole-cell SELEX strategy, they developed serum-stable RNA aptamers that bind to live *T. cruzi* trypomastigotes with an affinity ranging between 8 and 25 nM. The aptamer with the highest affinity, Apt68 (K_d 7.686 ± 1.63 nM), also showed high specificity and did not bind either insect stage epimastigotes of *T. cruzi*, *Leishmania donovani* promastigotes or *Trypanosoma brucei*. The authors also demonstrated that Apt68 was able to bind parasites from different strains, when immobilized in the solid phase and at parasite concentrations as low as 0.33 parasites/ml (five parasites in 15 ml). This approach could be useful for CD diagnosis

during the early phase of infection (the window period of PCR detection) or during the chronic phase when there is intermittent parasitemia in the blood [74].

More recently, the same group used the SELEX strategy to select aptamers that bind specifically to TESA (*T. cruzi* excreted-secreted antigens) aiming to develop a new direct non-serological, non-PCR-based assay to detect *T. cruzi* infection. After ten rounds of selection, Apt-L44 showed specific binding to TESA as well as to *T. cruzi* trypomastigote extract from three different strains but not to epimastigotes, host proteins, or *L. donovani* proteins. Using biotinylated Apt-L44 in an enzyme-linked aptamer (ELA) assay, the aptamer showed specific binding to TESA, and higher levels of binding were observed in the serum of *T. cruzi*-infected mice compared to non-infected mice. Additionally, Apt-L44 could detect circulating TESA in mice in both the acute and chronic phases. Apt-L44 ELA assay could be used as a qualitative assay in drug screening to detect *T. cruzi* antigens in infected mice and demonstrate that live parasites are present in the host, even if their direct detection in blood by PCR is negative [75].

Increasing the SELEX rounds and the stringency of the conditions, de Araujo et al. found an aptamer (Apt29) with a higher signal for TESA in infected mice and a higher signal-to-noise ratio compared to Apt-L44 [76]. This aptamer was also able to differentiate infected from non-infected mice and predict treatment failure. In infected and chronically infected mice, the TESA levels detected by Apt-29 ELA were reduced upon BZN treatment. However, levels did not return to those seen in non-infected treated mice, suggesting parasitemia was reduced but parasitological cure was not achieved. These results are in agreement with the detection of parasites in the heart and skeletal muscles by PCR and suggest that the assay can be used to assess treatment efficacy in vivo in murine drug discovery models [76]. However, its ability to predict parasitological cure needs to be confirmed. The ELA assay does not need sophisticated equipment or reagents but can be performed in high-throughput formats, and animals do not need to be sacrificed. In addition, aptamers can be used to evaluate patients' serum and can be coupled to different matrices to increase the detection limit, which makes them a promising tool for CD diagnosis and prognosis. Further validation using human specimens is, however, necessary before drawing conclusions.

6.2 Biosensors

The application of new technologies and miniaturization have led to new tools, biosensors, that can be applied to the field of Chagas diagnostics and that can help to detect the presence of *T. cruzi* in serum. A biosensor is an analytical device that converts molecular recognition of a target analyte into a measurable signal via a transducer. Depending on the type of transducer that is employed, they may be electrochemical, acoustic, or optical [77, 78]. To develop a biosensor, a biologically active component needs to be immobilized onto the surface of the transducer; once the target analyte is recognized, a signal response in the sensor is generated, and the

signal can be amplified and measured in an electronic system that acquires and records the signal [78]. Biosensors are easy to use, give results in real time and require small sample volumes and short assay times, and have a low energy consumption, which make them excellent tools for point-of-care (PoC) units. Furthermore, they are a sensitive and inexpensive technology platform compared with conventional diagnosis technologies. During the last 15 years, different electrochemical (amperometric or impedimetric) and optical sensors have been designed and tested for the indirect or direct (label-free) detection of *T. cruzi* in serum at acute and chronic stages for CD diagnosis.

In 2011, Pereira et al. optimized an electrochemical immunosensor to quantify IgG *T. cruzi* antibodies in serum patients using *T. cruzi* epimastigote membranes. In order to increase the sensitivity and efficiency of membrane immobilization, a screen-printed carbon electrode (SPCE) was used, and gold nanoparticles (AuNPs) were electrodeposited where the *T. cruzi* antigens were immobilized. The biosensor also uses anti-IgG antibodies coupled to horseradish peroxidase (HRP) and redox reagents to amplify (label) the immunodetection [79]. Their optimized biosensor showed a linear detection of IgG *T. cruzi* antibodies between 11 and 205 ng/mL and a detection limit of 3.065 ng/mL. In addition, the microfluid technology used allowed a fast response and short assay time (26 min). This biosensor is easy to operate and transport, but it still depends on the presence of anti-*T. cruzi* antibodies to predict infection.

More recently, a similar approach was used to develop a biosensor to detect and quantify anti-*T. cruzi* IgM antibodies in newborns and infants and to predict congenital CD [80]. In this case, a SAPA (shed acute-phase antigen) was immobilized in a SPCE together with AuNPs. IgM antibodies appear early in the acute phase of *T. cruzi* infection, and the SAPA has been shown to be a good marker for CD diagnosis by conventional serology. Moreover, anti-SAPA antibodies (IgM or IgG) have been detected in 90% of acute chagasic patients and in 7–10% of chronic patients [81]. This biosensor can distinguish between congenitally infected and non-infected infants when cord blood is tested, the sensitivity is in the ng/mL range (3.03 ng/mL) and the linear response between 10 and 200 ng/mL [80]. This device could facilitate and speed up the unequivocal diagnosis of congenital transmission, since it does not depend on the detection of parasite in newborns or the clearance of maternal antibodies in infants. To continue its validation, a large set of samples from newborns and infants at different ages needs to be tested.

A biosensor to be used in PoC units for serodiagnosis of infectious diseases was fully developed by Cortina et al. [82]. In this device, antigen-coated magnetic beads are used to detect antibodies in serum samples. Immunocapture is amplified using HRP-conjugated secondary antibodies, and the beads magnetically collected are placed on an electrode surface to detect peroxidase activity amperometrically [82]. For CD diagnosis, recombinant proteins of different *T. cruzi* antigens (Ag1, Ag36, SAPA, and TSSA) were used to coat superparamagnetic beads and tested with serum samples from CD patients and HC. Results showed that the magnetic bead-based biosensor discriminates infected from non-infected serum with a minimal overlap and excellent signal-to-noise ratio. It also showed a high level of accuracy

in diagnosis, similar to ELISA and IFA, as well as having similar sensitivity and selectivity [82]. Despite the different steps to be performed during the assay, it can be done in PoC units as it uses an eight-channel portable potentiostat powered by a rechargeable battery. The device is not yet commercialized, but there is potential for its use in the diagnosis of CD and other parasitic infections.

All the above biosensors detect *T. cruzi* antigens indirectly by peroxidase activity, which requires multiple steps of incubation with a secondary antibody and redox reagents. In contrast, a free-label biosensor that uses optical transducers needs fewer steps and a shorter assay time and still shows high sensitivity. An optical immunosensor (SPRCruzi) with a surface plasmon resonance (SPR) transducer was recently developed for CD diagnosis [83]. SPR sensors use surface plasmons, which are electromagnetic waves that can be excited by light at gold sensor interfaces, to transduce a biochemical interaction. In brief, the interaction changes the SPR baseline, and real-time measurement of specific analytes in unknown samples flushed over the sensor can be performed; for a review, see [77, 84].

To build SPRCruzi, Luz and collaborators used soluble antigens of *T. cruzi* epimastigotes immobilized on a sensor chip. The biosensor was able to discriminate positive from negative serum, including those infected with other related parasites, and detect antibodies in serum dilutions as high as 1280×. In 2016, the same group tested SPRCruzi with a higher number of positive and negative serum samples and compared their results with conventional serological tests. SPRCruzi showed 100% sensitivity (cutoff ΔθSPR 17.2°), 99.6% global accuracy, and a better specificity (97.2%) compared to ELISA [80]. Nonetheless, the use of this device is still limited to laboratories since expensive and heavy SPR equipment is required.

Lastly, a CD nanowire electrical sensor based on field-effect transistor (FET) technology was designed last year by Janissen et al. [85]. In FET sensors, the current-carrying capability of a semiconductor is used, and the sensor response is interpreted as a result of a shift in the threshold voltage of the field-effect structure [77]. In the Janissen et al. device, anti-*T. cruzi* IBMP8-1 antibodies were immobilized on a surface using a biocompatible ethanolamine and poly(ethylene glycol) derivate coating. This biosensor reached detection limits in the femtomolar range (6 fM) for a recombinant IBMP8-1 protein. This limit of detection is 1000-fold lower than ELISA (30 nM), PCR (10 nM), and even electrochemical immunosensors (20 pM). In addition, the assay is fast, taking less than 30 min, and label-free. This highly sensitive biosensor still needs to be tested in human samples and optimized for PoC unit use; but so far it is the only CD biosensor that does not depend on the detection of anti-*T. cruzi* antibodies.

6.3 MicroRNA

MicroRNAs play key roles in intracellular and extracellular protein expression and regulation of biochemical pathways. They may be associated with regulating cellular apoptosis, proliferation, differentiation, metabolism, invasion, and migration. Different studies have also shown that microRNAs may serve crucial functions in the progression of numerous cancers and other diseases and

consequently can be used as biomarkers for prognosis of disease progression [86]. For example, microRNA-208a (miR-208a) encoded by the α-myosin heavy chain (MHC) gene has been shown to be involved in pathological cardiac growth, fibrosis, and upregulation of β-MHC expression in human dilated cardiomyopathy (DCM) [87]. It is also an early diagnostic biomarker of acute myocardial infarction (AMI) and can be used for prognosis postinfarction and disease monitoring [88].

Different microRNAs and their mRNA targets appear dysregulated in chronic Chagas disease cardiomyopathy (CCC) patients and CD murine models during acute infection [89, 90]. This year, Linhares-Lacerda et al. suggested microRNA208a could be a potential biomarker of chronic indeterminate CD (CID) [91]. Their results have shown upregulation of miR-208a in serum of CD patients in the indeterminate stage compared to chronically infected cardiac patients with DCM. This suggests the microRNA is participating in the early-onset events responsible for activation of fibrosis and cardiac dysfunction processes in CD. The same microRNA has been reported to be downregulated in the heart muscle of CD patients with CCC or DCM compared to non-infected controls [89] but upregulated in endomyocardial tissues of non-infected DCM patients [87]. Clearly a better understanding and tests in a large number of patients is needed to validate this biomarker. Nevertheless, this study opens the door to looking at microRNA as a possible analyte for CD diagnosis and prognosis.

6.4 Omics-Based Applications

Omics-based applications are formidable new technological resources for investigating the status of human diseases and understanding the pathophysiology of disease processes. They can generate enormous amounts of data with high fidelity thanks to recent advances in chromatography, mass spectrometry, and bioinformatics. Furthermore, omics outputs have the advantage of complementarity, enabling cross-corroboration and cross-validation [92]. In the search for biomarkers and tools for CD diagnosis and drug treatment efficacy assessment, our group is using omics applications to detect changes in the proteome, metabolome, and lipidome of CD patients compared to healthy people (Fig. 1). We are using our omics studies to build assays that could be widely employed for diagnosis, prognosis, and evaluation of treatment efficacy of new drugs. To this end, we are focusing primarily on the identification of new host markers following comparative analysis of serum samples issued from patients diagnosed with CD, treated, and followed up several years after treatment and in some cases until they reach seroreversion, the only current surrogate marker for parasitological cure.

In our earlier work involving mass spectrometry serum protein profiling studies, we identified highly sensitive and specific host protein markers (see Sect. 5.2) [50, 51]. We started our proteomics studies using MS SELDI technology for intact serum proteins analysis; in spite of the promising results, the SELDI technology did not allow the direct identification of proteins by tandem MS and had low resolution. Recently, we have used an ultrahigh-resolution quadrupole time-of-flight (UHR-

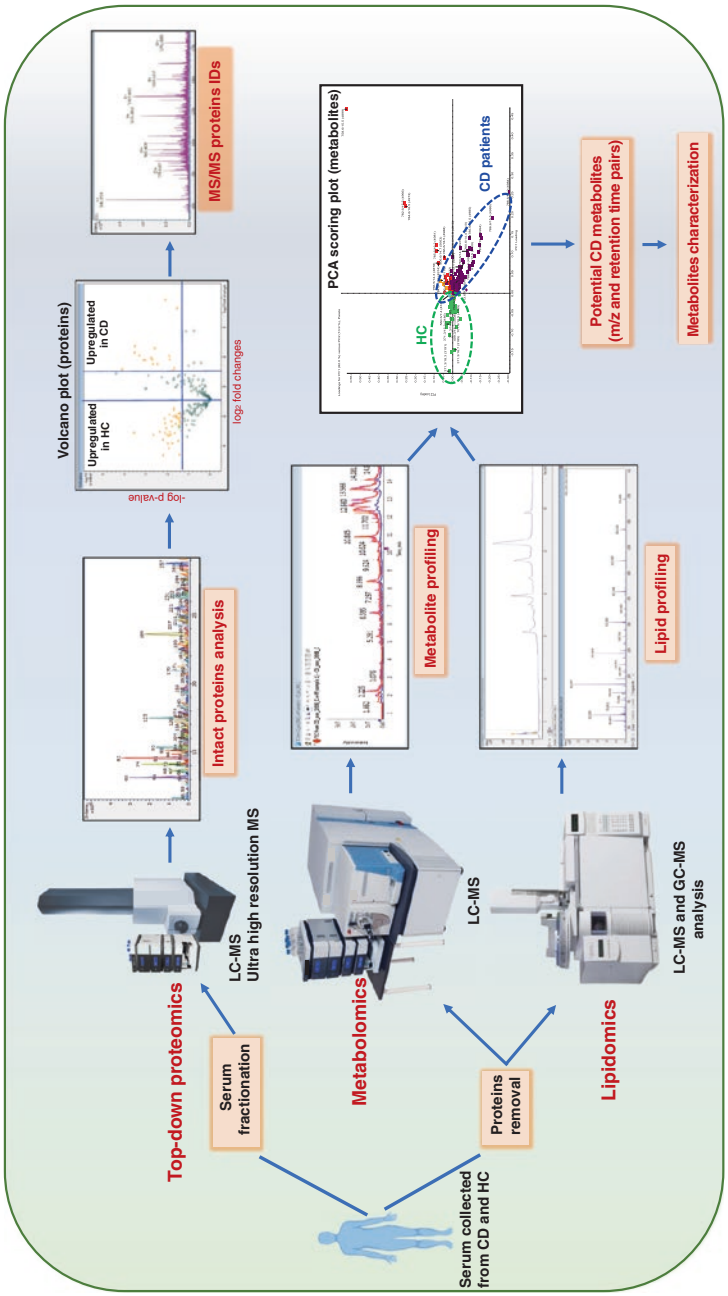

Fig. 1 Omics-based applications for CD biomarkers discovery. Serum proteins, all metabolites or only lipids from Chagas Disease (CD) and Healthy Control (HC) populations are analyzed by different LC-MS systems. Features (Intact proteins in *top-down proteomics*, candidates metabolites in *metabolomics* or candidate lipids in *lipidomics*) profiles are compared between population to find those that can discriminate CD of HC. Rigorous statistical analysis is done in all cases and candidates are further study by LC-MS/(MS^n) for identification and verification

QTOF) tandem mass spectrometer coupled to ultrahigh-performance liquid chromatography (HPLC) for the same purpose. Despite the differences between MS platforms and sample processing, we have been able to reproduce our previous findings, reinforcing the robustness of our data.

In order to validate some of the protein biomarkers identified (ApoA1 and FBN fragments) using our new MS platform, we recently attempted to correlate the presence/absence of these fragments with seroreversion, which is currently the gold standard of parasitological cure in a cohort study of CD children treated with BZN. Compared to adults, seronegative conversion in children occurs in a few months to a few years, which made children's serum samples ideal for the validation of these biomarkers. Our MS analysis and specific immunoblot results showed these fragments were absent in serum at seroreversion in the entire CD pediatric population and in some cases at the end of BZN treatment even when children remained seropositive [93]. Although still preliminary, these data suggest that ApoA1 and FBN fragments could be used as biomarkers of parasitological cure and predict cure earlier than serology, which will make them better endpoint surrogates. Additional studies are needed to further explore the real potential of these new biomarkers, for instance, an evaluation of fragment disappearance kinetics following treatment until seroreversion.

While the proteome is of great importance, the metabolome can also provide an excellent pathophysiological understanding of disease, as proteins have functions in a range of complex metabolic reactions and their activity ultimately affects the phenotype. Metabolites (low molecular weight organic and inorganic chemicals) are simpler to study compared to proteins, are the final downstream products, and give a sensitive and rapid measurement of the phenotype. Various disease states may be characterized by a specific metabolite or a pattern of metabolite changes. Metabolomics has been successfully applied to clinical conditions including inborn errors of metabolism, cardiovascular disease, and cancer to identify biomarkers related to diagnosis, assessment of disease severity, or drug toxicity/efficacy—for a review, see [94]. Changes in the human metabolome due to *T. cruzi* infection are as yet unknown, and metabolites have not been explored as biomarkers for CD. Taking advantage of our high-resolution platform, we are currently studying the serum metabolite profile of CD patients, looking for biological differences after treatment and compared to a healthy population.

Finally, considering the evidence that associates *T. cruzi* with the adipose tissue, ApoA1 and with HDL and LDL modifications, as well as its interaction with LDL receptors [95], the serum lipid profile is an interesting and important metabolome component in the search for possible CD biomarkers. Lipidomics has been defined as "the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation." Although lipids are not used in clinical applications yet, many individual lipids have been associated with the evolution of different cancers, cardiovascular, neuropsychiatric, respiratory, and kidney diseases [96] and can provide information on disease status. Together with our metabolomics study, we are presently characterizing the lipidome in CD patients using an untargeted approach.

We expect these holistic approaches lead to the identification of analytes that can discriminate between infected and non-infected populations.

Taken together, data issued from these different technologies will hopefully speed up the identification of markers suitable for clinical settings and proof of concept clinical studies and during drug development.

6.5 Other Technologies

Other no less important approaches to finding potential biomarkers rely on the identification of specific antibodies and the detection of anti-live *T. cruzi* antibodies by flow cytometry. To discover pathogen-specific linear B-cell epitopes from clinical samples, Carmona et al. used a highly multiplexed platform based on next-generation high-density peptide microarrays to map antibody specificities in CD [97]. In this approach, individual peptides (~180,000) are synthesized in situ on a glass slide at high densities, which reduces cost and allows a high-throughput and precise mapping of antibodies. After screening the arrays with antibodies purified from CD patients and HC, 2031 disease-specific peptides and 97 novel parasite antigens were identified, together with their linear B-cell epitopes [97]. Recently, Mucci et al. assessed the serological performance of 27 of these epitopes and their use in a multipeptide-based diagnostic method [98]. Seven peptides were evaluated in ELISA against 199 serum samples from CD and HC, including samples from leishmaniasis subjects. The assay showed a sensitivity of 96.3% and a specificity of 99.15% for CD, which suggests that the peptides could be used in CD diagnosis; however, their usefulness in treatment efficacy evaluation needs to be further studied.

As mentioned in Sect. 5.1, the detection of lytic antibodies against live parasites is an alternative to the evaluation of specific antibodies. In a double-blinded study with 94 coded samples, Martins-Filho et al. found that anti-live trypomastigote antibody (ALTA) measured by flow cytometry (FC) was able to discriminate not treated (NT), treated but not cured (TNC), and treated and cured (TC) patients when using a 1:256 serum dilution [99]. In a larger study population with four different cohorts, the same group demonstrated that anti-fixed epimastigote antibody (AFEA) discriminates the clinical status of CD patients after treatment at higher serum dilutions (1/2048). FC-AFEA-IgG showed 100% sensitivity (80.3–100%) and specificity (85.6–100%) with positive and negative predictive values of 100%. This suggests both antibodies measured by FC are not only good enough for diagnosis and prognosis but could be useful as criteria of cure.

7 The Way Forward

The identification and validation of biomarkers is in general is a very challenging process. For Chagas disease, the definition of cure or clinical benefit following treatment is clearly another major challenge. In fact, in principle, we are looking for a surrogate of a surrogate for clinical benefit, i.e., looking for a surrogate of serological cure assuming that the latter is a surrogate marker for clinical cure or halting

of progression of the disease, and searching for a surrogate marker of serological cure that allows the rapid determination of seroreversion, indicating that *T. cruzi* parasites have been eliminated from the patient's body. The identification and validation of new biomarkers for Chagas disease is, therefore, a major challenge but a serious and important one that needs to be tackled in the endeavor to develop drugs for CD. Unfortunately, no biomarkers have yet progressed to clinical validation. No correlation has been made between either the absence of *T. cruzi* DNA in blood (as assessed by PCR) or the reduction in titers of specific *T. cruzi* antigens or antibodies (as assessed using serological tests at specific timepoints after treatment) and seroreversion; which make these markers potential pharmacodynamic markers but not surrogates for parasitological cure at this point. Newly identified potential markers using different technology platforms, from either the host (−omics) or other antigens of the parasite (microarrays), are showing promise, but more work is needed to assess their validity. In particular, the development, optimization, and analytical verification and validation of tests for these new markers, either as single prototypes or in a multiplex, are needed before moving forward to clinical validation. It is also reasonable to believe that a set of biomarkers rather that a single "magic bullet" might be needed to ensure a correlation with parasitological cure.

Pending a robust validated multiplex assay with valid biomarkers, the challenge of being able to clinically validate these markers and obtain regulatory acceptance remains significant. In order to do this, a sufficient number of high-quality samples from well-defined cohorts are needed to move forward (biostatistical plan). The use of retrospective cohorts could be envisaged, but a large prospective study (clinical trial with long post-therapeutic follow-up of patients) might be required to verify that markers are surrogates for seroconversion. In either case, the entire Chagas community needs to make a concerted unified effort.

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