Title: State-of-the-art in host-derived biomarkers of Chagas disease prognosis and early evaluation of anti-*Trypanosoma cruzi* treatment response.

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Abstract:

Chagas disease is caused by infection with the parasite *Trypanosoma cruzi*, which might lead to a chronic disease state and drive to irreversible damage to the heart and/or digestive tract tissues. Endemic in 21 countries in the Americas, it is the neglected disease with a highest burden in the region. Current estimates point at ~6 million people infected, of which ~30% will progress onto the symptomatic tissue disruptive stage. There is no vaccine but there are two anti-parasitic drugs available: benznidazole and nifurtimox. However, their efficacy is variable at the chronic symptomatic stage and both have frequent adverse effects. Since there are no prognosis markers, drugs should be administered to all *T. cruzi*-infected individuals in the indeterminate and early symptomatic stages. Nowadays, there are no tests-of-cure either, which greatly undermines patients´ follow-up and the search of safer and more efficacious drugs. Therefore, the identification and validation of biomarkers of disease progression and/or treatment response on which to develop tests of prognosis and/or cure is a major research priority. Both parasite- and host-derived markers have been investigated. In the present manuscript we present an updated outlook of the latter.

Keywords: Chagas disease; *Trypanosoma cruzi*; biomarkers; host-derived; treatment response; disease prognosis.

Abreviations[1].

[1]Adenosine deaminase (ADA); angiotensin-converting enzyme 2 (ACE2); apolipoprotein A1 (ApoA1); brain natriuretic peptide (BNP); cardiac troponin T (cTnT); C-reactive protein (CRP); creatine kinase-MB (CKMB); deoxyribonucleic acid (DNA); endogenous thrombin potential (ETP); extracellular vesicles (EVs); fibronectin (FBN); fragment 1+2 (F1+2); galectin-1 (Gal-1); genome-wide association study (GWAS); glutathione peroxidase (GPx); human leukocyte antigen (HLA); interferon gamma (IFN-γ); interleukin 1 beta (IL-1β); interleukin 6 (IL-6); interleukin 10 (IL-10); loop-mediated isothermal amplification (LAMP); matrix metalloproteinase 2 (MMP-2); matrix metalloproteinase 9 (MMP-9); micro RNAs (miRNAs); nitric oxide (NO); N-terminal portion brain natriuretic peptide (NT-proBNP); plasmin-antiplasmin complexes (PAP); polymerase chain reaction (PCR); reactive oxygen species (ROS); recombinase polymerase amplification (RPA); ribonucleic acid (RNA); selenium (Se); single nucleotide polymorphisms (SNPs); S-nitrosylation (SNO); tissue inhibitor of metalloproteinase 1 (TIMP-1); tissue inhibitor of metalloproteinase 2 (TIMP-2); tumor necrosis factor alpha (TNF-α); transforming growth factor beta 1 (TGFB1); transforming growth factor beta 2 (TGFB2); vascular cell adhesion molecule-1 (VCAM-1).

1. Introduction.

Chagas disease is caused by infection with the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). It affects ~6 million people around the world, mostly in Latin America (1), and it is estimated that approximately ~30% of them will eventually progress to the symptomatic life-threatening stages of the disease that entail cardiac and/or digestive tissues disruptions (1). This organ involvement, which can lead to the Chagas disease characteristic mega-syndromes, does not occur immediately but it is rather observed upon several years of infection (even 10 to 30 years) (2,3). Unfortunately, at present there is not a way to predict this possible progression. Being able to anticipate it would be crucial to tailor the needs of patients based on their risk of progression. This would mean a breakthrough in the management of the disease considering the limited resources available. Thus, finding prognosis markers to assess the risks of progression and predict the early stages of organ involvement is one of the main objectives of biomarker research projects in the field.

On the other hand, despite there are two drugs to treat *T. cruzi* infection, benznidazole (BNZ) and nifurtimox (NFX) (2,3), both have a poor safety profile that forces clinicians to discontinue a high percentage of treatments (4–6). Besides, although BNZ and NFX have a very good efficacy against the acute stage of the disease, it is at the chronic stage that the infection is generally diagnosed and treated. By then drugs´ efficacy is reduced. Limitations of currently available drugs unveil the requirement of biomarkers for the early assessment of treatment response so that a closer and accurate follow-up of treated patients can be made. Availability of such biomarkers would allow the development of tests of cure based on them. These tests would also be very useful in the search of safer and more efficient new therapeutic options. A major obstacle for the clinical evaluation of new treatments is that the current "gold standard" of cure is the negative seroconversion of treated patients, which occurs years or decades after treatment (7,8). This time scale is impractical from the viewpoint of the daily clinical management of patients, as well as for the interpretation of the outcome of clinical trials with new drugs or with new regimens of existing ones. Thus, it is urgent to identify and validate biomarkers for the early assessment of therapeutic efficacy too.

Finding reliable biomarkers of disease progression and/or treatment response would mean the greatest leap forward in the history of Chagas disease since its discovery in 1909 by Dr. Carlos Chagas. For this reason there are numerous research groups devoted to the

search of both host-derived and *T. cruzi*-derived biomarkers (9). With the aim to host discussions on the subject and nurture joint investigations appeared the NHEPACHA network (10). Since its inception in 2012 it has produced some important advances in the field as a result of multidisciplinary and transnational collaboration, like a paradigm shift proposal for the treatment of chronically infected people (11), or the elaboration of the first Target Product Profile document for a test to early address treatment response (9). Standing on these and other works, it is the purpose of this article to review the research on host-derived biomarkers for Chagas disease providing an updated overview of the advances made, and outlining state-of-the-art investigations in the field.

2. Host-derived biochemical markers for the management of Chagas disease.

Research on biochemical markers gathered attention in the last years due to their potentially simple analysis and low cost, which makes them easy to implement in middle- and low-income countries (9). In the case of Chagas disease, several biomarkers have been proposed for the assessment of cardiac and/or digestive involvement (12) (Figure 1). In addition, there are also some examples of biochemical markers proposed for the early evaluation of treatment response (13). We summarize all biochemical markers identified so far in the context of Chagas disease and their proposed application in Table 1. Unfortunately, despite recent advancements, we are yet far from having one single molecule that meets all the required expectations. Due to the complexity of the chronic stage of the disease, the option of using a battery of biomarkers rather than relying on a single one to assess treatment response and/or anticipate pathological progression of the infection appears more likely (9).

2.1. Biochemical markers for the evaluation of treatment response.

Setting the focus on biomarkers of treatment response, there are two main groups of molecules identified up to now: those related to the metabolism of lipids (13,14), and those associated to the hypercoagulability state (15,16).

2.1.1. Markers based on lipid metabolism intermediates.

A study by Santamaria and co-workers with sera from 37 adult Chagas disease patients and 37 healthy subjects showed that apolipoprotein A1 (ApoA1) and certain fragments of ApoA1 and fibronectin (FBN) proteins were interesting for the evaluation of

treatment response. Higher levels of fragments of ApoA1 and FBN were found in serum samples of *T. cruzi* seropositive patients in comparison to the control group. Contrarily, full-length Apoa1 levels were lower in *T. cruzi* infected individuals compared to healthy donors (13). Such altered levels of ApoA1, its specific fragments thereof, and a fragment of FBN returned to normal in 43% of the studied *T. cruzi*-infected subjects three years after NFX treatment (13). These results have been recently validated in a cohort of 30 *T. cruzi* seropositive children treated with BNZ (14). In the latter study, ApoA1 and FBN fragments were absent at the end of BNZ treatment in a significant part of the cohort (66.6% and 53.3% of the children respectively for ApoA1 and FBN) (14). Also, correlation between seroconversion of the children upon treatment and absence of detection of ApoA1 and FBN fragments in serum samples was observed in 100% and 96.6% of the cases, respectively (14).

2.1.2. Hypercoagulability state biomarkers.

The presence of a hypercoagulability state in *T. cruzi*-infected patients was described a few years ago (15,16), which contrasted with other report by Melo and co-workers (17). Notwithstanding, at the clinical level, the presence of a coagulation state condition could be hinted upon the description of the occurrence of thromboembolic events in *T. cruzi*-infected patients who did not show any signs of cardiomyopathy (18). In 2016, some of us published the results of a clinical study with plasma samples from 56 chronically *T. cruzi*-infected adult patients and reported that a high percentage of them had statistically significant altered levels of the hypercoagulability markers prothrombin fragment 1+2 (F1+2) and endogenous thrombin potential (ETP) (19). These two markers, which had not been looked upon by Melo et al., were abnormally expressed in respectively 77% and 50% of the patients (19). Moreover, after BNZ treatment, both markers returned to and remained at their normal levels in respectively 76% and 96% of the patients by 36 months upon end of treatment (19). Amongst the rest of hypercoagulation markers that were evaluated in that study, plasmin-antiplasmin complexes (PAP) also showed good results in terms of percentage of patients that returned to normal levels upon treatment (94% of them) (19). However, PAP was found altered in only 32% of the studied *T. cruzi*-infected participants before treatment and it was thus discarded from further consideration (19).

2.2. Biochemical markers of disease progression.

Besides the identification and validation of biomarkers for the early evaluation of treatment response, the other main area of research encompasses a whole series of studies to find biomarkers of pathogenesis progression. Their availability would promote the development of diagnostic tools based on them that could allow clinicians to adequately triage those chronically infected patients that will likely develop cardiac and/or digestive tissue disruptions from those that will not. Having such tests would permit to administer the limited resources to follow-up and manage Chagas disease patients in a most efficient manner.

Cardiac damage is the most common clinical outcome observed in the symptomatic state of the disease (2,3). Thereby, markers of cardiac damage progression have been the most studied for Chagas disease (Figure 1). It is estimated that between 20% - 30% of *T. cruzi* chronically infected people will develop cardiomyopathy, and an early indicator of disease prognosis would help to prioritize treatment to those patients with a high risk of developing complications. Multiple biomarkers have been associated with Chagas disease cardiomyopathy (Table 1). However, although it has been proved that their levels increase accordingly to the severity of the damage, most of them are not able to distinguish between Chagas disease cardiomyopathy and other cardiomyopathies (20).

Natriuretic peptides (brain and atrial) were among the first markers of cardiac disease progression ever considered. These are released under conditions of myocardial stress and have been shown to be increased in *T. cruzi* experimentally infected animals (21), and in Chagas disease patients with cardiomyopathy (22). Furthermore, levels of natriuretic peptides correlated to clinical prognosis (23). The N-terminal portion of brain natriuretic peptide (NT-proBNP) could be a better predictor than BNP itself, due to its high stability (24). Both peptides are strong predictors of mortality, and are some of the most well characterized markers for assessing early cardiac damage and predicting heart failure outcome. Measurement of BNP levels has also been suggested for the prognosis of patients presenting left ventricular systolic dysfunction, one of the typical signs of cardiac Chagas disease (22,25-27).

The use of natriuretic peptides in the context of Chagas disease has also been studied in combination with other host-derived molecules. For example with cardiac troponin T

(cTnT), which is a marker of ischemia and inflammation (see next section for further details). A study assessing it in combination with NT-proBNP that included samples from 137 *T. cruzi*-infected patients with several forms of the disease found that levels of both markers were increased in those individuals with cardiomyopathy in comparison to the asymptomatic ones (28). Moreover, their values were increased accordingly to the severity of the cardiomyopathy (28). Plasma leptin levels and their relation to different forms of the disease were also studied in combination with NT-proBNP in 52 *T. cruzi*-infected patients, and those patients with heart failure had higher levels of NT-ProBNP and lower levels of leptin than controls (29).

Angiotensin-converting enzyme 2 (ACE2) is another potential prognostic biomarker that has been evaluated. Wang and colleagues showed that ACE2 activity was significantly increased in those patients with signs of heart failure, but it was not so in patients without systolic dysfunction (30). Moreover, plasma ACE2 levels significantly correlated with clinical severity and echocardiographic (ECHO) parameters indicative of this (30). Similarly to cTnT and leptin, ACE2 activity was also compared to BNP levels in order to predict cardiac death and need of heart transplant, finding an additive predictive value when both markers were used in combination (30). Other enzymes that have been suggested as possible markers for early cardiac damage are glutamic oxaloacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT), alkaline phosphatase (ALP), acid maltase (AM) and alpha-hydroxybutyric dehydrogenase (alphaHBDH), and their levels were found to be significantly altered in chagasic patients (31). Very interestingly, authors of that reference suggested that the finding of those released enzymes in patients without clinical evidence could represent good biomarkers of early myocardial damage (31).

On the other side, the measurement of selenium (Se) levels has also been valued as a disease progression marker for cardiac as well as also for digestive manifestations (Figure 1; Table 1). In a cohort of 170 *T. cruzi*-infected people, Rivera and co-workers found that Se levels were lower in patients presenting the cardiac form of the disease compared with healthy donors or asymptomatic individuals (32). Moreover, such decrease of normal levels of Se was significantly correlated with malfunction of the ventricular ejection fraction. Low Se levels were also found in 6 out of the 10 *T. cruzi*-infected patients with digestive mega-syndromes (32). In fact the use of Se as dietary supplementary was suggested as a possible

therapeutic strategy to preserve heart function in patients presenting the indeterminate form of the disease (33).

Finally, another biochemical biomarker that has been researched for Chagas disease is endothelin 1, but the available data about it is yet controversial. A study performed in 2001 showed that plasmatic levels of endothelin 1 were elevated in patients presenting *T. cruzi* infection related cardiomyopathy (34). However, Garcia-Alvarez and colleagues were not able to replicate those results, and reported in another study that similar plasmatic levels of endothelin 1 were observed in *T. cruzi* seropositive patients and control individuals (35). Moreover, the group of infected subjects presenting the undetermined form of the disease had even lower levels of endothelin 1 than controls (35).

2.2.1. Biochemical markers of inflammation.

Although the molecular mechanisms of Chagas disease pathogenesis are yet largely unknown, the presence of an inflammatory environment is a common feature to both cardiac and/or digestive tissue disruptions. Such continuous inflammation would lead to the occurrence of organ mega-syndromes, severe indicators of symptomatic chronic *T. cruzi*-infection. Thereby, the prognostic value of host biochemical markers with inflammatory mediator function has also been evaluated. For instance C-reactive protein (CRP), which is liberated during the acute phase of inflammation and its serum levels associate to vascular inflammation and development of cardiovascular events (36). Several studies have evaluated this protein as a marker for the progression of Chagas disease, showing an association between chronic inflammation, cardiac manifestations and CRP levels (37,38).

Other interesting marker under research is the enzyme adenosine deaminase (ADA). ADA regulates adenosine levels and its activity increases as a consequence of hypoxia and inflammation associated with immunologic events (39). In the same study, which included serum samples from 28 healthy individuals and from 82 *T. cruzi*-infected people presenting asymptomatic and symptomatic (cardiac) forms of the disease, it was shown that CRP and ADA levels linearly increased in connection to disease severity, and further correlated with the observed ECHO and electrocardiographic (ECG) parameters indicative of this state (39).

Galectin-1 (Gal-1), found in human heart tissue, is also involved in immunological and inflammatory processes. A study with serum samples from healthy donors and patients

in the acute and chronic phases of Chagas disease showed that anti-Gal-1 IgG auto-antibodies specifically correlated with cardiac damage severity caused by *T. cruzi* infection as they were shown to be absent in non-related cardiomyopathies (40). Moreover, levels of Gal-1 were upregulated in cardiac tissue from patients presenting chronic *T. cruzi* infection, compared to cardiac tissue from healthy individuals (40).

In the presence of excessive oxidative and nitric oxide (NO) stress, a post-translational modification of cysteine residues of host proteins can occur. This modification, called S-nitrosylation (SNO), can affect cellular homeostasis and contribute to disease development. Recently, Zago and co-workers have shown that SNO modifications found in peripheral blood mononuclear cells from 53 Chagas disease patients were differentially abundant according to disease state, having the potential to identify disease severity (41).

Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) have also been investigated in the context of *T. cruzi* infection, but their participation in the disease progression is yet subject to controversy. A cross-sectional study with plasma samples from 144 patients at different stages of Chagas disease and 44 samples from healthy donors showed that patients had increased levels of MMP-2 and MMP-9 (42). This work also reported that patients presenting ECG abnormalities had a significant increase of both enzymes compared to patients presenting the indeterminate form (42). In contrast, another study with serum samples from 193 *T. cruzi*-infected individuals observed an increase of the MMP-2/MMP-9 ratio that was associated with severity of the cardiac form of the disease (20). The study authors claimed that this ratio could be useful for assessing the progression from early inflammation to late fibrosis (20). *T cruzi*-infected subjects presented progressively higher levels of MMP-2, tissue inhibitor of metalloproteinase 1 (TIMP-1, inhibitor of MMP-9) and tissue inhibitor of metalloproteinase 2 (TIMP-2, inhibitor of MMP-2) that paralleled cardiac severity (20). In line with that, Clark and colleagues have shown that serum levels of MMP-2 and TIMP-1 increased progressively in 85 individuals presenting cardiac structural changes, either in early or late stages of the disease (43). In comparison, other studies performed using serum and whole blood samples suggest that MMPs are differentially involved in Chagas disease cardiomyopathy: while MMP-2 would be associated to regulatory cytokines, MMP-9 would be correlated with inflammatory ones (44,45). Thus, MMP-2 would present a cardiac-protective and regulatory function, favoring

the indeterminate form of the infection, and MMP-9 would promote an inflammatory atmosphere, favoring the development of the cardiac form (44,45).

Similarly to what occurs with BNP and NT-proBNP, high levels of creatine kinase-MB (CKMB) indicate extensive damage and worse prognosis in patients suffering heart failure. Thus, this compound is commonly used as a measure of heart failure severity, and has been suggested as a possible biomarker for Chagas disease (20,46). A first study by Okamoto and colleagues assessing serum levels of BNP, NTproBNP, CKMB, troponin I, MMP-2, MMP-9, TIMP-1, TIMP-2, transforming growth factor beta 1 (TGFB1), and transforming growth factor beta 2 (TGFB2), showed no differences in biomarkers levels when stratifying patients by cardiac stage and *T. cruzi* infection status (20). This was the first time that CKMB was examined as a prognostic marker in Chagas cardiomyopathy. In that study, Okamoto and co-workers saw that troponin I levels (among others) rose in relation with an increasing severity of the disease stage, but unfortunately it did not distinguish between Chagas cardiomyopathy and other cardiomyopathies (20). However, Sherbuk and colleagues showed that CKMB, together with BNP, NTproBNP and MMP2, were significantly associated with mortality among patients presenting severe Chagas cardiomyopathy (46). Similarly, the conclusions from the cross-sectional case control retrospective study by Keating and co-workers, which measured CKMB, troponin, myoglobin, VCAM, NTproBNP as well as the cytokines IFN-γ, IL-6, IL-10 and TNF-α (see next section for more information about studies involving cytokines) pointed out that a clear pattern of inflammatory biomarkers was solely observed in those subjects presenting with the more severe cardiac symptomatic stages (36).

As it was mentioned above, measuring serum levels of cTnT was analyzed as prognosis test of cardiac damage progression. Determining cTnT levels with a highly sensitive assay in serum samples from 26 healthy subjects and 179 chronically infected subjects concluded that those were significantly higher in patients suffering cardiomyopathy compared to the rest of groups (47). Moreover, authors of that work indicated that cTnT value correlated with the severity of that cardiomyopathy (47). Notably, CRP and IL-6 levels followed the same trend as cTnT changes (47).

Finally, NO levels have been also reported to be significantly higher in patients with cardiomyopathy compared with asymptomatic patients and healthy donors (48–51). This

increase of NO levels in serum correlated with an increase of TNF-α, and a reduction in glutathione peroxidase (GPx) and superoxide dismutase (48). Alterations in the oxidant/antioxidant balance had been previously reported in a cohort of 80 *T. cruzi* infected patients, 50 healthy individuals, and 20 non-chagasic cardiomyopathy subjects (49). That study showed that *T. cruzi*-infected patients presented higher levels of malonylaldehyde and lower levels of glutathione, glutathione peroxidase, superoxide dismutase and manganese superoxide dismutase than healthy individuals. Patients presenting cardiomyopathy but negative for *T. cruzi* infection presented insignificant higher plasma malonylaldehyde levels compared to healthy patients, and their plasma antioxidant defense capacity was not compromised (49). Years later, the same group of researchers studied the role of inflammatory mediators such as myeloperoxidase, inducible nitric oxide synthase (iNOS) and xanthine oxidase, in the stimulation and sustenance of oxidative and nitrosative stress response in plasma samples of *T. cruzi* seropositive and seronegative patients (50). Those infected presented a significant increase in myeloperoxidase activity and protein level, advanced oxidation protein products, and 3-nitrotyrosine levels compared to healthy donors. However, plasma levels of xanthine oxidase and nitrate/nitrite contents were not altered. A correlation between increased myeloperoxidase activity and protein 3-nitrotyrosine formation was found, suggesting that myeloperoxidase could contribute to protein nitration and thus to oxidative and nitrosative damage in Chagas disease patients (50). More recently, the role of other markers of inflammation and oxidant/antioxidant status was studied in a cohort of 116 *T. cruzi* seropositive patients characterized as clinically-symptomatic or asymptomatic, 45 seronegative healthy individuals, and 102 *T. cruzi* seronegative patients presenting cardiac problems. Consistent with previous findings, seropositive subjects showed an increase in sera or plasma levels of myeloperoxidase, advanced oxidation protein products, nitrite, lipid peroxides and malonylaldehyde, and a decrease in superoxide dismutase and glutathione compared to healthy controls. Interestingly, myeloperoxidase and lipid peroxides levels correlated with clinical disease state, being potential biomarkers candidates for evaluating Chagas disease clinical severity (51).

2.3. Markers associated with the host immune state.

The study of cytokines has also been suggested as a relevant tool for assessing cardiac disease progression (36). These and other biomarkers associated with the host immune state identified so far for Chagas disease are summarized in Table 2. In 2014, a study by Sousa et al. involving plasma samples of 176 *T. cruzi* infected people and 24 healthy individuals showed that the expression of plasma inflammatory cytokines, such as IFN-γ, TNF-α, IL-6, and IL-1β, was higher in those with cardiac form of the disease (52). These results were consistent with previous findings where high levels of TNF-α were found in patients suffering Chagas disease cardiomyopathy (48,53). Poveda and colleagues also found a higher expression of IFN-γ in serum samples from Chagas disease patients with cardiomyopathy compared to those with the indeterminate form (54). By contrast, indeterminate *T. cruzi* infected patients had a higher expression of IL-10 when compared with that of individuals with cardiac damage (54). Interestingly, IL-10 expression was associated with better cardiac function as determined by left ventricular ejection fraction and left ventricular diastolic diameter values (54). These results confirmed previous findings from Costa and co-workers, which suggested a cardiac-protective role for IL-10 in *T. cruzi* infection (55).

In line with the former, other work has shown that high levels of IL-17 could correlate with better cardiac function, although this is considered a pro-inflammatory cytokine (56). Nonetheless, the role of IL-17 is still open to discussion because a recent study assessing plasma samples from 57 children showed that IL-17A levels were significantly higher in *T. cruzi* infected in comparison to seronegative individuals. Interestingly, those higher IL17A levels decreased to normal one year after treatment (57).

Table 1. List of biochemical markers described up to date. Markers are ordered as they appear in the text. (Table 1 continues in next page).

Biomarker name (acronym)	**Application evaluated (higher levels correlate to)**	**References**
ApoA1, FBN	Therapeutic response[#]	(13,14)
F1+2 and ETP	Therapeutic response[#]	(19)
BNP	Cardiac damage progression	(20,22,23,25–27,30,43)

[#] Therapeutic response is associated with a decrease in the markers levels.
[*] These markers with inflammatory mediators function are described separately in their own section in the text.

NTproBNP	Cardiac damage progression	(20,24,28)
cTnT*	Cardiac damage progression	(28,47)
Leptin	Cardiac damage progression	(29)
ACE2	Cardiac damage progression	(30)
GOT, GPT, ALP, AM, alpha-HBDH	Cardiac damage progression	(31)
Selenium (Se)	Cardiac and digestive disease progression	(32)
Endothelin	Cardiac damage progression (controversial)	(34,35)
CRP*	Cardiac damage progression	(37–39)
ADA*	Cardiac damage progression	(37)
Gal-1, anti-Gal-1 auto-antibodies	Cardiac damage progression	(40)
NO* and SNO*	Cardiac damage progression;	(41,48)
MMP-2*, MMP-9*	Cardiac damage progression (controversial)	(20,42–46)
TIMP-1*, TIMP-2*	Cardiac damage progression	(20,46)
CKMB, troponin I, TGFβ1, and TGFβ2	Cardiac damage progression	(20,36,43,47)
Biomarker name (acronym)	**Application evaluated (higher levels correlate to)**	**References**
GPx, superoxide dismutase*	Cardiac damage progression	(48)
Malonylaldehyde, glutathione, glutathione peroxidase, superoxide dismultase, manganese superoxide dismutase	Cardiac damage progression	(49,51)

Myeloperoxidase, nitric oxide synthase, xanthine oxidase, oxidation protein products, 3-nitrotyrosine, nitrate/nitrite	Cardiac damage progression	(50,51)
Lipid peroxides	Cardiac damage progression	(51)

At present, despite the identification of cytokine signatures indicative of a pattern of pathogenesis progression has been pursued, the only tests that would independently have value for clinical decision would be the aforementioned NTproBNP (see section 2.2) and the detection of *T. cruzi* DNA by PCR, as far as they are accompanied with electrocardiographic (ECG) and ECHO clinical assessments (36).

Other studies have focused on the potential role of adaptive immune response mediators as biomarkers. In relation to humoral immunity, the possible impact of anti-troponin T and myosin autoantibodies has been suggested (58). Serum samples from 131 patients presenting different clinical forms of Chagas disease, healthy donors, and patients with ischemic cardiomyopathy were included in a study whose results showed that specific anti-*T. cruzi* antibodies and autoantibodies against myosin and troponin T are frequently co-detected in high levels in patients with chronic Chagas disease (58). Even though anti-troponin T autoantibodies levels were very similar in patients presenting the indeterminate form of the disease and in patients presenting cardiac symptoms, the study found a correlation between cardiac Chagas disease, the production of anti-troponin T and anti-myosin autoantibodies, and a diminished left ventricular ejection fraction, which is an important indicative of systolic dysfunction (58). Anti-*T. cruzi* specific antibodies and their correlation with disease progression and cardiac damage have also been evaluated. In a cohort of 55 *T. cruzi* infected patients (20 presenting the indeterminate form of the disease and 35 suffering cardiac damage) an inverse correlation between anti-*T. cruzi* IgG1 titers and left ventricular ejection fraction was found in patients presenting the cardiac form of the disease, indicating a worse prognosis for those *T. cruzi*-infected patients presenting high titers of anti-*T. cruzi* IgG1 (59). Thus, anti-*T. cruzi* IgG1 levels could be an interesting biomarker to predict the severity of chronic Chagas disease cardiomyopathy.

In terms of T cell immune responses, it has been recently described that Chagas patients with cardiac tissue damage present higher expression levels of T-cell inhibitory receptors and lower antigen-specific capacity compared with that of asymptomatic patients (60). These features were partially reversed by BNZ treatment in both asymptomatic and symptomatic patients: the co-expression of inhibitory molecules was reduced, the multifunctional antigen-specific response of $CD8^+$ T cells was enhanced, and an increase in the subset of cells with cytotoxic proprieties and production of IFN-γ was observed. These results point at a potential application of the analysis of those immunological signatures as biomarkers of disease progression and treatment response.

3. Other types of host-derived Chagas disease biomarkers.

3.1. Extracellular vesicles.

Extracellular vesicles (EVs) are cell-derived membranous nanoparticles released by most cells, present in all body fluids, and implicated, as intercellular communicators, in diverse pathological processes (61). Mainly for these reasons, EVs hold an enormous potential as predictive biomarkers for human diseases, including several parasitic diseases (62,63).

In Chagas disease, the possible role of EVs as active communicators between *T. cruzi* and its host, as well as the implications of this interaction in parasite tropism and changes in immune status during the chronic phase of the disease, has been recently reviewed (64). Moreover, the use of EVs as potential diagnostic biomarkers during the

Table 2. List of immune-related molecules studied as markers for the evaluation of cardiac damage progression.

Name of the marker or combinations of markers	**Observed effect of higher marker expression**	**Reference**
TNF-α	Progression	(48,52,53)
Chemokine C-C ligands 2 and 3 (CCL2, CCL3)	Progression	(53)
IL-6	Progression	(37,47)

IL-10	Protection	(55)
Anti-troponin T antibodies, myosin autoantibodies	Progression	(58)
IFN-γ, IL-1β	Progression	(52)
IL-2, IL-4, *IL-5*, IL-9, IL-12p70, *IL-13*, IL-22	Progression/*Protection*	(54)
IL17, IL-17A	Controversial	(55-57)
$CD8^+$ T cell inhibitory receptors and antigen-specific capacity	Cardiac damage progression and therapeutic response	(60)
Anti-*T. cruzi* IgG1 levels	Cardiac damage progression	(59)

course of the disease has been recently reported in several studies. Diaz Lozano and collaborators showed that EVs secreted by the infective forms of *T. cruzi* are targeted by the immune system forming immune complexes that could be used as biomarkers of prognosis for digestive Chagas disease (65). In addition, EVs released by human THP-1 cells after interacting with different stages of *T. cruzi* parasites are differently recognized by antibodies present in sera from infected individuals with the indeterminate or the cardiac form of the disease (66). Moreover, it has also been shown that circulating micro-particles secreted by Chagas disease patients induce pro-inflammatory activation and nitrosative response in THP-1 macrophage cells, which is dependent of the clinical stage of the patients (67).

Proteomic analysis to determine the molecular composition of EVs secreted by different *T. cruzi* parasite stages have been reported in recent years (68–70). However, to the best of our knowledge the protein-cargo associated to EVs circulating in peripheral blood of *T. cruzi*-infected subjects has not been evaluated in the context of therapeutic response. In this regards, unpublished results by Cortes-Serra et al. describe for the first time, human and *T. cruzi* proteins present or upregulated in plasma-derived EVs from a chronic Chagas disease patient before chemotherapy and that are absent or downregulated following treatment. Although these results derive from the analysis of a single heart-transplanted patient

presenting severe cardiac complications, they represent a proof-of-principle of the potential of this approach to discover new biomarkers of therapeutic response.

3.2. Markers derived from human genetic studies.

Chagas disease has a multifactorial etiology that involves complex host-parasite interactions governed by parasite and host genetics, which can be as well influenced by environmental factors. As it was mentioned before, the mechanisms of pathogenesis of chronic *T. cruzi* infection are yet largely unknown. But immune system mediators have been described to participate in driving heart and/or gut tissues inflammation, either through response to the parasite presence and, to a certain level, by autoimmune reactions (71). Thus, most of the genetic studies performed so far have searched for sequence variations that could be associated to chronic Chagas cardiomyopathy susceptibility in immune system related genes. These searches followed a hypothesis-driven approach to find single nucleotide polymorphisms (SNPs) in genes known or suspected to play a role in those inflammatory phenomena (72). Amongst the genes studied there are: human leukocyte antigen (HLA) class I and class II alleles, cytokines (e.g.: IL-1β, IL-10, TNF-α, IL-17, IL-18) and chemokines and their receptors (MCP1/CCL2, CCR5, MIG/CXCL9, IP10/CXCL10) (reviewed in (72)), as well as inflammasome genes (73). Some variations were associated to chronic Chagas cardiomyopathy, but in other traits like TNF-α controversy is open mainly due to the limited sample size analyzed and the ample genetic heterogeneity of the studied cohorts. These features have also limited the only genome-wide association study (GWAS) performed so far in Chagas disease (74). With the power of its "hypothesis-free" and "hypothesis-generating" nature, unfortunately this study did not report any significant associations with chronic cardiomyopathy at the genomic level. However it interestingly suggested that SNPs in the solute carrier family gene *SLCO1B1* associated to a cardiomyopathy phenotype (74).

Other kind of genetic studies, such as transcriptomics and epigenetics works, will be also required in order to functionally expand and integrate the aforementioned genomics data, as well as to comprehend the impact of environmental factors in the susceptibility to the disease. In this regard, a whole-blood transcriptome of *T. cruzi*-infected subjects and uninfected controls identified a signature of 27 genes, mainly related to natural killer (NK) and $CD8^+$ T cells, which would mark a disease progression pattern (75). Whereas Frade et

al. analyzed the whole-transcriptome of heart biopsies from chronic Chagas cardiomyopathy patients and identified a long non-coding ribonucleic acid (RNA) molecule that had been associated to heart failure (76). Long non-coding RNAs are >200 nt long RNA transcripts that have been described to have a broad functionality in the regulation of gene expression at transcriptional, post-transcriptional and epigenetics levels (76).

Other series of studies analyzed the differential expression of micro RNAs (miRNAs) in chronic cardiomyopathy Chagas patients, either versus those suffering from idiopathic dilated cardiomyopathy (77), or comparing them to *T. cruzi*-infected subjects at the indeterminate stage and a group of non-infected subjects (78,79). These miRNAs are small non-coding RNA molecules with a cell and tissue specific expression pattern that are involved in post-transcriptional regulation and might target up to 60% of the human genes (80). A work integrating miRNA and gene expression profiles of *T. cruzi* acutely infected mice heart tissues suggested a correlation between those miRNAs and the observed pathobiology (81). Such miRNAs can be epigenetic regulators and be regulated epigenetically at the same time (82). One of the most common epigenetic modifications is deoxyribonucleic acid (DNA) methylation, and the results from a whole-genome cardiac fingerprinting study revealed that up to 399 genes were differentially methylated and expressed in chronic Chagas cardiomyopathy patients in comparison to healthy controls (83).

The above mentioned studies are very necessary to achieve a deeper understanding of Chagas disease complex pathogenesis events. However, so-called genomic medicine yet lies far from being applicable for Chagas disease. The usefulness of those genetic markers in the field as potential markers of disease prognosis will largely depend on a much awaited generalization of molecular-based diagnostics, or the development of easier-to-use molecular based detection methods such as loop-mediated isothermal amplification (LAMP) or recombinase polymerase amplification (RPA) assays based on them.

4. Conclusions.

We are still far from having a licensed test for the early assessment of treatment efficacy or to accurately anticipate Chagas disease progression. Many candidate molecules, parasite- and host-derived, have been evaluated so far. Most of the studies have generally been limited to the evaluation of a few dozen of samples. Larger study cohorts, which should

ideally involve individuals from varied geographic origins and accurate clinical stratification according to the distinct disease stages, should be pursued in order to obtain robust results as well as to shed light onto those markers that still have controversial results. In addition, the length of the follow up periods should be extended to up to 5 years post-treatment particularly for studies on therapeutic response biomarkers. In relation to studies on biomarkers to evaluate pathogenesis progression, an even longer longitudinal follow-up of participants would be desirable. This is because in the majority of studies performed so far cardiac alterations (assessed by ECG and ECHO) are already present at the time of triaging the study groups. With current designs it would not be possible to assign a clinical decision on the patients´ management upon observed changes in the markers´ levels, as these were found in patients already symptomatic. Thus, those markers that appear altered at indeterminate stages with no evidences of clinical signs should be therefore the most promising.

Regardless of their application, assessment of the markers with the same cohort of samples could also contribute to draw a clearer picture. In this sense, multinational scientific networks like NHEPACHA (10) are very interesting initiatives so as to bring together academic, clinical and industry groups, providing them with the environment to collaboratively work towards the identification of the most useful biomarkers.

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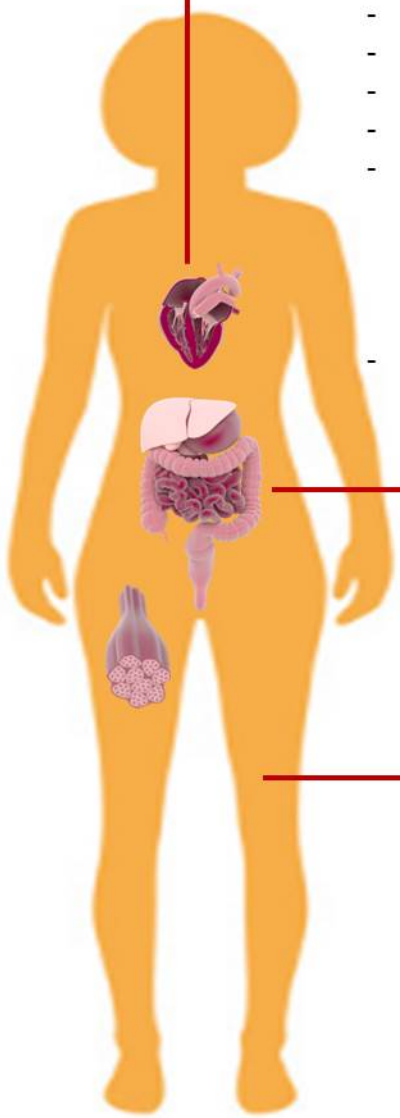

CARDIAC MARKERS

- Natriuretic peptides (ANP, BNP, NTproBNP)
- Cardiac troponin T
- Leptin
- Endothelin
- CCL2, CCL3
- Enzymes (angiotensin-converting enzyme 2, creatine kinase-MB, glutamic oxaloacetic transaminase, glutamic–pyruvic transaminase, alkaline phosphatase, acid maltase, alpha-hydroxybutyric dehydrogenase)
- Selenium levels

DIGESTIVE MARKERS

- Selenium levels

INFLAMMATORY BIOMARKERS

- C-reactive protein
- Adenosine deaminase
- Galectin-1 (Gal-1),
- Ig G anti-Gal-1 autoAb
- Oxidative stress related compounds (malonylaldheide, nitric oxide, S-nitrosylation, glutathione peroxidase, superoxide dismutase, manganese superoxide dismutase, catalase, glutathione reductase, glutathione, myeloperoxidase, nitric oxide synthase, xanthine oxidase, oxidation protein products, 3-nitrotyrosine, nitrate/nitrite, lipid peroxides)
- MMP-2 and MMP-9
- TIMP-1 and TIMP2
- Cytokines (TNF-a, IFN-c, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL17, IL-17A, IL-22, TGFb1, TGFb2)

BIOCHEMICAL COMPOUNDS

- ApoA1
- FBN
- Hypercoagulability biomarkers (ETP, F1+2, PAP)

IMMUNOLOGICAL MARKERS

- Anti-troponin T antibodies
- Myosin autoantibodies
- CD8 + T cell inhibitory receptors and antigen-specific capacity
- Anti- *T. cruzi* IgG1 levels

OTHER COMPOUNDS

- Extracellular vesicles
- Human genetic markers

T. cruzi MARKERS