

Circulating Serum Markers and QRS Scar Score in Chagas Cardiomyopathy

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Abstract. Approximately 8 million people have *Trypanosoma cruzi* infection, and nearly 30% will manifest Chagas cardiomyopathy (CC). Identification of reliable early indicators of CC risk would enable prioritization of treatment to those with the highest probability of future disease. Serum markers and electrocardiogram (EKG) changes were measured in 68 *T. cruzi*-infected individuals in various stages of cardiac disease and 17 individuals without *T. cruzi* infection or cardiac disease. *T. cruzi*-infected individuals were assigned to stage A (normal EKG/chest x-ray [CXR]), B (abnormal EKG/normal CXR), or C (abnormal EKG/cardiac structural changes). Ten serum markers were measured using enzyme-linked immunosorbent assay (ELISA)/Luminex, and QRS scores were calculated. Higher concentrations of transforming growth factor- β 1 (TGF β 1), and TGF β 2 were associated with stage B compared with stage A. Matrix Metalloproteinase 2 (MMP2), Tissue Inhibitors of MMP 1, QRS score, and Brain Natriuretic Protein rose progressively with increasing CC severity. Elevated levels of several markers of cardiac damage and inflammation are seen in early CC and warrant additional evaluation in longitudinal studies.

INTRODUCTION

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*. Endemic transmission by triatomine vectors occurs in many Latin American countries, where an estimated 8–11 million people are currently infected.¹ Although many infected individuals are asymptomatic, approximately 20–30% will progress to clinically significant heart disease, with onset occurring years to decades after the initial infection.^{1,2} Early cohort studies in highly endemic areas showed 2–3% annual incidence of cardiac disease,^{3,4} and a recent retrospective cohort analysis of blood donors in Brazil showed a 1.85% annual cardiomyopathy incidence attributable to *T. cruzi* over a 10-year period.⁵

The earliest signs of Chagas cardiomyopathy (CC) are typically conduction system abnormalities, with subsequent progression to ventricular arrhythmias, dilated cardiomyopathy, apical aneurysm, and thrombus formation.^{6,7} Ventricular arrhythmias and left ventricular dysfunction are strongly associated with premature mortality risk in infected individuals but relatively late indicators of cardiac dysfunction.⁸

The pathogenesis of CC is thought to occur through inflammation caused by parasite persistence, which over time, leads to fibrotic changes.^{9,10} The literature suggests several molecules, such as Brain Natriuretic Protein (BNP), that might rise with CC progression; most are known to be associated with dilated cardiomyopathy and/or congestive heart failure of other etiologies.¹¹ Matrix Metalloproteinase 2 (MMP2), MMP9, and Tissue Inhibitors of MMP 1 (TIMP1) have been linked to matrix remodeling in heart failure, cardiomyopathy, and other cardiac diseases.^{12–14} MMPs activate transforming growth factor- β (TGF β), an important regulator of inflammation, and TGF β 1 has been shown to play a central role in the

pathogenesis of *T. cruzi* heart disease in animal models.^{15–17} Serum TGF β correlates well with CC severity in humans¹⁸ and induces expression of Connective Tissue Growth Factor (CTGF), a secreted profibrogenic cytokine that is correlated with increasing diastolic dysfunction in humans by promoting myofibroblast differentiation and extracellular matrix synthesis.^{19–21} Procollagen Type I C-terminal Propeptide (PICP) is formed when procollagen is cleaved in the process of forming fibrils. In heart disease patients, PICP levels correlate with severity of myocardial fibrosis.²² Procollagen Type III N-terminal Propeptide (PIINP) reflects collagen synthesis and extracellular matrix turnover, and it was found to correlate with cardiac damage in an experimental guinea pig model.²³ Taken together, this body of work suggests that the presence of serum molecules elicited by escalating cardiac damage could function as an early biomarker for CC.

In addition to serum molecules, we also calculated a QRS scar score from each subject's EKG. This score summarizes EKG abnormalities based on Q-, R-, and S-wave durations, amplitudes, and morphologies following published methods of calculation, with modifications in the presence of conduction system abnormalities.²⁴ Each point corresponds to scarring of 3% of the left ventricle.²⁵ In patients with CC, the QRS score has been shown to provide an accurate estimate of myocardial scar size as measured by cardiac magnetic resonance imaging (MRI) and shows a correlation with history of ventricular tachycardia and worsening heart failure symptoms.²⁶ Our objective was to investigate the association of these serum proteins and QRS scar score with presence and severity of CC to identify promising markers for future rigorous evaluation.

METHODS

Ethics statement. This protocol was approved by the institutional review boards of the Centers for Disease Control and Prevention (CDC; Atlanta, GA), Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura (Lima, Peru), and Hospital Universitario Japonés (Santa Cruz,

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Bolivia). All participants provided written informed consent as well as assent and parental consent for children less than 18 years old. Both adults and children participated in the initial epidemiological study, but only adults 17 or older are included in this analysis. All research activities adhered to the principles expressed in the Declaration of Helsinki.

Study population. Blood specimens for this analysis came from two studies: a community-based survey in the Bolivian Chaco and a hospital-based study of heart disease in Santa Cruz, the closest large city to the Chaco.^{27,28} The Chaco is a sparsely populated, hot, and semiarid lowland region that encompasses parts of Bolivia, Argentina, Paraguay, and Brazil that has the highest prevalence of *T. cruzi* infection in the world.^{28,29} Serum samples from adults in the community study were eligible for inclusion if confirmed *T. cruzi* infection status and complete EKG and chest x-ray (CXR) data were available. Individuals from the hospital-based study in Santa Cruz were positive for *T. cruzi* by serology, had complete EKG and CXR data, and were diagnosed with New York Heart Association (NYHA) class III or IV heart disease.²⁷ In total, 85 individuals (64 individuals from the community study and 21 individuals from the hospital study) were included in this study.

Sample collection. A 6- to 12-mL blood specimen was obtained from each participant using clot-activator tubes (BD Vacutainer, Franklin Lakes, NJ). The samples were transported to the nearby Camiri Hospital Laboratory on ice, centrifuged, and separated into serum and clot using standard protocol. Sera were immediately stored at -20°C . Serum specimens were then stored at the Division of Parasitic Diseases and Malaria, CDC Laboratory for use in enzyme-linked immunosorbent assay (ELISA) and Luminex as described below.

***T. cruzi* diagnosis.** *T. cruzi* serologies were determined by the indirect hemagglutination assay (Chagas Polychaco Kit; Lemos Laboratories, Buenos Aires, Argentina) and Chagatest parasite lysate ELISA (Wiener Laboratories, Rosario, Argentina). A third assay, either the Chagatest Recombinante 3.0 ELISA (Wiener Laboratories, Rosario, Argentina) or the immunofluorescence antibody test with a titer of 1:32 as the positive cutoff, was performed on samples with discordant results. A subject was considered to have confirmed *T. cruzi* infection if the serum specimen showed positive results by at least two serological assays.³⁰ Individuals with discordant serological results that could not be resolved were excluded from this analysis.

Electrocardiographic analysis and QRS scoring. All patients underwent standard 12-lead EKGs, which were read by two different cardiologists. A third reader was used for EKGs with discordant findings. EKG analysis was performed

blinded to all clinical data except age and gender, which are considered when performing QRS scoring. QRS scar scoring was calculated following published methods.²⁴

Clinical classification. The American College of Cardiology/American Heart Association (ACC/AHA) heart failure classification system was applied to all patients with available CXRs.³¹ For *T. cruzi*-infected patients, we also assigned a severity classification based on a slight modification of published methods.^{27,32} An abnormal EKG was defined by the presence of right bundle branch block (RBBB), left anterior fascicular block (LAFB), left posterior fascicular block (LPFB), left bundle branch block (LBBB), incomplete RBBB, atrioventricular block (AVB), junctional rhythm, multifocal or paired ventricular pre-mature beats, atrial fibrillation or flutter, or bradycardia ($< 50/\text{minute}$). Based on *T. cruzi* serologic results and EKG and CXR findings, individuals were categorized into four stages: (1) uninfected control: *T. cruzi*-negative with normal EKG and CXR; (2) stage A: *T. cruzi*-positive with normal EKG and CXR; (3) stage B: *T. cruzi*-positive with abnormal EKG findings but normal heart size on two-dimensional (2D) echo or CXR and no signs of congestive heart failure; and (4) stage C: *T. cruzi* infection with abnormal EKG findings plus indication of increased left ventricular size and/or decreased left ventricular ejection fraction on CXR and/or 2D echo. EKG changes found in stages B and C participants are listed in Supplemental Table 1.

Analysis of serum markers. Ten serum molecules were measured. TGF β 1 and TGF β 2 were tested using TGF β 1 and TGF β 2 Quantikine ELISAs (TGF β 1 sensitivity = 15.4 pg/mL, interassay coefficient of variation [%CV] = 5.7–8.4; TGF β 2 sensitivity = 7 pg/mL, interassay %CV = 4.3–5.0; R&D Systems, Minneapolis, MN), respectively. CTGF was tested using the Human CTGF “Super-X” Pre-Coated ELISA Kit (sensitivity = 40 pg/mL, %CV < 10 ; Antigenix, Huntington Station, NY). MMP2 and MMP9 were tested using the Fluorokine MAP Multiplex Human MMP Panel Base Kit (MMP2 sensitivity = 12.6 pg/mL, interassay %CV = 10.0–13.3; MMP9 sensitivity = 13.7 pg/mL, interassay %CV = 9.3–11.7), and TIMP1 was tested using the Human Fluorokine MAP Base Kit, Cardiac Panel B (sensitivity = 5.05 pg/mL, interassay %CV = 7.1–12.6; both from R&D Systems, Minneapolis, MN). BNP was tested using BNP Human ELISA (sensitivity = 1.02 pg/mL, interassay %CV < 15 ; RayBiotech, Norcross, GA). Mannose Binding Lectin (MBL) was tested using the MBL Human ELISA Kit (sensitivity = 0.41 ng/mL, interassay %CV < 7.4 ; Hycult Biotech, Plymouth Meeting, PA). Finally, PICP and PIIINP were tested using ELISA kits (sensitivity = 0.50 ng/mL, interassay %CV < 10 ; Mybiosource, San Diego, CA).

TABLE 1
Demographic characteristics and EKG and CXR findings

	Uninfected controls (N = 17)	<i>T. cruzi</i> -infected participants		
		Stage A (N = 21)	Stage B (N = 20)	Stage C (N = 27)
Median age (IQR)	18 (17–20)	33 (26–37)	39 (33–44)	50 (46–57)
Female, n (%)	12 (71)	18 (86)	14 (70)	12 (44)
Heart rate median (IQR)*	71 (65–77)	67 (63–71)	68 (59–74)	68 (57–81)
PR interval median (IQR)†	153 (150–160)	155 (148–160)	157 (129–166)	182 (151–192)
QRS duration median (IQR)	85 (82–90)	89 (83–92)	143 (120–158)	124 (112–154)
CTI median (IQR)‡	45.9 (43.1–48.6)	45.5 (43.0–46.4)	44.5 (41.6–46.6)	57.7 (57.1–57.9)

* In beats per minute.

† In milliseconds.

‡ Cardiothoracic index (CTI) in percentage.

IQR = interquartile range; PR.

Statistical analysis. Measured values of each marker were log-transformed. Statistical significance of the differences across all four disease groups was determined by Kruskal–Wallis test. Wilcoxon rank sum tests were used to compare pair-wise differences in the median value of each marker between two disease stages. Multinomial logistic regression was used to calculate relative odds ratios (RORs) and 95% confidence intervals for the association of serum marker concentration and QRS scores with disease stage. For regression analysis, marker values were dichotomized at the median value for stage A and treated as binary. Stage A was used as the reference group. Wald’s test was used to determine the difference in post-estimation RORs between stages B and C. All models were adjusted for age. A $P < 0.05$ was considered significant. All analyses were performed using STATA 12.1 (College Station, TX).

RESULTS

Demographic characteristics, EKG, and CXR findings. The median age increased significantly with progressive cardiac stage, and females outnumbered males in all groups except stage C (Table 1). QRS duration and cardiothoracic index increased significantly with progressive cardiac stage.

Differences in QRS score and serum protein concentrations by disease stage. Compared with *T. cruzi*-negative controls, *T. cruzi*-infected individuals in stage A had lower concentrations of MMP2 ($P = 0.020$) and TIMP1 ($P = 0.039$) and higher concentrations of TGFβ2 ($P = 0.008$) (Table 2). Of 11 markers considered, 6 markers showed significant differences when all CC severity groups were compared. QRS score ($P < 0.001$), BNP ($P < 0.001$), MMP2 ($P < 0.001$), and TIMP1 ($P < 0.001$) were higher among those in stage C compared with those in stage A. TGFβ1 ($P = 0.003$) and TGFβ2 ($P < 0.001$) concentrations were significantly higher among those in stage B compared with stage A. Individuals in stage B had higher QRS scores ($P = 0.001$) and higher concentrations of MMP2 ($P = 0.013$), TIMP1 ($P = 0.003$), TGFβ1 ($P = 0.023$), and TGFβ2 ($P < 0.001$) compared with those in stage A. Furthermore, individuals in stage C had higher QRS scores compared with those in stage B ($P < 0.001$), whereas TGFβ1 and TGFβ2 concentrations were significantly lower in those in stage C compared with stage B patients ($P = 0.002$ and $P < 0.001$, respectively).

Logistic regression analysis. In multinomial logistic regression models, higher QRS scores were associated with stages B ($P = 0.005$) and C ($P = 0.008$) compared with stage A (Table 3). Higher concentrations of serum BNP also were associated with stages B ($P = 0.018$) and C ($P = 0.042$). Higher concentrations of MMP2 ($P = 0.025$), TIMP1 ($P = 0.016$), TGFβ1 ($P = 0.011$), and TGFβ2 ($P = 0.004$) were associated with stage B only compared with stage A. The RORs for the association of TGFβ1 and TGFβ2 with stage B compared with stage A were significantly higher than the RORs for the association of these markers with stage C compared with stage A (Wald’s $P = 0.022$ and $P = 0.007$, respectively).

DISCUSSION

The most feared consequence of *T. cruzi* infection is CC, which is estimated to occur in 20–30% of infected individuals.^{6,8} The current norms of the Bolivian national program include antitrypanosomal treatment only up to age 15 years. However, the impetus for adult treatment has grown in the

TABLE 2
Candidate biomarker levels among study participants by severity of CC

Biomarker	Median (interquartile range)				P value*			
	Uninfected controls (n = 17)	Stage A (n = 21)	Stage B (n = 20)	Stage C (n = 27)	Control vs. A	A vs. B	B vs. C	A vs. C
QRS score	3 (0–9)	0 (0–3)	6 (3–9)	15 (9–24)	0.099	0.001	0.003	< 0.001
BNP	1,183 (640–1,337)	661 (327–1,586)	1,289 (1,074–1,903)	2,845 (2,209–4,007)	0.849	0.183	0.001	< 0.001
MBL	95,461 (57,354–174,577)	144,440 (88,698–284,726)	184,568 (111,405–228,773)	165,263 (95,924–285,029)	0.271	0.638	0.561	0.795
MMP2	285,349 (213,954–371,903)	216,859 (192,329–264,988)	277,556 (234,829–330,336)	324,623 (296,421–666,490)	0.020	0.013	0.089	< 0.001
MMP9	481,996 (332,814–749,818)	361,305 (239,520–708,749)	375,039 (197,756–591,389)	238,169 (117,153–379,862)	0.202	0.375	0.204	0.045
TIMP1	185,370 (137,014–201,683)	151,800 (131,475–181,649)	199,684 (170,818–208,972)	257,752 (183,586–319,857)	0.039	0.003	0.102	< 0.001
PIGP	1,937 (1,110–2,651)	1,644 (1,354–1,895)	1,913 (1,591–2,183)	1,829 (1,343–2,655)	0.918	0.076	0.914	0.228
PIIINP	154 (103–333)	241 (190–715)	430 (188–615)	420 (9–843)	0.297	0.794	0.738	0.975
TGFβ1	29,513 (27,935–33,227)	28,799 (22,881–38,591)	36,135 (32,247–40,875)	23,479 (19,455–35,431)	0.547	0.023	0.002	0.069
TGFβ2	458 (348–689)	687 (554–824)	1,186 (925–1,327)	261 (176–413)	0.008	< 0.001	< 0.001	0.001

* Wilcoxon rank sum test. Serum biomarker concentrations are in picograms per milliliter.

TABLE 3
Multinomial logistic regressions and RORs of biomarker percentile category change

Median value*	N (%)†			ROR (95% confidence interval)				P value‡ (B vs. C)
	Stage A (N = 21)	Stage B (N = 20)	Stage C (N = 27)	B vs. A	P value	C vs. A	P value	
QRS score								
0	13 (62)	3 (15)	2 (8)	1		1		
> 0	8 (38)	17 (85)	24§ (92)	9.78 (2.01, 47.5)	0.005	32.6 (2.52, 421)	0.008	0.349
BNP								
≤ 661	11 (52)	3 (15)	1 (4)	1		1		
> 661	10 (48)	17 (85)	26 (96)	6.50 (1.38, 30.6)	0.018	11.9 (1.09, 129)	0.042	0.631
MMP2								
≤ 216	11 (52)	4 (20)	4 (15)	1		1		
> 216	10 (48)	16 (80)	23 (85)	5.38 (1.23, 23.5)	0.025	5.09 (0.85, 30.5)	0.075	0.951
MMP9								
≤ 361	11 (52)	9 (45)	18 (67)	1		1		
> 361	10 (48)	11 (55)	9 (33)	1.41 (0.39, 5.06)	0.602	0.89 (0.19, 4.24)	0.891	0.535
TIMP1								
≤ 151	11 (52)	2 (10)	3 (11)	1		1		
> 151	10 (48)	18 (90)	24 (89)	8.34 (1.49, 46.6)	0.016	1.94 (0.28, 13.5)	0.504	0.198
TGFβ1								
≤ 28	11 (52)	3 (15)	18 (67)	1		1		
> 28	10 (48)	17 (85)	9 (33)	7.91 (1.61, 39.0)	0.011	1.15 (0.22, 5.95)	0.864	0.022
TGFβ2								
≤ 687	11 (52)	3 (15)	18 (67)	1		1		
> 687	10 (48)	17 (85)	9 (33)	30.3 (2.92, 314)	0.004	1.28 (0.24, 6.97)	0.773	0.007

* All median values are in picograms per milliliter except for QRS score.

† The number and percentage of participants with biomarker values less than or equal to and greater than the median value for stage A.

‡ P value comparing the difference in the ROR for the association of each biomarker with stage B versus stage C.

§ QRS score was not calculable for one patient with a ventricular pacemaker.

Stage A is the referent for all analyses.

last decade because of patient demand and data suggesting that treatment, even in patients with early cardiac morbidity, decreases CC progression and possibly, mortality.^{33–35} A clinical trial is currently underway to assess benznidazole efficacy to prevent or slow progression in adults with early signs of CC.³⁶ However, in the absence of an effective algorithm to prioritize those at high risk of progression to CC, adult treatment will remain beyond the resources of poorer countries. With the goal of identifying preliminary markers to identify individuals at risk of progression to CC, this study evaluated the association of 10 serum markers and the QRS scar score with presence and severity of CC. We found that there were higher concentrations of MMP2, TIMP1, TGFβ1, and TGFβ2 in *T. cruzi*-positive individuals with early cardiac structural changes (stage B) compared with those with normal EKGs (stage A). MMP2, TIMP1, QRS score, and BNP were also higher in patients with late-stage disease compared with those with early or no cardiac findings.

This study found that QRS score was associated with specific CC disease stage, which is consistent with the progressive fibrosing myocarditis that is the main pathological finding in CC.⁹ Focal myocarditis is found even in early stages and intensifies as the disease progresses. The loss of cardiomyocytes and substitution of fibrotic tissue induce disruption of muscle fibers, which leads to dysfunction of cardiac muscle tissue and conduction pathways. This pathophysiology explains the tendency of Chagas disease patients to develop heart failure, conduction system defects, and ventricular arrhythmias.^{9,37} QRS scar score may, therefore, be useful as a yardstick to differentiate between serum markers that indicate existing structural abnormalities and those that may suggest future cardiac disease. BNP and QRS scar score both increased across cardiac severity groups.¹¹ These two markers may simply represent the amount of cardiac damage that has already occurred.

We found lower MMP2 and TIMP1 among those with stage A disease compared with the uninfected control group followed by increasingly elevated levels in stages B and C. The higher levels seen in those with heart disease may reflect cardiac remodeling occurring in response to inflammatory damage to heart tissue.^{38,39} However, longitudinal follow-up will be necessary to determine if these serum proteins have predictive value.

TGFβ1 and TGFβ2 concentrations were highest among those individuals with stage B disease. TGFβ1 has been independently associated with hospitalization and mortality caused by heart failure or arrhythmias in patients with CC,⁴⁰ but TGFβ2 has not been widely studied. TGFβ is an important regulator of inflammation, acting as a suppressor at high concentrations.^{15–17} TGFβ has been identified as acting at several points in the *T. cruzi* lifecycle and pathogenesis of CC.¹⁵ The TGFβ signaling pathway is involved in parasite invasion into mammalian cells and the transformation of intracellular amastigotes into trypomastigotes, and there is evidence suggesting that the parasite causes up-regulation of the pathway.¹⁵ At the time of transformation, blocking TGFβ signaling enhances parasite apoptosis, suggesting a parasite-protective role for TGFβ.⁴¹ These effects operate during the earlier stages of *T. cruzi* infection and may explain the elevation seen in stage B. In the chronic phase, TGFβ is involved in cardiac fibrosis.¹⁵ However, TGFβ plays a similar role in other cardiac fibrotic conditions and is not specific to *T. cruzi*-induced cardiac damage.⁴² In immunological studies of CC, the phenomenon of immune exhaustion has been reported (seen for inflammatory cytokines such as interferon-γ) and may possibly explain the decrease in TGFβ levels from stage B to C.⁴³ In previously published human data, TGFβ levels rose from the asymptomatic category to the category for mild CC but fell slightly from the mild to the more severe class of CC.¹⁸

This study had several limitations. First, the lack of an uninfected group with heart disease precluded evaluation of the specificity of the markers for CC. Because > 90% of adults in the community study site and > 80% of advanced cardiac patients in the hospital study were infected with *T. cruzi*,^{27,28} it was difficult to find uninfected patients with heart disease. Second, the relatively small sample size of this study limited the ability to observe differences in marker levels across different disease states. Third and most important, this study was cross-sectional and therefore, cannot address the temporal relationship between marker concentrations and disease outcomes. Future studies to confirm the use of the markers identified in this study will use a longitudinal design to evaluate the predictive ability of each marker for development of CC.

Nevertheless, our data represent a preliminary step in the search for a marker to predict progression from the indeterminate form of Chagas disease to CC. Markers of fibrosis and tissue remodeling (QRS score, BNP, MMP2, and TIMP1) likely indicate advancing cardiac structural change. The elevated concentrations of immunoregulatory markers (TGFβ1 and TGFβ2) among those in stage B show promise as clinically useful indicators of eventual development of CC. This information will be particularly important in the countries where Chagas disease is most prevalent and medical resources are limited.

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