Expert Reviews

Biological markers for evaluating therapeutic efficacy in Chagas disease, a systematic review

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The most neglected aspects of Chagas disease (CD) have been patient care and treatment. Despite recent progress in the development of potentially improved drugs, there is no consensus among different research groups on the lack of therapeutic response markers to evaluate efficacy of newly proposed drugs early after treatment. A systematic review of current evidence regarding molecules which are potential biomarkers for therapeutic response has been conducted using quality assessment and target responses as primary criteria. The review provides a panorama of the cumulative evidence and specific needs for development of a battery of complementary biomarkers which together fulfill ideal or acceptable criteria to evaluate early responses to treatment for chronic CD. There are several marker candidates which together may fulfill acceptable criteria to indicate the efficacy of a trypanocidal treatment. Data from ongoing studies are considered essential to improve assessment of existing markers and to identify those for early follow-up of treated patients.

KEYWORDS: biological marker • biomarker • Chagas disease • cure marker • humoral and cellular immune response • PCR • treatment • Trypanosoma cruzi

A lack of appropriate clinical and biomarker tools limits the direct measurement of treatment impact for any infectious disease. This is a common limitation for many of the 17 internationally recognized neglected diseases. One striking example is Chagas disease (CD), an endemic zoonosis caused by the protozoan parasite *Trypanosoma cruzi*, which affects 7–8 million people currently, not only in Latin America where the disease is autochthonous [1] but also is worldwide due to population migrations [2,3].

Despite the burden of CD morbidity and lower cost of timely diagnosed and treated patients [4], only two drugs, benznidazole and nifurtimox, are currently available for treatment. Both drugs have variable efficacy depending on the disease stage, drug dose, patient age and geographical origin. *T. cruzi* infection treatment is currently strongly recommended in both acute and chronic stages of the infection [5–7]. The association of *T. cruzi* infection and disease progression, and

therefore its clinical cure, remains unclear mainly because physiopathological changes develop slowly and symptoms may appear several years after infection. Disease symptoms may appear when there is an imbalance between the host immune response and parasite proliferation in tissues. Along with tissue damage caused by the presence and persistence of the parasite [8], there are inflammatory processes and cross-reactivity with host molecules [9]. Currently, the association of parasite persistence and symptom development is a recurring controversy.

In the chronic stage, drug therapy efficacy is variable and is difficult to compare since most studies use different treatment regimens and response assessment methods (variable assays, frequency and duration of follow-up) [10], in addition to the lack of class I studies [11,12]. Even in successful treatment, the gold standard for evaluating efficacy (seroconversion using conventional serological tests) may take years to decades to assess [13,14]. Hence, long-term

Table 1. Keywords used for literature searches using the NLM gateway.

Category	MeSH term	MeSH subheading
Response to treatment by biochemical biomarkers	CD OR *Trypanosoma cruzi*	AND biological markers AND treatment
		AND cure markers AND treatment
		AND biomarker AND treatment
Response to treatment by immunological biomarkers	CD OR *T. cruzi*	AND biological markers
		AND biomarkers
		AND immunological markers
		AND biological markers AND treatment
		AND biomarkers AND treatment
		AND immunological markers AND treatment
		AND biological markers AND immunology
		AND biomarkers AND immunology
		AND immunological markers AND immunology
PCR for the evaluation of treatment	CD	AND polymerase chain reaction

CD: Chagas disease.

treatment of chronic CD cases has been neglected in two primary areas:

- A lack of interest since the 1970s to develop new drugs specifically targeting intracellular parasites in general and for *T. cruzi* in particular. Fortunately, in the last 5 years, new drugs or new schemes of current drugs have been proposed as potential alternatives and are being tested in different phases of development for safety and efficacy against *T. cruzi* in humans.
- A lack of consensus regarding therapeutic response markers for early assessment of antitrypanocidal drug efficacy for disease management and of clinical trials with new drugs for *T. cruzi* treatment.

Several prognosis and progression markers for *T. cruzi* infection have been proposed during the last 20 years. However, only a few of these have been evaluated using appropriately designed studies for therapeutic response. In order to assess molecules as potential biomarkers for therapeutic response and their disease stage-specific characteristics, a systematic review of current evidence was conducted focusing on the quality of biomarker studies in both aspects. Only studies assessing biological markers (immunological, biochemical and molecular biomarkers or nucleic acid-based biomarkers) during treatment follow-up of chronic *T. cruzi* patients have been included. On the basis of review of current evidence, a target product profile (TPP) was developed for an 'ideal' and/or an 'acceptable' biomarker for anti-*T. cruzi* treatment response in different epidemiological and clinical scenarios.

Methods

The reviewed publications addressing CD-specific treatment response markers were organized into three areas of analysis:

immunological markers, biochemical biomarkers and nucleic acid amplification strategies.

Acquisition of evidence

The literature was reviewed based on electronic searches in The Cochrane Central Register of Controlled Trials on The Cochrane Library, NLM GATEWAY (PubMed/MEDLINE, Clinicaltrials.gov, Bookshelf and Meeting Abstracts), WHOLIS, BVS (BIREME and LILACS), SCIELO (1990–2012). The indexing terms used in the searches are presented in TABLE 1.

A secondary search was performed using the first or last article's author or the marker under analysis as keywords AND 'CD' OR 'Chagas' AND 'treatment', AND 'human', AND 'patient', OR 'patients'.

We considered reports only of original research, mainly but not exclusively intervention trials, specificity of diagnostic methods and observational studies, with scope targeted at biomarkers of treatment response for patients with chronic CD. Articles published in Spanish, English and Portuguese from 1990 through 31 December 2012 were reviewed according to the following inclusion criteria:

- Studies concerning development, standardization and/or validation of:
  - Biochemical biomarkers
  - Immunological biomarkers
  - Nucleic acid amplification strategies
- The aim of the study was to:
  - Monitor antiparasitic treatment
  - Test the response to antiparasitic treatment or to testing antiparasitic treatment outcomes
- Studies performed in humans

Articles that had the following characteristics were excluded:

- Studies previous to 1990
- Studies performed in experimental models or PCR studies using vector samples
- Studies not specifically designed to evaluate treatment impact by specific biomarkers and/or PCR techniques
- Studies that evaluated response to antiparasitic treatment with image techniques (x-ray, echocardiography, scintygraphy, etc.)
- Nucleic acid amplification studies, if the purpose of the technique was genotyping, DNA cloning or genomics and retrotranscription–PCR assays for gene expression.

Systematizing results

A matrix was constructed with the following information categories for each biomarker and article: which test was evaluated, distribution by sex, age of study population, geographical area, times of study patients follow-up, sample size, missing data, study design, stages of the disease included in the study, treatment (drug, dose, length of treatment), reference test performed, values of the reference standardized test, dispersion values of the standardized test, sensitivity and specificity of the biomarker, biomarker efficacy evaluation and study biases (TABLE 2). The main characteristics and limitations of each biomarker are highlighted.

Results of the searches

The results of the searches are summarized in FIGURE 1 and a review of the titles, abstracts and in some cases the full texts were examined to select relevant papers for the review. A more detailed review and data collection from each study were conducted after reviewing title and/or abstracts, in order to evaluate its relevance.

Evidence synthesis

The results of the searching have been summarized in TABLE 2.

Analysis of the results by type of molecules & technique

Immunological molecules

Four categories of 25 markers have been used to measure therapeutic efficacy for CD. The first group includes four markers that detected specific antibodies for host antigens. The second group (14 markers) involves methods to detect antibodies generated against parasite antigens. The third group includes those that measure the cytokine level and/or cytokine pattern in a patient's serum (three markers), and a fourth group includes four markers to quantify cellular immune response populations or populations expressing specific cytokines.

Host antigens

Several human antigens have been proposed as biomarkers of treatment response in CD. Increased levels of sP-selectin and soluble vascular cell adhesion molecule '1' (sVCAM-1) have been observed in 41 asymptomatic chronic CD pediatric patients. Before treatment, 83 and 71% of these exceeded the cut-off control value for sP-selectin and sVCAM-1, respectively. There was a significantly greater decrease in the titers of sP-selectin (66.7%) and sVCAM-1 (41.0%) in those children who received benznidazole therapy compared with a control group receiving placebo [15].

Levels of anti-R3 antibodies, a peptide encoded in the human autoantigen 'Cha', increased with the progression of clinical manifestations of chronic CD. Anti-R3 antibody titers decreased in 19 patients treated with antiparasitic drugs (benznidazole or nifurtimox), despite the fact that all had higher titers than those observed in healthy donors [16].

The production of anti-M2 muscarinic receptor autoantibodies (anti-M2R Ab) and IFN-γ profiles was characterized in 30 *T. cruzi*-infected children in the early stage of chronic CD, before and after trypanocidal benznidazole chemotherapy [17]. Before treatment, anti-M2 receptor autoantibodies were detected in 56% of *T. cruzi*-infected patients and none of the 19 uninfected control subjects. Infected children also exhibited a significantly higher serum IFN-γ level than that observed in healthy controls. At 6 months post-treatment with benznidazole, there was a significant decrease in anti-M2R Ab and IFN-γ levels in all patients, throughout follow-up, with a 29.7–88.1% decrease in anti-M2R Ab and 10–100% decrease of IFN-γ.

Parasite antigens

A complement-mediated lysis test (CoML) using living trypomastigotes was compared with conventional serological methods at different times following treatment [18]. Seroconversion of the CoML occurred in 8 out of 21 patients (38%) between 6 and 24 months following treatment, in 4 out of 21 patients (19%) between 24 and 36 months and in one patient within 4 years post-treatment. The use of the CoML test has, however, several limitations, in particular the need for living infective trypomastigotes. A possible substitute for the CoML test, an ELISA technique based on a low-molecular weight-recombinant protein of *T. cruzi*, rTc24 was also developed [19]. All patients with active infection (positive CoML) recognized rTc24 using ELISA and western blot, while 80% of seropositive patients with negative CoML were seronegative to rTc24. There was a decrease in anti-rTc24 antibodies in 38% of patients using ELISA between 6 and 24 months post-treatment and in 19% of patients at 36 months post-treatment.

Three groups of *T. cruzi*-infected patients, untreated cases, patients with treatment failure and successfully treated patients, were tested for antiparasite antibodies using an immunoflourescence assay of fixed trypomastigotes (referred as ISIFA) [20]. A successfully treated patient was defined as a case with undetectable parasitemia using xenodiagnosis at 6 years post-treatment. ISIFA was able to differentiate successfully treated cases from untreated or those with treatment failure [21,22]. Treatment efficacy was monitored by using disappearance of antibodies by serological methods (complement fixation, indirect immunofluorescence, indirect hemagglutination and ELISA using total *T. cruzi* protein as antigens). Only 8% of 113 patients in the

Table 2. Review results.

Study (year)	Test	Sex distribution	Age (rank/mean–SD)	Geographical region (country)	Follow-up (months: rank/mean–SD)	Missing (%)	N	Study design	Chagas disease stage[†]	Treatment regime
Biochemical and metabolic biomarkers										
Pinazo et al. (2011)	ETP, F 1+2	YES	20–45	Latin America	6	23	43	Prospective	ALL	BZD, 5 mg/kg/day, 60 days
PCR techniques										
Britto et al. (1995)	PCR	NO	ND	Brasil	48	0	34	Prospective	C/ I	BZD, 50–600 mg/day, 45–180 days
Lauria-pires (2000)	PCR	NO	31–60	Brasil	120	15.50	45	Prospective	ND	BZD/NFX, ND, 60/30/20 days
Braga MS (2000)	PCR	YES	ND	Brasil	120	ND	17	Prospective	ND	BZD/NFX,10 mg/kg/day, at least 30 days
Britto et al. (2001)	PCR	ND	ND	Brasil	84–420/240	0	100	Retrospective	ND	BZD, 5–6 mg/kg/day, 30–60 days; NFX, 7–8 mg/kg/day, 60–90 days
Solari et al. (2001)	PCR	ND	0–10/6	Chile	36	15	66	Prospective	ASYMTOMATIC	NFX, 7–10 mg/kg/day, 60 days
Galvao et al. (2003)	PCR	NO	7 a 12	Brazil	36	13	127	Prospective	ND	BZD 7.5 mg/kg/day, 60 days
Schijman (2003)	PCR	YES	0–17/4.2	Argentina	24/36	0	40	Prospective	ND	BZD,10–16 mg/kg/day, 60 days; NFX,10–15 mg/kg /day, 60 days
Sánchez et al. (2005)	PCR and FC-ALTA	YES	22 (MEAN)	Chile	120	0	54	ND	ND	ALLO 8.5 mg/kg, 60 days; ITRA 6 mg/kg, 120 days
Lacunza et al. (2006)	PCR	YES	18–30	Argentina	6	17	23	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Meira et al. 2006	HEMOCULTURE, LMCo, PCR	ND	ND	ND	6–48/27.7	ND	31	ND	ND	BZD
Fernandes et al. (2009)	PCR	YES	17–42/30	Brasil	36	0	130	Prospective	ASYMTOMATIC	BZD, 10 mg/kg/day, 60 days
Lana et al. (2009)	PCR, SEMIQUANTITATIVE ELISA, FC-ALTA IgG	YES	6–37/27–8	Brasil	108	0	28	Retrospective	C/D/I	BZD, 5–10 mg/kg/day, 40–60 days
Murcia et al. (2010)	PCR	NO	ND/33–11	Bolivia	14	68	181	Prospective	ALL	BZD, 300 mg/day (adults), 60 days 5–7 mg/kg/day (pediatrics), 60 days
Perez-ayala et al. (2010)	PCR	YES	29–44/36	Bolivia	12	41	195	Prospective	ALL	BZD, 5 mg/kg/day,60 days; NFX, 8–10 mg/kg/day, 90 days
Aguiar et al. (2012)	PCR	YES	8–56/36–7.24	Brasil	12–348	0	39	Retrospective	ND	BZD, 5–7 mg/kg/day, 60 day
Machado de assis et al. (2012)	PCR, Hemoculture, REC-ELISA, TESA-BLOT	YES	2–60/32,9–10,9	Brasil	120–432/16.9–6.8	0	94	Retrospective	ALL	BZD, 5 mg/kg/day, 60 days
Ramos (2012)	PCR	YES	0–72/35	Bolivia	ND	25	76	Retrospective	ALL	BZD, 5 mg/kg/day, 40–60 days

[†]Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ND	ND	ND	ND	ND	No	Yes	Yes	No	No	ETP: 73,3 F 1 +2: 80	ETP: 100 F 1 + 2: 73.3	[69]
Serology	Yes	No	ND	ND	No	Yes	No	No	No	ND	71.8	[71]
Serology	No	No	ND	ND	No	No	No	No	No	ND	3.15	
Serology	No	No	ND	ND	No	No	Yes	No	No	ND	0	
Serology	No	No	ND	ND	No	No	No	No	No	ND	65	[72]
Serology	Yes	No	ND	ND	No	No	No	No	No	100	100	[85]
Serology	Yes	ND	ND	ND	No	No	No	No	No	85.9	60.4	[81]
Serology	Yes	No	ND	ND	No	Yes	No	No	No	77.5	100	
Serology	Yes	No	ND	ND	No	No	No	No	No	ND	14.8	[76]
Serology	Yes	No	ND	ND	No	No	No	No	No	100	85.7	[78]
Serology	No	No	ND	ND	ND	ND	ND	ND	ND	77.4	50	[27]
Serology	Yes	No	ND	ND	No	ND	No	No	No	100	11.3	[73]
Serology	Yes	No	ND	ND	No	No	No	No	No	ND	14.8	[74]
Serology	Yes	No	ND	ND	ND	ND	No	No	No	68	90	[79]
Serology	No	No	ND	ND	No	Yes	No	No	No	63	100	[80]
Serology	No	No	ND	ND	Yes	Yes	No	No	No	ND	41.4	[83]
Serology	No	No	ND	ND	No	ND	No	No	No	ND	47.2	[84]
Serology	No	No	ND	ND	No	ND	No	No	Yes	65.8	100	

Table 2. Review results (cont.).

Study (year)	Test	Sex distribution	Age (rank/mean–SD)	Geographical region (country)	Follow-up (months: rank/mean–SD)	Missing (%)	N	Study design	Chagas disease stage[†]	Treatment regime
Immunological biomarkers										
Vitelli avelar *et al.* (2008)	IL-12, IL-10, IL-13, TNF-α	YES	43–70	Brasil	12	ND	29	Prospective	I/C	BZD
Laucella *et al.* (2009)	INF-γ	NO	23–55	Argentina	36	ND	43	Prospective	I/C	BZD, 5 mg/kg/day, 30 days
Gironés *et al.* (2001)	R3-CHA	NO	ND	Venezuela, Argentina	ND	ND	19	Prospective	I	BZD, NFX
Cancado Jr *et al.* (2002, 1999)	Parasite total protein (STc)	YES	69–9	Brasil	72–216	ND	113	Prospective	ALL	BZD (18 GR)
Viotti *et al.* (2011)	Parasite-recombinant proteins (14)	YES	8–41.4	Argentina	36	ND	142	Prospective	I/C	BZD, 5 mg/kg/day, 30 days
Galvao *et al.* (1993)	CoML	NO	ND	Brasil	120	ND	82	Prospective	ND	BZD, 5–7 mg/kg/DÍA, 30–60 days; NFX, 8–10 mg/kg/day, 30–60 days
Meira *et al.* (2004)	recombinant complement regulatory protein	NO	ND	Brasil	37–12	13 (12 m), 48 (24 m), 32 (36 m), 80 (> 37 m)	31	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Levy *et al.* (1996)	ISIFA	YES	25–50	Brasil	ND	ND	26	Retrospective	ND	BZD, NFX
Moretti *et al.* (1998)	IV Fraction, EXO	NO	4–53	Argentina	24–240	ND	44	Prospective	I	BZD, NFX
Sanchez-negrete *et al.* (2008)	13 Antigen	YES	19–41	ND	36	28 (36 m), 17 (42–60 m), 1.5 (66 m)	18	Prospective	I/C	BZD
Andrade *et al.* (2004)	AT	YES	7–12	Brasil	72	9.4	53	Prospective	I	BZD, 7.5 mg/kg/day, 60 days
De Andrade *et al.* (1996)	AT	YES	7–12	Brasil	36	25.0	64	Prospective	I	BZD, 7.5 mg/kg/day, 60 days
Sosa estani *et al.* (1998)	F29	NO	6–12	Argentina	48	13.7	51	Prospective	I	BZD, 5 mg/kg/day, 60 days
Krautz *et al.* (1995)	rTc24	NO	ND	Brasil	ND	ND	72	Retrospective	I/C	BZD, NFX
Fernandez-villegas *et al.* (2011)	KMP11, HSP70, PAR2, Tgp63	YES	18–68	Latin America	24	24.0	46	Prospective	I/C/D	BZD, 5 mg/kg/day, 60 days
Cooley *et al.* (2008)	16 *T. cruzi* recombinant proteins	NO	29–61	Argentina	36	ND	38	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Fabbro *et al.* (2011)	P2β	YES	29–35	Argentina	240–300	ND	78	Retrospective	I/C	BZD, 5 mg/kg/day, 30 days; NFX, 8–10 mg/kg/day, 45–60 days

[†]Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ND	NO	ND	ND	ND	No	No	No	No	No	ND	ND	[36]
Serology	Yes	ND	ND	ND	No	No	No	No	No	71.0	IFN-γ decrease: 34.6% (12 m), 46.8% (24 m), 71.8% (36 m)	[41]
Serology (R3 and Shed Acute Phase Antigen Ag, total extract)	Yes	ND	92.4	100	No	No	No	No	No	92.4	100.0	[16]
ELISA, IFI, HAI, Xenidiagnosis. Hemoculture	ND	ND	ND	ND	No	No	No	No	No	100.0	8.0	[21,22]
HAI, IFI	Yes	ND	ND	ND	No	No	Yes	No	No	ND	40.0	[33]
IFI, Hemoculture	Yes	ND	ND	ND	No	No	No	No	No	ND	38% (6 m), 38% (24 m), 19% 36 (m)	[18]
CoML, Hemoculture, PCR, serology	Yes	ND	ND	ND	No	No	No	No	No	100.0	29.7% (12 m), 37.5% (24 m), 28.6% (36 m), 66.6% (48 m)	[27]
CoML, MbIFA, XENODIAGNOSIS	Yes	Yes	80–98.6	98	No	No	Yes	No	No	ND	ISIFA: 84%	[20]
IFI, HAI, SEROLOGY	Yes	ND	ND	ND	No	Yes	Yes	Yes	No	ND	F IV: 36% EXO: 44%	[26]
HAI, CMA ELISA, IFI	Yes	Yes	72.2	ND	No	Yes	Yes	No	No	72.2	66.6%	[31]
ND	No	ND	ND	ND	No	No	No	No	No	100.0	88.7%	[24]
HAI, ELISA, IFI	Yes	Yes	ND	ND	No	No	No	No	No	100.0	57.8%	[23]
HAI, ELISA, SEROLOGY	Yes	Yes	ND	ND	No	No	No	No	No	100.0	35.7% (6 m), 62.1% (48 m)	[25]
GST ELISA	Yes	ND	ND	ND	Yes	No	No	No	No	ND	ND	[19]
ELISA	Yes	ND	KMP11:90 HSP70: 90 PFR2: 75 Tgp63: 30	KMP11:85 HSP70: 90 PFR2: 92 Tgp63: 70	No	No	No	No	No	100.0	KMP11: 67% HSP70: 50% PFR2:34% K11-HP70-PFR2-Tgp63: 80%	[34]
ELISA, IFI, HAI	No	ND	ND	100	No	No	No	Yes	No	ND	ND	[42]
Xenodiagnosis	No	ND	ND	ND	No	Yes	Yes	No	Yes	ND	75.4% in I and 45% in C (23 years)	[35]

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Table 2. Review results (cont.).

Study (year)	Test	Sex distribution	Age (rank/ mean–SD)	Geographical region (country)	Follow-up (months: rank/ mean–SD)	Missing (%)	N	Study design	Chagas disease stage[†]	Treatment regime
Cutrullis *et al.* (2011)	M2	YES	8–17	Argentina	6	33.3%	30	Prospective	I	BZD, 5–8 mg/kg/day, 60 days
Dutra *et al.* (1996)	CD3+ y CD5+/ CD19+, CD3+HLAR+	NO	29–66	Brasil	60	ND	ND	Prospective	ND	BZD, 5–7 mg/kg/day, 30–60 days; NFX, 8–10 mg/kg/ day, 30–60 days
Argüello *et al.* (2012)	CD4$^+$/LIR-1	NO	36–68	Argentina	12–50	ND	87	Prospective and retrospective	I/C	BZD, 5 mg/kg/day, 30 days
Laucella *et al.* (1999)	sVCAM y sPLectina	NO	6–12	Argentina	48	ND	23	Prospective	I	BZD, 5 mg/kg/day, 60 days
Sathler-avelar *et al.* (2012)	IL12, IL10 e IFN-γ	YES	33–56	Brasil	84	ND	14	Prospective	I	BZD, 5 mg/kg/day, 60 days
Sathler-Avelar *et al.* (2008)	Monocytes: CD16/ CD14 NK: CD3/CD16/CD56	NO	9–14	Brasil	12	ND	6	Prospective	I	BZD, 8 mg/kg/day, 60 days
Guedes *et al.* (2012)	IL17, IL10, TNF-α e IFN-γ	NO	ND	Brasil	ND	ND	8	Retrospective	I/C	BZD, 5 mg/kg/day, 60 days

[†]Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

chronic stage of the disease were considered cured after 6–18 years following treatment. Unfortunately, potential reinfection for patients living outside the transmission area could not be evaluated.

Therapeutic efficacy in children with early chronic CD has also been studied. Sera from 130 children treated with benznidazole or placebo were analyzed using a purified trypomastigote glyconjugate in a chemiluminescent ELISA [23]. At 6 months post-treatment, 37 of 64 treated patients (56%) compared with 3 of 65 (5%) placebo cases were negative using this test [24]. However, although at 6 years post-treatment, 47 out of 53 patients (88.7%) and only 12 of 46 placebo subjects (26.1%) were negative in the same test. Short-term monitoring using a recombinant *T. cruzi* flagelar calcium-binding protein (F29) in an ELISA was used in a study of Argentinean chronic stage children [25]. Results from the latter study indicate that 35.2 and 62.1% among 44 benznidazole-treated children were seronegative for F29, after 6 and 48 months post-treatment, respectively.

Antibody levels against fractions obtained from *T. cruzi* extracts (FI–FV) and against exo-antigens obtained from trypomastigote-infected mice (EXO) were studied in 42 treated patients (between 2 and 20 years after treatment with benznidazole or nifurtimox) and in 42 untreated controls [26]. Negative serology using FI-V antigen in an ELISA was observed in 64% of treated versus 33% untreated patients. In addition, an ELISA using EXO as antigen was negative in 44% treated versus 8% of untreated patients.

Several researchers have used recombinant antigens for monitoring post-therapeutic cure. Antibody levels for recombinant

complement regulatory protein (rCRP) were evaluated using an ELISA in 31 patients before and after treatment, monitoring an average 27.7 months after treatment [27]. There was an inverse relationship between rCRP ELISA positivity and period of follow-up, decreasing from 100% at treatment to 70.3, 62.5, 71.4 and 33.4% in the first, second, third and fourth years after treatment, respectively. Additionally, antibody levels against antigens 1, 2, 13, 30, 36 and Shed Acute Phase Antigen (SAPA) [28–30] were assessed in sera from 18 CD patients before and after 3 years follow-up post-treatment. Antigen 13 was shown to be a good marker of treatment efficacy using ELISA, since negative conversion occurred in 67% (6 of 9) of patients (p = 0.002) [31].

Sera had a distinctive but highly consistent reactivity pattern using a panel of 16 *T. cruzi* proteins (recognized by sera from CD patients living in endemic areas) in a multiplex system [32]. There was a decreased response to the panel in six patients followed by 36 months after treatment. Two treatment failures did not have a change in antibody response pattern over time. Seronegative conversion, as well as a decrease in antibody titers, was measured serially in 53 benznidazole-treated and 89-untreated chronic patients, with a median follow-up of 36 months using conventional serological assays (IA, IFI and ELISA) and the above-mentioned multiplex assay [33]. Remarkably, there was a strong correlation between results from both conventional serological tests and the multiplex assay [33]. A decrease in conventional serology titers against *T. cruzi* was measured in 64% of treated patients versus 21% of untreated patients, while there was negative seroconversion in 40% treated versus 7% of untreated patients.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ELISA, HAI	Yes	YES	ND	56.70	No	No	No	No	No	56.7	antiMR2: 29.7–88.1% IFN-γ: 10–100% (6 m)	[17]
Hemoculture, CoML ELISA	Yes	ND	ND	ND	No	No	Yes	No	No	ND	ND	[39]
ND	ND	ND	ND	ND	No	No	Yes	No	No	60.0	60.0%	[43]
ND	ND	ND	ND		No	No	No	No	No	sPselectin: 83 sVCAM: 71	sPselectin: 66.7% sVCAM: 41%	[15]
Cytokine measure in stimulation culture	ND	ND	ND	sPselectin: 83 sVCAM: 71	No	Yes	No	No	No	ND	ND	[37]
ND	ND	ND	ND	ND	No	No	No	No	No	ND	ND	[40]
ND	ND	ND	ND	ND	No	Yes	Yes	No	No	ND	ND	[38]

A serological test using the KMP11, HSP70, PFR2 and Tgp63 recombinant proteins was evaluated in 35 treated patients before and after benznidazole administration [34]. A statistically significant decrease in reactivity against KMP11 occurred 6 months post-treatment in 26 out of 35 of patients (74%), at 9 months post-treatment against PFR2 in 26 out of 35 (74%) and against HSP70 in 25 out of 35 (71%) CD patients. When the response against only two of these antigens was evaluated, the decrease in specific antibody titer occurred in 80% of patients at 9 months post-treatment and continued during the 2-year post-treatment follow-up period. The overall decrease in titers against KMP11, HSP70 and PFR2 24 months post-treatment was 67, 50 and 34% of patients, respectively.

Antibody levels against *T. cruzi* ribosomal acidic protein P2β (Tc P P2β) were analyzed in: 30 asymptomatic CD patients having received specific treatment with clinical follow-up for more than 20 years (group A); 37 asymptomatic CD patients not having been treated (group B); and 11 untreated chronic CD patients (group C) [35]. Antibody levels against TcP2β were significantly lower only in patients from group A.

Cell markers: cytokines
Active parasite-specific B and T cell responses and specific cytokine profiles in patient sera are associated with development of CD pathology and have been proposed as immune markers for disease progression and for treatment assessment and follow-up. Circulating leukocytes from asymptomatic *T. cruzi*-infected patients (without stimuli) secreted a predominant regulatory cytokine profile, whereas symptomatic cardiac patients had a

predominant inflammatory cytokine pattern [36]. This cytokine profile reverts after treatment, with asymptomatic patients shifting to predominant inflammatory profiles, and symptomatic cardiac patients upregulate regulatory cytokine production. A similar profile is observed following *in vitro* stimulation of leukocytes with *T. cruzi* trypomastigotes. Untreated asymptomatic patients have a type-1 regulated cytokine profile (innate immune compartment) and a predominantly type-2 adaptive profile in *ex vivo* analysis. Following treatment, they have a downregulated cytokine profile in both innate and adaptive immune compartments [37].

Patients with moderate and severe cardiomyopathy produce high levels of TNF-α and IFN-γ and low levels of IL-10 and IL-17 compared with mild cardiomyopathy or cardiomyopathy-free patients [38]. Treated patients with mild or free cardiomyopathy produced high levels of IFN-γ compared with untreated patients with mild or free cardiomyopathy. Deficient suppressor activity controlling myocardial inflammation by regulatory T cells may cause the altered immune response observed in patients with moderate and severe cardiomyopathy.

Cellular surface markers
The phenotype of T and B lymphocytes from peripheral blood mononuclear cells (PBMCs) was analyzed in untreated, treated (uncured and cured) CD patients and healthy donors. The patients were considered cured when hemoculture and the above-mentioned CoML tests were negative [39]. Untreated patients had a lower proportion of CD3+ T lymphocytes and a higher proportion of CD5+ B cells than healthy donors, while
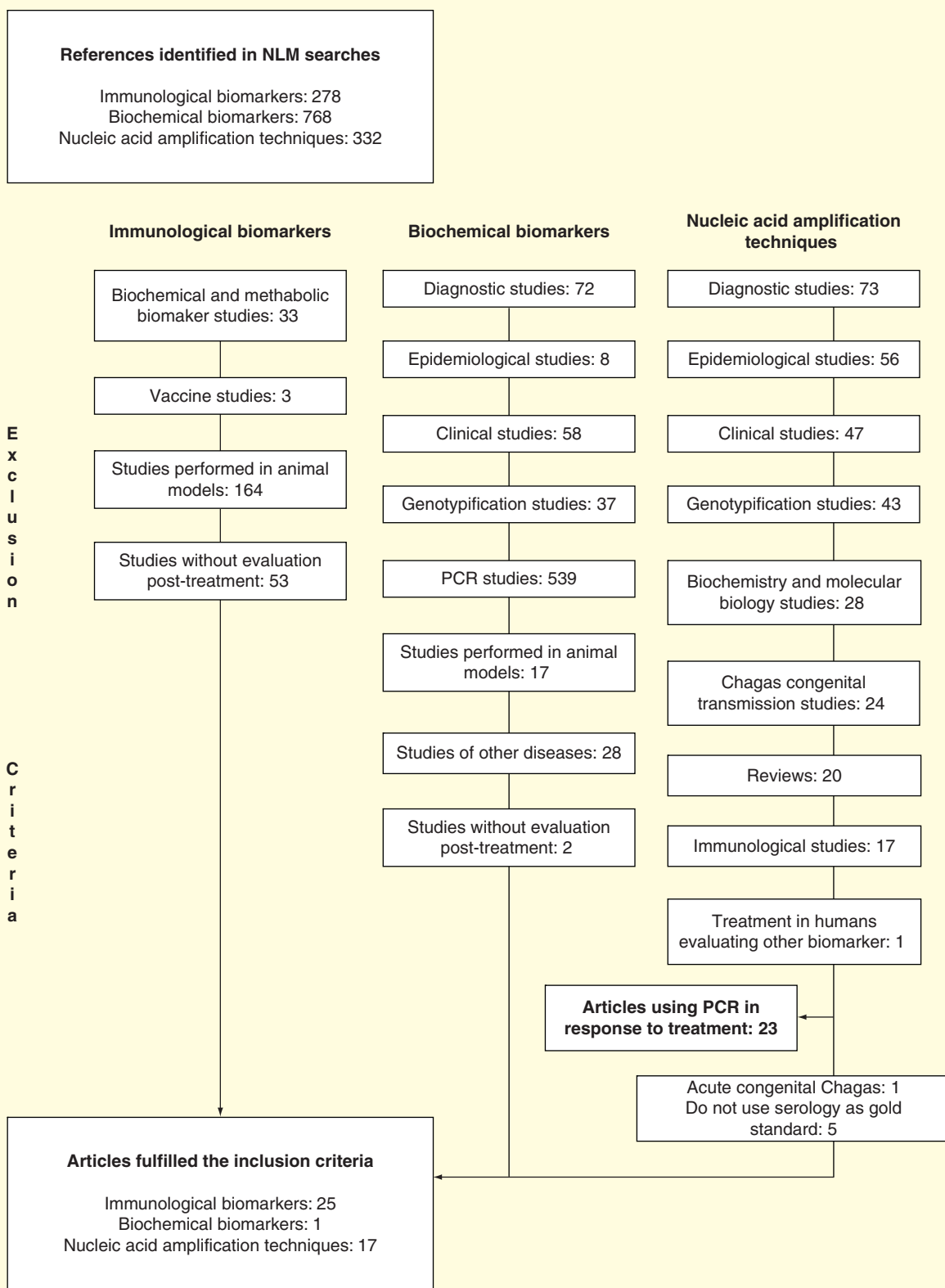
References identified in NLM searches

Immunological biomarkers: 278
Biochemical biomarkers: 768
Nucleic acid amplification techniques: 332

Immunological biomarkers

Biochemical and methabolic biomaker studies: 33

Vaccine studies: 3

Studies performed in animal models: 164

Studies without evaluation post-treatment: 53

Biochemical biomarkers

Diagnostic studies: 72

Epidemiological studies: 8

Clinical studies: 58

Genotypification studies: 37

PCR studies: 539

Studies performed in animal models: 17

Studies of other diseases: 28

Studies without evaluation post-treatment: 2

Nucleic acid amplification techniques

Diagnostic studies: 73

Epidemiological studies: 56

Clinical studies: 47

Genotypification studies: 43

Biochemistry and molecular biology studies: 28

Chagas congenital transmission studies: 24

Reviews: 20

Immunological studies: 17

Treatment in humans evaluating other biomarker: 1

Articles using PCR in response to treatment: 23

Acute congenital Chagas: 1
Do not use serology as gold standard: 5

E x c l u s i o n   C r i t e r i a

Articles fulfilled the inclusion criteria

Immunological biomarkers: 25
Biochemical biomarkers: 1
Nucleic acid amplification techniques: 17

Figure 1. Flow of inclusion of studies on biological markers for evaluating.

treated patients (uncured and cured) had intermediate values. Treated or untreated patients had approximately 2.5-fold more CD3$^+$/HLA-DR$^+$ cells than those detected in uninfected individuals. The mean proliferative response in vitro of PBMC from cured patients to parasite-related stimuli was equivalent to the low levels detected in healthy donors.

Analysis of the immune response changes following etiological treatment of CD with benznidazole was analyzed in children at an early indeterminate stage [40]. Treated patients had a higher activation status of circulating monocytes, inversely associated with the level of IL-12$^+$CD14$^+$ cells. Moreover, benznidazole treatment triggered a high proportion of circulating CD3$^-$/CD16$^+$/CD56$^-$ NK cells associated with a type 1-modulated cytokine pattern. The benznidazole treatment induced substantial T and B cell activation associated with an overall IL-10-modulated type 1 cytokine profile.

The proportion of PBMC-expressing IFN-γ was measured in 67 indeterminate patients (treated or untreated) and in 8 treated patients with abnormal electrocardiographic findings [41]. Following treatment, there was either nil or a three-fold decrease of IFN-γ expression compared with pretreatment levels, from 9 of 26, 15 of 32 and 23 of 32 patients, at 12, 24 and 36 months after treatment, respectively. The antibody response of these patients to a pool of recombinant T. cruzi proteins using a multiplex system also decreased over time [42]. The increase in cells not expressing IFN-γ is associated with an early increase in IFN-γ-producing T cells with effector/effector memory cell phenotype (result observed in 7 of 19 patients analyzed).

The proportion of total CD4$^+$LIR$^+$ T cells decreases significantly in 60% of patients (6 of 10) with indeterminate stage CD, after benznidazole treatment [43]. The decrease is observed as early as 2–6 months after treatment and is sustained at least for 2 years.

In summary, cellular markers are not direct markers of treatment as they do not pretend to detect parasite presence. However, they may be used to asses the influence of the treatment on the clinical improvement in a particular CD patient group, by comparing the presence or expression level of the marker, in other words, in symptomatic versus asymptomatic patients. Most of the data on immunological biomarkers were published before the development of nucleic acid amplification techniques.

Biochemical & metabolic molecules

CD is a chronic infection, which stimulates a continuous inflammatory immune response. Molecules that are inflammatory mediators of metabolic processes are altered in chronic states of inflammation and/or infection such as chronic CD. Even though many biochemical biomarkers are easily accessible and easy to use at a reasonable cost, we found that few of them have been considered as potential treatment response biomarkers, until recently.

Certain biomarkers of cardiac damage, troponin I and T and natriuretic peptides, have been analyzed to determine progression of the primary complication of CD and are also being proposed as diagnostic tools for T. cruzi infection progression [44]. Atrial natriuretic peptide, brain natriuretic peptide (BNP) and N-terminal proBNP have been assessed to discriminate myocardial involvement in early stages of CD [45–50]. Results from these studies are contradictory, indicating that serum BNP levels are similar in T. cruzi-infected and uninfected individuals [51]. Other natriuretic peptides (NT-proBNP [52–54], BNP [55–57] and atrial natriuretic peptide [46,49]) and troponin I and T [58] have been proposed to differentially diagnosis late stages of CD.

Clinical trials have assessed the serum enzymes glutamic oxaloacetic transaminase, glutamic–pyruvic transaminase, alkaline phosphatase, acid maltase and alpha-hydroxybutyric dehydrogenase (alpha-HBDH or LDH1) for early detection of myocardial involvement [59]. Patients with chagasic cardiomyopathy have low leptin [54], adipokynes [60] and angiotensin-converting enzyme levels [57], which suggest their potential use as progression markers. Low selenium levels have also been suggested as a progression marker for chronic digestive and cardiac CD [61]. Using animal models, caveolin-3 (Cav-3) [62], myocardial and peripheral protein-3-nitrotyrosine (3NT) and its protein carbonyl formation [63], the higher level of catalase, glutathione peroxidase, glutathione reductase and reduced function of glutathione and Mn(2+) superoxide dismutase have been reported in T. cruzi-infected individuals [64].

Hypercoagulability markers such as prothrombin fragments 1+2 (F 1+2), thrombin–antithrombin complex (ATM complex), fibrinogen/fibrin degradation products, D-dimer [65] and lipid bodies [66] in addition to apolipoprotein A1 (ApoA1) [67] have been measured in T. cruzi patients. Despite the important number of biomarkers studied from this latter group, response to treatment has not been evaluated except in two of these in a single study. These unpublished studies demonstrated the usefulness of ApoA1 and fibronectin [68].

Hemostatic biomarkers such as endogenous thrombin potential (ETP) and 1+2 prothrombin fragments 1+2 (F 1+2) have altered levels in T. cruzi-infected patients compared with controls (73 and 80% of patients, respectively), which decreased significantly 6 months after treatment (100 and 73%, respectively) [69]. Evaluation of ETP and F 1+2 was conducted in a nonendemic area on all stages of CD, thereby controlling for possible reinfection. Nevertheless, since the results are unpublished from a preliminary phase of a larger study, the small sample size, short follow-up period after treatment, large number of cases lost to follow-up (due to high mobility of the study population) and lack of a standardized test to compare proposed biomarkers argue for caution in data interpretation.

In summary, biochemical and metabolic molecules could be useful surrogates for response to treatment of T. cruzi infection due to their easy analysis and low cost. However, only four of these (ETP, F 1+2, potentially ApoA1 and fibronectin) have been evaluated after treatment of chronic CD patients, which implies that further studies will be required to assess their specificity and validate their use in diagnosis or prognosis.

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Table 3. Target product profile for an 'ideal' or/and an 'acceptable' biomarker criteria for anti-*T. cruzi* treatment response.

	Acceptable	Ideal
Indications and usage	Chronic CD in the symptomatic and asymptomatic form	Acute and chronic CD in all the stages
Samples (Sample collection, conservation)	Peripheral blood (cubital puncture) Collection in stabilizing buffer for conservation and transportation at room temperature or at 4°C	Peripheral blood (digital puncture) Urine Umbilical cord blood for congenital CD Collection in stabilizing buffer for conservation and transportation at room temperature
Number of samples and volume	Three samples • One pretreatment • Two post-treatment Maximum volume: 5 ml in adults; 2 ml in children, 1 ml neonates and newborns	Two samples • One pretreatment • One post-treatment Maximum volume: 2 ml in adults, 1 ml in children, 0.5 ml umbilical cord blood in newborns
Storage conditions technology • Equipment required • High technology required • Human resources	Storage at room temperature, 4°C or -20°C • Laboratory equipment • No high technology required • Specialized human resources (second/third-level center)	Storage at room temperature or 4°C • Point of care • No high technology required • Nonspecialized human resources (primary care center)
Time to processing	48–72 h	<24 h
Methods	Qualitative Semi quantitative	Qualitative Quantitative
Sensitivity and specificity	≥95%, ≥95%	100%, ≥98%
Time of response	12–24 months	3 months
Percentage of expression of altered BMK values (nontreated *T. cruzi*-infected patients)	50%	100%
Percentage of response in treated *T. cruzi*-infected patients	70%	100%
Precautions	None	None
Costs	Low	Low
Availability	In endemic countries	In all countries

CD: Chagas disease

Nucleic acid amplification techniques

Amplification of *T. cruzi* DNA has been tested and is being evaluated as a reliable marker of therapeutic response in clinical trials for efficacy of trypanocidal drugs in *T. cruzi*-infected patients. When parasite DNA amplification was compared with other parasitological diagnostic methods, such as hemoculture and xenodiagnosis, the sensitivity obtained clearly favors the PCR technique for patient treatment follow-up [70–74]. PCR alone or combined with DNA hybridizations was used to evaluate efficacy in patients treated with itraconazole or allopurinol, having a significantly higher sensitivity compared with xenodiagnosis [75–77].

Although *T. cruzi* DNA amplification in the blood of chronic *T. cruzi*-infected and treated patients proved to be in most cases a useful tool to demonstrate failure of treatment,

different research groups reported variable success in complete negativization. Unsuccessful specific elimination of *T. cruzi* was reported in 85–89% of patients positive for PCR after trypanocidal treatment [70,73,74] although in other studies, a high percentage (>70%) of patients given trypanocidal treatment converted to negative when tested by PCR [71,78–82]. After an average follow-up of 20–35 years after trypanocidal treatment, 30–60% of *T. cruzi*-infected patients continued to be positive using DNA amplification [72,83,84].

Pharmacological treatment does not correlate with parasite elimination or sustained elimination of parasite DNA, which may be related to many factors such as CD stage, patient pathology at the time of treatment, drug and treatment protocols, parasite strain and load, DNA amplification technique and/or the number of patients studied. The most sensitive

method, unfortunately not used in all studies, is to test several samples from each patient at different intervals, which increases the sensitivity of the PCR to detect *T. cruzi* DNA in blood samples. Nevertheless, while PCR-negative conversion could be achieved in many studies and in a variable proportion of treated patients, a significant seroconversion has not been recorded [71,73,74,78–80,83,85]. It is important to note that obtaining a negative PCR result does not guarantee parasitological cure, since parasitemia may fluctuate, at least in the chronic phase of the infection and parasitemia may be below the PCR detection level, especially after a long period of follow-up after trypanocidal treatment [79,80,86]. In order to avoid false negatives and measure test confidence, repeated PCR of new patient samples is necessary over time.

T. cruzi nucleic acid amplification techniques offer the most sensitive parasitological diagnostic method for infection. Early detection of parasite susceptibility to drugs, and therefore, the use of PCR as a method to promptly detect failure for lack of adherence or parasite resistance to chemotherapy, far out-weighs serology as a tool for patient follow-up after trypanocidal treatment. Since *T. cruzi*-specific antibodies could persist for many years after the etiological treatment in chronic chagasic patients, parasite DNA amplification provides a more rapid, sensitive and cost-effective test to avoid very demanding long patient follow-up, which is always very difficult to accomplish.

Recommendations based on the review: what to expect from early markers of therapeutic response – a definition based on the TPP model

A biomarker is defined as a sign that can be measured accurately and reproducibly to reflect the status of a disease process. Effective markers quantitatively correlate (either directly or inversely) with disease progression. A surrogate marker could be defined as a sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint, that is a direct measure of how a patient feels, functions or survives and is expected to predict the effect of the therapy [87,88]. One of the major reasons for identifying early biomarkers of cure/progression of chronic CD is to improve patient management and has available tools to evaluate clinical trials. Currently, the efficacy of a drug cannot be evaluated in a short period of time after treatment, since parasitological clearance cannot be measured except if it correlates with a significant decrease in titers from conventional serology, which may take many years if at all.

Early biomarkers and some surrogate markers of therapeutic response for chronic CD should be molecules that fulfill specific quality criteria. 'Acceptable' and 'ideal' characteristics that are necessary for biomarkers used to evaluate response to treatment in patients with chronic CD are proposed in TABLE 3 according to TPP models used for development and evaluation of drugs. There are two primary and essential technical aspects to fulfill for biomarkers: the level of biomarker expression before treatment and the elapsed time in which the marker begins to decrease. There is no consensus regarding the

definition of an 'early' therapeutic response for chronic CD, and there is dearth of evidence to define the optimum timing for marker assessment. Evidence suggests that the parasite genotype will have a direct influence on the treatment efficacy, but a biomarker will assess the patient's response to treatment and hence the biomarker effectiveness would not be influenced by *T. cruzi* genotype. The ideal biomarker must be expressed at high levels in chronic *T. cruzi* patients before treatment, but evidence for proportional change after treatment will depend on normal population variation and statistical difference for each individual marker.

Future strategies in order to validate early biomarkers of response to treatment

There are several key questions in order to drive future studies regarding specific response biomarkers to treatment with benznidazole and/or nifurtimox in chronic *T. cruzi*-infected patients. Validation and use criteria have also been included in the TPP model (TABLE 3). It is important to highlight the heterogeneity of data from the markers studied by different groups, which do not evaluate the same parameters. There are, however, several markers that do have the ability to detect treatment response and can be classified in two groups:

• Parasite biomarkers

There are two main classes of recombinant proteins that are effective at different ages and stages of the disease:

• a 16 protein group [42]
• a combination of four recombinant proteins [34]: KMP11, HSP70, PAR2 and Tgp63.

Other parasite biomarkers include the CoML marker purified from trypomastigotes [18]; and in pediatrics, the AT antigen, which is treatment response indicators still needing to be tested in adults [24] and the F29 protein, recently tested in adults [25,89]. In this field, the disaccharide Galα(1,3)Galβ as an immunodominant glycotope of a synthetic glycoarray containing nonreducing α-galactopyranosyl moieties related to mucin O-glycans, evaluated by a chemiluminescent enzyme-linked immunosorbent assay, has showed its usefulness in the diagnosis of the infection in chronic stages, but it has not been tested to assess response to treatment [90].

• DNA amplification techniques

Recently, Nagarkatti *et al.* (2014) used short RNA ligands called aptamers to detect biomarkers of *T. cruzi* infection in the plasma of infected mice [91]. Aptamers were generated against *T. cruzi* excreted/secreted antigens (TESA) purified from *in vitro* culture supernatants of infected host cells and used as specific ligands in enzyme-linked aptamer assays. TESA molecules could be detected in the blood of infected mice during both the acute and the chronic phases of the disease. Although the identity of the TESA biomarkers is currently not known, these molecules represent novel markers of *T. cruzi* infection. Their detection in clinical samples is currently being assessed (personal communication). These assays also have great

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potential in drug development applications and/or to help evaluate treatment efficacy and possibly parasitological cure in human clinical trials.

• Host response/damage biomarkers

Biochemical biomarkers such as F 1+2, ETP [69], apolipoprotein 1 and fibronectin [68] (Ndao *et al.*, personal communication) detect early treatment response at different stages of chronic CD in adults. Muscarinic receptor antigen M2 is effective to detect treatment response, but it has only been studied in patients under 18 years old. Cytokines and surface markers that characterize host cellular responses need to be further assessed although the ELISPOT for IFN-γ is standardized [41].

Expert commentary & five-year view
Based on current published data, there are certain biomarkers that have shown their effectiveness assessing responses to specific treatment with benznidazole and nifurtimox in different stages of CD. Nucleic acid amplification techniques have demonstrated their effectiveness to assess therapeutic failure. Immunological and biochemical biomarkers have not been fully developed as tools to monitor treatment response, even if they are considered interesting research paths. There is heterogeneity in methodologies and scarce data evaluating specificity and sensitivity of assays using these biomarkers.

In our opinion, the availability of suitable biomarkers would open the door for new drugs with better tolerance profiles and greater efficacy in clinical trials. Standardized methods for evaluation of diagnostic tools (specificity, sensitivity, precision and reproducibility) are currently needed to improve biomarker assessment and develop new markers and to motivate research on CD diagnosis and treatment.

The present article reviews the landscape of existing evidence in biomarkers of chronic CD, and specific needs, to develop a battery of complementary biomarkers which together fulfill ideal or acceptable criteria to evaluate response to treatment for chronic CD. Currently, there is no published data to support the use of a single biomarker to monitor treatment efficacy. DNA amplification techniques and other marker candidates show promise and are currently being tested in different population groups. New studies are necessary to improve assessment of existing markers and to identify those that could be useful for early follow-up of treated patients.

Key issues

- There is a lack of biomarkers for early therapeutic response to antitrypanocidal drugs.
- Accurate and early biomarkers that assess the effectiveness of new drugs or which are useful for management of patients with Chagas CD are needed.
- Certain biomarkers have shown their effectiveness to assess treatment response in different CD stages including DNA amplification techniques.
- There is heterogeneity of treatment response and therapeutic failure biomarkers available.
- Data suggest that no current biomarker may be sensitive enough to be used as a single tool to monitor the efficacy of a trypanocidal treatment.
- Early surrogate markers of therapeutic response in chronic CD should be defined following quality criteria. Using the target product profile model, acceptable and ideal characteristics for a biomarker are proposed to evaluate response to treatment in patients with chronic CD.

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