Biomarkers of therapeutic responses in chronic Chagas disease: state of the art and future perspectives

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The definition of a biomarker provided by the World Health Organization is any substance, structure, or process that can be measured in the body, or its products and influence, or predict the incidence or outcome of disease. Currently, the lack of prognosis and progression markers for chronic Chagas disease has posed limitations for testing new drugs to treat this neglected disease. Several molecules and techniques to detect biomarkers in Trypanosoma cruzi*-infected patients have been proposed to assess whether specific treatment with benznidazole or nifurtimox is effective. Isolated proteins or protein groups from different* T. cruzi *stages and parasite-derived glycoproteins and synthetic neoglycoconjugates have been demonstrated to be useful for this purpose, as have nucleic acid amplification techniques. The amplification of* T. cruzi *DNA using the real-time polymerase chain reaction method is the leading test for assessing responses to treatment in a short period of time. Biochemical biomarkers have been tested early after specific treatment. Cytokines and surface markers represent promising molecules for the characterisation of host cellular responses, but need to be further assessed.*

Key words: biological marker - Chagas disease - *Trypanosoma cruzi* - therapeutic response - parasite marker - host markers

The definition of a biological marker (and its portmanteau, "biomarker") is still under discussion. A biomarker is a medical sign that can be measured accurately and reproducibly (Strimbu & Tavel 2010). The International Programme on Chemical Safety led by the World Health Organization (WHO 1993) has defined a biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence or outcome of disease" (WHO 2001). A broader definition by the same working group included not only the incidence and outcome of disease, but also the effects of treatments, interventions and environmental exposures. Although they are commonly used in clinical research (even as primary endpoints), laboratory-measured biomarkers in clinical research are still being developed and their use for the diagnosis and evaluation of responses for some diseases is still in progress.

Currently, there is a lack of validated early markers for therapeutic responses to chronic Chagas disease. The gold standard for evaluating treatment efficacy is the seroconversion of conventional serological tests, which may take years to decades to assess (Viotti et al. 2006, Fabbro et al. 2007). Currently, biomarkers are potentially useful for two purposes: to evaluate the efficacy of current drugs in a short period of time, raising the possibility of precisely informing patients about their therapeutic progress and to provide valid tools for use in clinical trials with new drugs. The use of biomarkers will allow the improvement of therapeutic options for people who suffer from this neglected disease.

Recently, several prognosis and progression markers for *T. cruzi* infection have been developed or discovered, but only a few of them have been evaluated after specific treatment and using appropriately designed studies. Two systematic reviews were conducted over the last year to evaluate the following criteria: (i) the usefulness of the various types of blood-derived biomarkers that are currently under study to predict the progression of Chagas disease in patients with the indeterminate form, to assess the efficacy of antiparasitic drugs and to identify early cardiac or gastrointestinal damage (Requena-Méndez et al. 2013) and (ii) the therapeutic use of potential biomarkers and their disease stage-specific characteristics (Pinazo et al. 2014). Both reviews were based on current evidence and focused on the quality of biomarker studies. Additionally, the second review (Pinazo et al. 2014) established that molecules should fulfill several specific quality criteria for evaluating responses to treatment for chronic Chagas disease. A target product profile was developed based on the available evidence concerning biomarkers and the experience of an expert group on the field (New Tools for Diagnosing and Evaluating Chagas Disease Patients Working Group).

The conclusion of the reviews revealed that even if there is heterogeneity in the data concerning biomarkers studied by different groups, certain biomarkers have

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TABLE
Summary of major biomarkers described in the review

Parasite biomarkers	
Parasite molecules	
rTc24	Krautz et al. (1995)
tGPI-mucins (F2, F2/3 or AT antigen)	Almeida et al. (1993), de Andrade et al. (1996), Andrade et al. (2004)
Complement regulatory protein	Meira et al. (2004)
F-IV fraction and EXO	Moretti et al. (1998)
F29	Sosa-Estani et al. (1998), Fabbro et al. (2013)
Ag13	Sanchez-Negrette et al. (2008)
P2β	Fabbro et al. (2011)
Recombinant proteins set (multiplex)	Cooley et al. (2008), Viotti et al. (2011)
KMP11, H70, PFR2, Tgp63	Fernández-Villegas et al. (2011, 2014)
Parasite DNA/RNA amplification techniques	
Polymerase chain reaction techniques	Britto et al. (1999, 2001), Fernandes et al. (2009), de Lana et al. (2009), Gomes at al. (2009), Murcia et al. (2010), Pérez-Ayala et al. (2011), Aguiar et al. (2012), Machado-de-Assis et al. (2012)
Aptamers L44	Nagarkatti at al. (2012, 2014)
Host response/damage biomarkers	
Immunological markers	
TSKb20	Bustamante et al. (2008), Costa et al. (2009)
CD62L, CD127	Bustamante et al. (2014)
CD27, CD28	Appay et al. (2008)
TEM CD45RA+ CCR7	Albareda et al. (2006)
CD40L	Chamekh et al. (2005), Habib et al. (2007)
Interferon-γ	Romanha et al. (2002), Ferraz et al. (2007), Laucella et al. (2009), Poveda et al. (2014), Sousa et al. (2014)
Interleukin (IL)-10	Sousa et al. (2014)
IL-17	Kolls and Lindén (2004), Miyazaki et al. (2010), Magalhães et al. (2013), Costa et al. (2009)
CCL2/monocyte chemotactic protein-1	Paiva et al. (2009), Tuñón et al. (2014)
Macrophage inflammatory protein-1α/CCL3	Roffê et al. (2010), Falcão et al. (2002)
Ab amastigotes, trypomastigotes, epimastigotes	Alessio et al. (2014)

Host response/damage biomarkers	
Biochemical biomarkers	
Cardiological markers [troponin I, T, atrial natriuretic peptide, brain natriuretic peptide (BNP) proBNP]	Puyó et al. (2002, 2005), Ribeiro et al. (2003), Heringer-Walther et al. (2005), Machado et al. (2005), Moreira et al. (2008), García-Alvarez et al. (2010), Lima-Costa et al. (2010)
Selenium	Rivera et al. (2002)
Apolipoprotein A1 and fragments (F)	Santamaria et al. (2014)
Endogenous thrombin potential, F1+2	Pinazo et al. (2011)

been shown to be effective in assessing responses to specific treatment with benznidazole (BZ) and nifurtimox (NFX) in different stages of Chagas disease. Biomarkers for evaluating responses to specific treatments for people suffering from chronic Chagas disease can be classified into two groups: parasite biomarkers and host response/damage biomarkers.

The aim of this article is to provide an update on advances in the research of several of these molecules. Table summarises the major biomarkers described in this report and the related references. However, this review will be mainly focused on parasite-derived protein and DNA biomarkers and host-derived biochemical and immunological biomarkers. Due to its complexity, glycoconjugate biomarkers that effectively measure lytic, protective anti-α-galactosyl antibody levels have been extensively discussed elsewhere (Almeida 2014).

Parasite biomarkers

Parasite biomarkers include several proteins and glycoproteins isolated from the parasite (i.e., the F29 protein) (Fabbro et al. 2013), as well as protein groups, recombinant proteins, proteins purified from different forms of the parasite (Cooley et al. 2008, Fernández-Villegas et al. 2011) and parasite-derived glycoproteins and synthetic neoglycoconjugates (Almeida et al. 1993, 1994, Ashmus et al. 2013). Nucleic acid amplification techniques, such as real-time polymerase chain reaction (PCR) or the use of RNA ligands (aptamers) (Nagarkatti et al. 2014), are also included in this group.

Parasite proteins - Current tests for the diagnosis of *Trypanosoma cruzi* infection are mainly reliant on conventional serological techniques that have high sensitivity and specificity (Britto et al. 2001, Cançado 2002, Coura & de Castro 2002). However, these methods of diagnosis are not effective in determining the evolution of chronic disease in Chagas patients after treatment. Consequently, there are no early markers to detect recovery after treatment, making both the clinical follow-up of the affected patients and clinical trials with new drugs difficult. In this context, several systems have been tested as markers of therapeutic efficacy with relative success in prospective and retrospective trials. These assays are based on the detection of specific antibodies against parasite antigens.

In one retrospective study, a 24-kDa recombinant protein from *T. cruzi* (rTc24) was evaluated by ELISA and western blot tests. The study pursued the identification of treated chagasic patients who were considered uncured or cured based on a positive or persistent negative lytic antibody test (by complement-mediated lysis, CoML), respectively (Krautz et al. 1995). Forty sera samples from treated patients who were considered uncured because they presented a positive CoML test reacted with rTc24. Fourteen of these samples had negative haemocultures. Moreover, 22 out of 28 (79%) sera samples from treated patients with negative CoML and haemoculture tests, but with a positive result in the indirect immunofluorescence test, showed no reactivity against rTc24. The authors concluded that the rTc24 molecule could be used to assess the cure of treated Chagas patients.

A similar retrospective study evaluated an ELISA based on the recombinant *T. cruzi* complement regulatory protein (rCRP) as a method for determining parasite clearance. The study included sera from 31 patients collected before and after treatment (Meira et al. 2004). The results were compared to those obtained by other methods, such as CoML, conventional serology and haemoculture. The results showed that the percentage of patient samples that were positive by rCRP ELISA was reduced from 100-70.3%, 62.5%, 71.4% and 33.4% in the first, second, third and fourth years after treatment, respectively. The authors also showed that the percentage of positive samples by CoML was reduced to 85.2%, 81.2%, 71.4% and 33.4% during the same period of time, demonstrating the same tendency in the reduction of positive samples.

The serological reactivity of 42 chronic chagasic patients treated with NFX or BZ for two-20 years and 42 untreated patients (Moretti et al. 1998) against the F105 antigen, the F-III and F-IV fractions from *T. cruzi* extracts and the exo-antigens from trypomastigote-infected mice (EXO) was analysed by ELISA. The results showed a significant decrease in serologic reactivity against the F-IV fraction and EXO antigen in 64% and 44% of treated vs. 33% and 8% of untreated patients, respectively. No differences in reactivity against the F105 antigen and F-III fraction were detected between the two groups of patients.

Purified trypomastigote-derived glycosylphosphatidylinositol-anchored mucin glycoproteins (tGPI-mucins), also known as F2, F2/3 or AT antigen, were previously used in a chemiluminescent ELISA (AT CL-ELISA) to measure lytic anti-α-Gal antibody titres to confirm the efficacy of BZ treatment of chronically infected children and adolescents from an endemic area in a placebo-controlled randomised trial (de Andrade et al. 1996). Although all conventional serology tests remained positive after a three-year observation period, 58% of 64 children were considered cured according to the AT CL-ELISA. In a follow-up of that study, Andrade et al. (2004) confirmed successful BZ chemotherapy in ~65% and ~85% patients by intention-to treat and by per protocol analysis, respectively, as measured by seroconversion using AT CL-ELISA six years after the end of treatment. In a subsequent study, reactivity against the recombinant *T. cruzi* flagellar calcium-binding protein (F29) was analysed by ELISA for use as a short-term monitoring method to test the efficacy of BZ in children in the indeterminate phase of Chagas disease. The results from this double-blind, randomised, clinical field trial study indicated that 35.2% and 62.1% of the 44 BZ-treated children were seronegative for the F29 antigen six and 48 months post-treatment, respectively (Sosa-Estani et al. 1998). Recently, the ELISA-F29 test was also used to compare the time at which negative seroconversion against the F29 antigen was detected vs. conventional serology. The study included 29 patients who received NFX or BZ treatment and 37 untreated subjects (Fabbro et al. 2013). The data obtained showed that seroconversion was detected significantly earlier using the ELISA-F29 test compared to conventional serology, with values of 14.5 ± 5.7 and 22 ± 4.9 years, respectively. Likewise, regression in antibody levels against different recombinant molecules (Ag1, Ag2, Ag13, Ag30, Ag36 and SAPA antigens) was used to detect early markers of treatment effectiveness (Sanchez-Negrette et al. 2008). The specific-antigen antibody levels against these molecules were monitored in sera from 18 adult patients after three years post-treatment. Before treatment, most of the patients had specific reactivity against these antigens; after treatment, the specific reactivity against some of these antigens was reduced in 50% (9/18) of the patients. The best results for this technique as a marker of treatment efficacy were obtained for Ag13 due to the occurrence of negative seroconversion in six out of the nine patients who presented specific antibodies against this antigen before treatment.

The dynamics of reactivity against the *T. cruzi* ribosomal protein (P2β) were evaluated in a retrospective study using sera from treated and untreated chronic Chagas disease patients who were followed for more than 20 years. The obtained data showed that the levels of antibodies against P2β decreased from their initial values in treated asymptomatic patients, but did not decrease in asymptomatic or untreated cardiomyopathy patients (Fabbro et al. 2011).

In addition to the decrease in antibody titres, seronegative conversion was measured in 53 BZ-treated and 89 untreated chronic patients with an average follow-up time of 36 months using conventional serological assays (immunoassay, indirect fluorescent immunoassay and ELISA) and a set of 16 *T. cruzi* proteins incorporated into a multiplex bead array (Cooley et al. 2008). A decrease in antibody titres against *T. cruzi* was detected by conventional serology tests in 64% of treated patients vs. 21% of untreated patients and seronegative conversion against the set of proteins was detected in 40% of treated vs. 7% of untreated patients. Moreover, a strong correlation between the results from conventional serological tests and the multiplex assay was detected (Viotti et al. 2011).

A prospective study performed using sera from 46 adult chronic Chagas disease patients and 22 healthy donors showed that the set comprising four recombinant proteins (KMP11-H70-PFR2-Tgp63) could serve as a useful tool for monitoring the effectiveness of Chagas disease treatment (Fernández-Villegas et al. 2011). Regardless of the phase of Chagas disease, the sera from chagasic patients reacted against the above mentioned antigens with statistical significance relative to the recognition level of sera from healthy donors, patients with autoimmune diseases or patients suffering from other related infectious diseases, such as tuberculosis, leprosy or malaria. Shortly after BZ treatment, a drop in reactivity against three of these antigens (KMP11, PFR2 and HSP70) was detected in an antigen-specific manner. Thus, a statistically significant decrease in reactivity against KMP11 occurred six months post-treatment in 74% of patients, a drop in reactivity against PFR2 occurred at nine months post-treatment in 74% of patients and a drop in reactivity against HSP70 occurred at nine months post-treatment in 71% of Chagas disease patients. The drop in reactivity remained constant or continued to decrease during the post-treatment follow-up period (24 months). A further decrease in reactivity was detected in 67%, 50% and 34% of patients for KMP11, PFR2 and HSP70, respectively. However, no statistically significant drop in reactivity was observed against total soluble *T. cruzi* proteins.

The KMP11-H70-PFR2-Tgp63 serological biomarker was recently employed in a blind post-treatment follow-up of twin brothers congenitally infected with the same *T. cruzi* strain (Fernández-Villegas et al. 2014). Remarkably, a drop in reactivity against these proteins was correlated with treatment success in brother I. However, no modification in the antibody titre against these proteins (which would have allowed a treatment interruption) was detected in brother II. A drop in reactivity against these molecules that was correlated to treatment success was observed 45 days after administration of the second treatment.

Parasite DNA/RNA amplification techniques - Amplification of *T. cruzi* DNA is currently the leader test for assessing the response to treatment in a short period of time in patients with chronic *T. cruzi* infection. To date, this method has been mainly used in clinical trials for this purpose.

PCR techniques have been mainly used to assess treatment failure when a positive result was obtained, to diagnosis patients in the acute stage of the disease and for the early detection of reactivation of the disease in organ transplant patients. Compared with other parasitological diagnostic methods (i.e., haemoculture and xenodiagnoses), the sensitivity obtained by the PCR tech-

nique is higher for patient treatment follow-up (Britto et al. 1999, 2001, de Lana et al. 2009, Fernandes et al. 2009). Currently, due to improvements in PCR techniques the possibility of performing quantitative PCR in real time quantitative reverse transcription-PCR has provided more specificity for these tests.

However, blood parasitaemia fluctuates during the chronic phase of infection and may be below the PCR detection level, especially during the long period of follow-up after trypanocidal treatment (Gomes et al. 2009, Murcia et al. 2010, Pérez-Ayala et al. 2011). Several studies have used this technique, which has greatly improved the detection of treatment failure (85-89% of patients) (Britto et al. 1999, de Lana et al. 2009, Fernandes et al. 2009). However, a negative PCR result does not guarantee a parasitological cure or a response to a specific treatment. Nevertheless, PCR-negative conversion was achieved in some studies in a variable proportion of treated patients (Britto et al. 2001, Murcia et al. 2010, Pérez-Ayala et al. 2011, Aguiar et al. 2012, Machado-de-Assis et al. 2012) in a manner that did not always correlate with negative seroconversion (Britto et al. 1995, Lacunza et al. 2006, Solari et al. 2001, Fernandes et al. 2009, Murcia et al. 2010, Pérez-Ayala et al. 2011, Aguiar et al. 2012, Machado-de-Assis et al. 2013). Thus, obtaining several samples from each patient at different intervals to increase the probability of detecting *T. cruzi* DNA in blood samples is recommended to increase the sensitivity of the PCR test.

Although parasite DNA amplification techniques provide a more rapid and sensitive test and could avoid very demanding long-term patient follow-up, this technique is not available in regular health care centres.

Short RNA ligands called aptamers have been recently developed as potential biomarkers that could be used to test the response to specific treatments in chronic Chagas disease patients (Nagarkatti et al. 2012, 2014). These molecules have not yet been evaluated in humans, but have been used as diagnostic tests of both the acute and chronic stages of the disease in murine models. Aptamers generated against *T. cruzi* excreted/secreted antigens (TESA) were purified from in vitro culture supernatants of infected host cells and used as specific ligands in enzyme-linked aptamer assays. Aptamer L44 showed significant and specific binding to both TESA and a *T. cruzi* trypomastigote extract, but not to host proteins, proteins from *Leishmania donovani* (a related trypanosomatid parasite) or samples from noninfected mice.

Although they are still in an early stage of development and further investigations should be performed, aptamers have a great potential for the evaluation of treatment efficacy and possibly a parasitological cure in human clinical trials.

Host response/damage biomarkers

Biochemical biomarkers such apolipoprotein (APO) and fibronectin fragments (Santamaria et al. 2014) and hypercoagulability markers (Pinazo et al. 2011) have been tested early after specific treatments in patients in different stages of Chagas disease. Cytokines and surface markers are promising molecules that can be used to characterise host cellular responses, but their use in gauging the response to treatment during *T. cruzi* infection needs to be further assessed.

Immunological markers of treatment efficacy - The development of markers to measure therapeutic responses in *T. cruzi* infected patients is currently the subject of intense research. Drug discovery and the development of improved therapies for Chagas disease rely on the ability of assays to determine treatment outcomes. However, monitoring treatment efficacy in individuals infected with *T. cruzi* is still a significant hindrance. The major complication is associated with the fact that the parasite loads in chronically infected subjects (the main population in need of trypanocidal treatment) are controlled at extremely low levels. This situation has forced the need for an essential amplification step (blood culture or DNA isolation from the parasite) for the detection of parasites in patients who suffer from the chronic form of the disease (Bustamante & Tarleton 2011, Gilber et al. 2013, Muñoz et al. 2013).

Based on this complex scenario, the development of biomarkers to measure the treatment efficacy of Chagas disease is an imperative need in cases where the failure to detect *T. cruzi* is the most common result. Recently, work on animal models has generated a widely accepted endpoint immunosuppression procedure for the assessment of cure following drug treatment (Bustamante et al. 2008, 2014, Canavari et al. 2010, Villalta et al. 2013). Using this system, the generation of anti-*T. cruzi* T cell responses based on the detection of major histocompatibility complex (MHC)-peptide tetramers that are uniquely specific for $CD8^+$T cells recognising a transialidase peptide (TSKb20) were monitored as biomarkers of treatment success (Bustamante et al. 2008, 2014). Drug-cured mice showed a lower frequency of *T. cruzi*-specific $CD8^+$ T cells and an increased number of memory T cells displaying a central memory phenotype (T_{CM}) compared to their untreated counterparts (Bustamante et al. 2008). T_{CM} cells as well as T effector memory cells (T_{EM}) can be distinguished by their patterns of surface molecule expression. These molecules include the L-selectin receptor CD62L, which is essential for the ability of lymphocytes to traverse high endothelial venules and enter lymph nodes and the interleukin (IL)-7 receptor alpha (CD127), which is involved in the homeostatic maintenance of memory T cells. T_{CM} cells generally express both of these receptors, whereas T_{EM} cells express neither (Sallusto et al. 1999, 2004). Therefore, the phenotype of *T. cruzi*-specific $CD8^+$ T cells can be used as a potential marker for the determination of treatment efficacy and cure. Interestingly, conversion of *T. cruzi*-specific $CD8^+$ T cells from the largely T_{EM} phenotype ($CD62L^{lo}$, $CD127^{lo}$) characteristic observed in persistent infection to a majority T_{CM} cell population ($CD62L^{hi}$, $CD127^{hi}$) has been observed when a cure is established with different treatment protocols, such as the combination of the anti-fungal posaconazole with BZ and the use of intermittent regimens (Bustamante et al. 2014).

Although an immunosuppression assay cannot be performed in humans for obvious ethical reasons, an accidental validation of this finding was recently reported

in an immunosuppressed patient with systemic lupus erythematosus who was treated with trypanocidal therapy (posaconazole) to clear the infection (Pinazo et al. 2010). Nonetheless, the tracking and phenotyping of *T. cruzi*-specific T cells in humans are complicated by their low frequency and largely unknown antigen specificity (Laucella et al. 2004, Alvarez et al. 2008). The frequency of peripheral interferon (IFN)-γ-producing T cells specific for *T. cruzi* declined in BZ-treated individuals 12 months after follow-up; these cells subsequently became undetectable in a substantial proportion of the treated subjects (Laucella et al. 2009). In a previous work (Appay et al. 2008), a linear model of T cell differentiation based on the expression of the T cell surface markers CD27 and CD28 was proposed. In this model, $CD27^+$ $CD28^+$ T cells were defined as "early" differentiated memory $CD8^+$ T cells, as opposed to the $CD27^-$ $CD28^-$ T cells considered to be "late" or "fully" differentiated memory $CD8^+$ T cells. Using this model of differentiation, Albareda et al. (2006) found that the frequency of early-differentiated $CD8^+$ $CD27^+$ $CD28^+$ T cells in the entire $CD8^+$ T cell memory compartment in *T. cruzi* infected subjects decreased as the disease become more severe, while the proportion of fully differentiated memory ($CD27^-$ $CD28^-$) $CD8^+$ T cells increased. Additionally, they also reported a significant increase in the total effector memory $CD8^+$ T cell (TEM $CD45RA^+$ $CCR7^-$) population in *T. cruzi* infected subjects with mild heart disease compared with uninfected controls. However, whether the early-differentiated $CD8^+$ T cells ($CD27^+$ $CD28^+$) that decreased in the more severe clinical picture will increase after trypanocidal treatment and whether this T cell subset can be used as a surrogate marker for treatment success remain to be explored.

CD40L (CD154) is a T cell marker that has been reported to have prognostic value in some parasitic infections. CD40L, a member of the tumour necrosis factor superfamily of molecules, is primarily expressed on activated T cells and can also be found in a soluble form (sCD40L). CD40L plays a role as a co-stimulatory molecule and induces activation in antigen presenting cells (APCs) in association with T cell receptor stimulation by MHC molecules on the APC (van Kooten & Banchereau 2000). A previous work proposed that CD40L played a significant role in the control of *T. cruzi* infection in mice (Chamekh et al. 2005, Habib et al. 2007). High levels of sCD40L in serum have been associated with favourable clinical evolution in human visceral leishmaniasis, suggesting a potential use of this marker for the prognosis of this disease and other related diseases (de Oliveira et al. 2013). However, the use of CD40L as a clinical marker for treatment efficacy in *T. cruzi* infection remains to be explored.

Cytokine profiles have been proposed as potential biomarkers of disease progression for some infectious diseases (i.e., malaria and human immunodeficiency virus) (Armah et al. 2007, Boulware et al. 2010, Erdman et al. 2011). Cytokines modulate the balance between the humoral and cell-based immune responses and play a pivotal role in the control of *T. cruzi* infection (Savino et al. 2007). The balance between the pro-inflammatory [T-helper (Th)1 immune response] and antiinflammatory (Th2 immune response) cytokines has been suggested to be critical in the development of the chronic phase of *T. cruzi* infection (D'Ávila et al. 2009, Dutra et al. 2009).

IFN-γ is one the main cytokines that regulate Th1 immune responses. It is critical for innate and adaptive immunity against infection by viral and intracellular pathogens (Schoenborn & Wilson 2007). IFN-γ is essential for the control of *T. cruzi* infection and mediating a parasitological cure in experimental models (Romanha et al. 2002, Ferraz et al. 2007).

In human infections, changes in the number of peripheral IFN-γ-producing T cells specific for *T. cruzi* have been documented after BZ treatment (Laucella et al. 2009). Other studies have found a relatively high expression of IFN-γ in the plasma or serum of patients with Chagas cardiomyopathy compared to those with a noncardiac or indeterminate form of Chagas disease (Poveda et al. 2014, Sousa et al. 2014). These results suggest a potential use for this cytokine as a biomarker of treatment outcome. IL-10 is a major cytokine that modulates Th2 immune responses. It is produced by monocytes and T cells and possesses antiinflammatory and immunoregulatory actions (Moore et al. 2001). IL-10 has been suggested to be crucial for the control of *T. cruzi* infection because IL-10-deficient mice infected with *T. cruzi* showed a higher mortality rate compared to wild type infected animals (Abrahamsohn & Coffman 1996, Hunter et al. 1997). In humans, the association between high expression levels of IL-10 in serum and *T. cruzi* infected individuals displaying better cardiac function has been reported (Sousa et al. 2014), suggesting that the study of this cytokine and its expression profiles in *T. cruzi*-infected individuals before and after treatment would be useful. IL-17 is the central component of the Th17 immune response. IL-17 functions as a proinflammatory cytokine that responds to the invasion of the immune system and induces the destruction of the pathogen's cellular matrix (Kolls & Lindén 2004). Recent studies have shown that IL-17 plays a protective role in experimental *T. cruzi* infection as well as in human Chagas disease (Miyazaki et al. 2010, Magalhães et al. 2013). As observed with IL-10, high expression levels of IL-17 were correlated with better cardiac function (Costa et al. 2009, Magalhães et al. 2013). The potential use of changes in IL-17 as a surrogate marker of parasitological cure has not yet been explored.

Chemokines are key players in the control of the migration of specific cell types bearing their receptors to sites of tissue inflammation (Le et al. 2004). Monocyte chemotactic protein (MCP)-1 (or CCL2) is a small cytokine that belongs to the CC chemokine family. CCL2 recruits monocytes, memory T cells and dendritic cells to the sites of inflammation produced by either tissue injury or infection (Carr et al. 1994). A previous work found that CCL2/MCP-1 played a substantial role in controlling parasite burden, cell infiltration and mononuclear activation during acute *T. cruzi* infection (Paiva et al. 2009). CCL2/MCP-1 has been suggested as a marker of prognostic value in other cardiovascular diseases (Tuñón et al. 2014).

Another chemokine known as macrophage inflammatory protein (MIP)-1α (or CCL3) is involved in inflammation and the recruitment and activation of poly-

morphonuclear leukocytes (Menten et al. 2002). MIP-1α/CCL3 plays a substantial role in the control of *T. cruzi* infection (Roffê et al. 2010). Moreover, this chemokine has also been proposed as a marker for disease progression in a parasitic infection (Falcão et al. 2002). However, the potential value of MCP-1/CCL2 and MIP-1α/CCL3 as indicative markers of treatment outcomes in *T. cruzi* infection has not yet been studied.

The simultaneous detection of antibodies specific to the three evolutive forms of *T. cruzi* has recently been described (flow cytometric analysis of anti-live trypomastigote and anti-fixed epimastigote antibodies). Antibodies directed against live amastigotes (AMA), live trypomastigotes (TRYPO) and fixed epimastigotes (EPI) were found in 100% of individuals with *T. cruzi* infection using differential fluorescence staining; moreover, the 6% of false positives detected in healthy individuals were attributed to samples collected from leishmaniasis patients. The applicability of this technique in the post-therapeutic monitoring of Chagas disease has also been tested; 100% of nontreated and treated noncured samples tested positive against the three *T. cruzi* evolutive forms, while 100% of samples collected from the treated cured patients were negative against AMA, 93% were negative against TRYPO and 96% were negative against EPI (Alessio et al. 2014).

Other host biomarkers - Several inflammatory mediators have been proposed as biomarkers of the progression and/or response to treatment in patients with chronic Chagas disease. Some of these markers have been proposed to identify early cardiac, gastrointestinal or potential neurological damage. Markers of cardiac damage have been the most studied among the biomarkers of progression and some have been proposed as biomarkers of response to treatment.

Specific cardiological markers, such as troponin I and T and natriuretic peptides [i.e., atrial natriuretic peptide, brain natriuretic peptide (BNP) and N-terminal proBNP], have been proposed to determine disease progression when myocardial involvement was assessed both in the early (Puyó et al. 2002, 2005, Ribeiro et al. 2003, Heringer-Walther et al. 2005, Moreira et al. 2008, García-Alvarez et al. 2010, Lima-Costa et al. 2010) and late (Puyó et al. 2002, Heringer-Walther et al. 2005, Machado et al. 2005) stages of the disease.

Biochemical molecules, such as glutamic oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, acid maltase, alpha-hydroxybutyric dehydrogenase (or LDH1), leptin, adipokines and angiotensin-converting enzyme, have also been tested in humans to assess early cardiological damage (Alarcon-Corredor et al. 2002, Combs et al. 2005, Fernandes et al. 2007, Wang et al. 2010). Nevertheless, there are no reports of studies using these biomarkers to assess therapeutic efficacy.

Selenium levels have been studied as a progression marker for chronic digestive and cardiac manifestations, but have not been tested as a progression biomarker (Rivera et al. 2002). Other molecules and enzyme systems, including caveolin-3, myocardial and peripheral protein-3-nitrotyrosine and its protein carbonyl formation, catalase, glutathione peroxidase, glutathione reductase, glutathione and $Mn(2^{+})$ superoxide dismutase, have been reported to exhibit alterations in their regular function and/or expression in *T. cruzi*-infected animals. (Wen et al. 2004, Dhiman et al. 2008, Adesse et al. 2010)

Recently, some APO and several of their fragments [mature human APOA1 (28.1 kDa), fragments of human APOA1 (24.7, 13.6 and 9.3 kDa)] and a fragment of human fibronectin FN1 (28.9 kDa) were demonstrated to be useful for assessing the response to treatment with NFX in patients with chronic Chagas disease by surface-enhanced laser desorption/ionization analysis (Ndao 2012). The levels of these biomarkers in blood samples obtained three years after NFX treatment returned to levels similar to those seen in healthy controls and the pattern of these biomarkers between healthy and diseased samples was consistent with those observed in our previous study. The sensitivity of this group of biomarkers was 89-100% and the specificity was up to 98% (Santamaria et al. 2014). Overall, further studies are needed to correlate these changes with serological negativisation and seriated negative PCRs.

Other groups of potentially useful biomarkers include the hypercoagulability factors prothrombin fragments 1+2 (F 1+2) and endogenous thrombin potential (ETP). Despite the large number of hypercoagulability biomarkers that have been tested in patients with *T. cruzi* infection in different stages, only one study tested these biomarkers after specific treatment with BZ (Pinazo et al. 2011). ETP and F 1+2 showed altered levels in *T. cruzi*-infected patients compared with controls (73% and 80% of patients, respectively); these levels decreased significantly six months after treatment (100% and 73%, respectively). The evaluation of ETP and F 1+2 was conducted in a nonendemic area for all stages of CD, thereby controlling for possible reinfection.

One of the advantages of hypercoagulability biomarkers compared with the rest of the molecules and techniques described in other sections of this paper is that they are easily accessible at a reasonable cost in laboratories around the world because they represent regular tests performed in health care centres' laboratories. Most of them do not require an expert technician and highly technological equipment. However, the two groups that have shown the most potential usefulness after treatment were recently tested and presented some limitations. Further studies will be required to validate their use in diagnosis or prognosis.

Concluding remarks

There are currently several groups of biomarkers that have exhibited potential usefulness for the assessment of responses to specific *T. cruzi* treatments; these biomarkers are related to the parasite itself or the host response to the parasite presence. The research on parasite biomarkers is currently more developed than the research on host response biomarkers. Among parasite biomarkers, nucleic acid amplification techniques are the most common and have demonstrated their effectiveness in assessing therapeutic failure and cure after specific treatments in several experiments.

Several studies concerning immunological and biochemical markers related to the host response to the parasite have been recently published, but further studies must be performed to determine their usefulness in different clinical and epidemiological settings.

The availability of different types of new biomarkers combined with standardised criteria will be extremely useful in clinical trials involving the development of new and improved drugs to properly treat this neglected tropical disease.

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