

# Detection of *Trypanosoma cruzi* DNA in blood by PCR is associated with Chagas cardiomyopathy and disease severity

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| <b>Background</b> | The significance of detection of <i>Trypanosoma cruzi</i> DNA in blood of antibody-positive patients for risk of development of Chagas heart disease is not well established. The objective of this study was to compare detection of <i>T. cruzi</i> DNA with known clinical and laboratory markers of Chagas cardiomyopathy (CC) severity.   |
| <b>Methods</b>    | This is a case–control study nested within a retrospective cohort developed in Brazil to understand the natural history of Chagas disease. The study enrolled 499 <i>T. cruzi</i> seropositive blood donors (SP-BD) and 488 frequency matched seronegative control donors (SN-BD) who had donated between 1996 and 2002, and 101 patients with clinically diagnosed CC. In 2008–2010 all enrolled subjects underwent a health questionnaire, medical examination, electrocardiograms and echocardiograms and polymerase chain reaction (PCR) analyses. A blinded panel of three cardiologists adjudicated the outcome of CC. <i>Trypanosoma cruzi</i> kinetoplast minicircle sequences were amplified by real-time PCR using an assay with a sensitivity of one parasite per 20 mL of blood. All testing was performed on coded samples. |
| <b>Results</b>    | Rates of PCR detection of <i>T. cruzi</i> DNA were significantly ( $P=0.003$ ) higher in CC patients and SP-BD diagnosed with CC (79/105 [75.2 %]) compared with SP-BD without CC (143/279 [51.3%]). The presence of parasitaemia was significantly associated with known markers of disease progression such as QRS and QT interval duration, lower left ventricular ejection fraction, higher left ventricular index mass, and elevated troponin and NTpro-BNP levels.   |
| <b>Conclusion</b> | <i>Trypanosoma cruzi</i> PCR positivity is associated with presence and severity of cardiomyopathy, suggesting a direct role of parasite persistence in disease pathogenesis.  |
| <b>Keywords</b>   | <i>Trypanosoma cruzi</i> • PCR • cardiomyopathy  |

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## Introduction

Chagas disease is caused by the parasite *Trypanosoma cruzi*, which infects up to 10 million people in Latin America, causing approximately 12 500 deaths annually. The evolution from the time of infection to Chagas cardiomyopathy (CC) and/or the megacolon and megaesophagus syndromes occurs over 10–20 years in a slow but progressive fashion, although only in a proportion of chronically infected individuals.<sup>1–4</sup>

The pathogenic mechanisms of CC are not fully understood and the importance of parasitaemia for disease progression is poorly defined.<sup>1–3,5</sup> The parasite can be detected in blood by classical methods such as haemoculture and xenodiagnosis, but the sensitivities of these techniques are low, they require fresh blood and are expensive, and hence not practical for large-scale research or clinical applications. Non-commercial polymerase chain reaction (PCR) assays have been developed but have been difficult to optimize and standardize, and discrepant results are common when techniques are compared.<sup>6,7</sup> The lack of a reliable assay to detect and quantify parasitaemia is an obstacle to advancing our understanding of the impact of persistent parasitaemia on the natural history of the disease as well as to characterization of parasite load for prognostic and therapeutic assessments.<sup>6,7</sup>

We recently developed a target-capture real-time PCR assay that can detect as few as one parasite per 20 mL of processed blood, has a high degree of specificity and yields reproducible quantitative results across a wide dynamic range of parasite load.<sup>8</sup> We describe here the results obtained employing this assay to detect and quantify parasitaemia in blood specimens from a well-characterized retrospective cohort of *T. cruzi* seropositive blood donors (SP-BD), frequency matched seronegative control donors (SN-BD), and CC clinical cases developed as part of the National Heart, Lung and Blood Institute (NHLBI) Retrovirus Epidemiological Donor Study-II (REDS-II).<sup>9,10</sup> The aim of this analysis was to compare the presence and the levels of parasitaemia in blood and assess if parasitaemia is associated with the development of Chagas cardiomyopathy and with clinical parameters and laboratory markers of severity of cardiac disease. In addition, the study addressed the controversial issue of whether serosilent *T. cruzi* infections exist<sup>11–13</sup> by performing sensitive PCR analysis of samples from ~500 matched SN-BD.

## Methods

### Ethics

The study follows the Declaration of Helsinki of the Ethical Principles for Medical Research Involving Human Subjects, was approved by the Brazilian National Ethical Committee (CONEP# 1312/2006), and all subjects gave written informed consent.

### Study design

This is case–control study nested within a retrospective cohort that enrolled 499 *T. cruzi* SP-BD identified by blood bank screening in 1996–2002 (255 from the city of São Paulo and 244 from the city of Montes Claros in the State of Minas Gerais, Brazil) and 488 SN-BD

who were frequency matched to the SP-BD by site, donation date (year), age, and gender. This retrospective blood donor cohort was supplemented by parallel enrolment and evaluation of 101 previously diagnosed cases of CC from the Heart Institute of the University of São Paulo Medical School; inclusion criteria included a physician diagnosis of CC, confirmed *T. cruzi* seropositivity, no previous treatment with benznidazole, and no co-morbidities such as diabetes, hypertension, or renal failure. These individuals were recruited by letter and telephone call using the blood centre and hospital databases. From July 2008 to October 2010, recruited individuals (BD and CC patients) underwent standardized health questionnaires and medical evaluations including electrocardiogram (ECG), echocardiogram (ECHO), and phlebotomy with processing and cryopreservation of samples for subsequent batched blinded analyses of cardiac markers, PCR, and other biomarkers in the USA (see below).

Results of ECG and ECHO were reviewed blindly by centralized reading centres in the USA. All data were centralized by the REDS-II Data Coordinating Center (Westat). A pre-defined set of abnormalities in the ECHO or ECG measurements triggered the expert panel composed of three Brazilian cardiologists to review cardiac findings blinded to the subject's serostatus.<sup>10</sup> The expert panel was asked to reach a consensus regarding the following question 'If this patient were seropositive for *T. cruzi* how would you classify them: definite CC, probably CC, possible CC, or no CC.<sup>10</sup> The definition used by the expert panel were defined by an expert consensus and can be accessed at <http://circ.ahajournals.org/content/suppl/2013/02/07/CIRCULATIONAHA.112.123612.DC1.html>. Further details of the cohort procedures and rates and clinical correlates of CC have been reported previously.<sup>10</sup> The local physician also received all test results and counselled the participants when necessary.

## Blood processing and PCR procedures

At the time of interviews and medical examinations 20 mL of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood was collected from each enrolled subject and was immediately mixed with an equal volume of 6 M guanidine HCl–0.2 M EDTA solution. The guanidine–EDTA blood mixture was maintained at room temperature until boiled for 15 min, followed by vortexing and aliquoting (1.0 mL). Aliquots were frozen in Brazil at –20 °C until shipped to the US REDS-II Central Laboratory (BSRI) on dry ice, followed by maintenance at –70 °C.

The target-capture (TC) real-time (RT) PCR assay used in this study<sup>8</sup> was developed based on the PCR method described by Virreira *et al.*<sup>14</sup> that targets kinetoplast minicircle *T. cruzi* DNA. The DNA extraction was improved through use of a TC step that employed magnetic beads coated with a *T. cruzi*-specific 20-mer capture oligonucleotide (CaptureTc\_121 AAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAATAATGTACGGGKGAGATGCATGA; CaptureTc\_122 GGTTCGATTGGGGTTGGTGTAAATATAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAA; CaptureTc-S35 AAAAAAAAAAAAAAAAAAAAAA AAATAATGTACGGGKGAGATGCATG; CaptureTc-S36 GGGTTCGATTGGGGTTGGTGTAAAAAAAAAAAAAAAAAAAAAAA). Four replicate TC-RT-PCR assays were performed and results interpreted as positive if two to four of four replicates were found to have specific products and negative if one or zero replicates yielded specific product. As previously reported,<sup>8</sup> this TC-RT-PCR assay can detect a single parasite spiked into a 20 mL blood sample with a mean cycle threshold (CT) of 35 cycles, relative to the cut-off CT value of

40 cycles. The sensitivity of the assay was validated by spiking a serial dilution of lysed *T. cruzi* parasites into 20 mL of blood. The blood was heated at 95°C, aliquoted and frozen at -20°C. Each dilution was tested in quadruplicate. The CT values were lower (reflecting increased kinetoplast target DNA levels before amplification) for samples with higher input parasite concentrations, with a linear increase in CT values with the serial dilutions, indicating capacity to quantify parasite load in clinical samples. Quantification of parasitaemia was based on the difference between the  $C_T$  of a well-characterized positive run control and the average  $C_T$  of the sample ( $n = 4$ ). The test was performed on coded samples so the testing laboratory (BSRI, San Francisco, CA, USA) was blinded to the serological and clinical status of the donors and CC patients, with coded results including categorical positivity/negativity and parasite concentrations for positive samples sent to the data coordinating centre (Westat) for decoding of results and analysis.

## Other measurements

Resting 12-lead ECGs were recorded using the same model of machine at both sites (General Electric MAC 1200 electrocardiograph; GE Healthcare, Waukesha, WI, USA) using standardized procedures. All ECGs were processed blindly by the central ECG laboratory (Epidemiological Cardiology Research Center, Wake Forest University, Winston-Salem, NC, USA). The ECGs were analysed electronically, with manual over-reading by trained cardiologists to ensure quality control, and were classified by Minnesota code criteria using variables that were derived from the median complex of the Marquette measurement matrix.<sup>15,16</sup>

Echocardiogram studies were performed using a Sequoia 512 ultrasound instrument (Acuson, Mountain View, CA, USA) at the Sao Paulo site and by GE Vivid3 (GE Healthcare, Waukesha, WI, USA) at the Montes Claros site. Cardiac measurements were performed according to the guidelines of the American Society of Echocardiography.<sup>17</sup> Studies were recorded in digital format and all measurements were performed on digital loops using a Digisonics offline analysis station (version 3.2 software; Digisonics, Houston, TX, USA) at the Cardiovascular Branch, Echocardiography Laboratory, National Heart, Lung, and Blood Institute (Bethesda, MD, USA). The left ventricular (LV) ejection fraction was calculated based on modified form of Simpson's biplane method.<sup>17</sup>

Troponin, CKMB, and NT-proBNP levels in plasma were measured using US FDA cleared assays on the VITROS System (Ortho Clinical Diagnostics, Raritan, NJ, USA).

## Statistical methods

Statistical analyses were conducted using SAS (SAS 9.2; Cary, NC, USA), SPSS (SPSS for Windows 7; SPSS Chicago, MI, USA), and R (Version 2.14; Foundation for Statistical Computing, Vienna, Austria). The associations between dichotomous ECG and ECHO parameters and PCR status (+/-) were tested using logistic regression with case status (SN-BD, SP-BD, CC patients) included as covariates (see Table 1 and the Supporting Information, Table S1). The PCR and cardiac biomarkers differences between SP-BD with CC and SP-BD with no CC were compared using the Wilcoxon-Mann-Whitney test and Fisher's exact test (Table 2). Key ECG, ECHO and laboratory parameters (BNP, Troponin) were compared between total PCR- and total PCR+ SP subjects, between SP-BD who tested PCR- vs. PCR+ and between CC patients who tested PCR- vs. PCR+, all using

multivariate linear regression with case status included as covariates (Figures 1-3). Within all PCR+ samples, the Spearman correlation was calculated to compare the parasite concentrations and continuous ECG, ECHO, and laboratory variables.

## Results

Of the 499 SP-BD, 488 SN-BD and 101 CC patients who participated in the study, TC-RT-PCR results could be obtained for 493 (98.8%), 483 (99.0%) and 101 (100%) subjects, respectively. Of the 493 SP-BD, 48 had history of previous treatment with benznidazole and were excluded from the analyses owing to treatment-induced clearance of parasitaemia (see below). Table 1 summarizes the demographics and ECG and ECHO results from the SN-BD, SP-BD, and CC patients according to their PCR status. The CC patients presented more severe cardiac disease with all ECG and ECHO parameters significantly different from the SP-BD group irrespective of PCR status. Within the SP-BD group the following ECG abnormalities were significantly more common among PCR+ compared with PCR- subjects: complete right bundle branch block ( $p < 0.001$ ), pathological q waves ( $P = 0.02$ ), major isolated ST-T abnormalities ( $P = 0.01$ ), and left anterior hemi-block ( $P = 0.02$ ).

We evaluated if TC-RT-PCR-positivity rates and levels of parasitaemia were associated with clinical and laboratory parameters that are known to be related with severity of CC (Figures 1-3). The figures present the comparison of PCR+ vs. PCR- for all seropositive subjects (BD and patients) as well as for SP-BD and CC patients separately. Positive *T. cruzi* PCR results in the combined group were associated with longer durations of the QRS and QTc (corrected QT using Bazett's formula) intervals (Figure 1), lower LV ejection fractions and higher LV mass index values (Figure 2), and higher troponin and NTpro-BNP blood levels (Figure 3). All of these parameters were also associated with PCR positivity when SP-BD were analysed separately. With the exception of troponin, none of these parameters were associated with PCR status when CC patients were evaluated separately.

Within all PCR positive subjects the parasite concentration in blood samples was weakly but significantly correlated with LV ejection fraction ( $\rho = -0.11$ ,  $P = 0.04$ ) and troponin levels ( $\rho = 0.15$ ,  $P = 0.007$ ) (see the Supporting Information, Figure S1).

Table 2 summarizes the comparison of parasitaemia and clinical diagnosis of CC. In this analysis we have included the SP-BD who reported usage of benznidazole ( $n = 48$ ) before recruitment into the study as a separate group. TC-RT-PCR was positive in 81% of the CC patients and 75% of the SP-BD classified as having CC; these rates were similar and significantly higher than the 51% rate of detection of *T. cruzi* DNA among untreated SP-BD who did not have CC ( $p = 0.003$  for comparison of CC SP-BD [75%] vs. non-CC SP-BD [51%]; and  $P < 0.0001$  for comparison of CC patients [81%] vs. non-CC SP-BD [51%]). Furthermore, *T. cruzi* DNA concentrations in blood of TC-RT-PCR+ subjects appeared to be higher among patients with CC (median 1.8 parasites/20 mL) and SP-BD with CC (median 0.3 parasites/mL), compared with non-CC SP-BD (median 0.05 parasites/mL), although the latter difference (0.3 vs. 0.05) was non-significant ( $P = 0.08$ ). The benznidazole-treated SP-BD had a TC-RT-PCR+ rate of 27%,

**Table 1 Clinical, laboratory, electrocardiogram (ECG) and echocardiogram findings for each study group [seronegative control blood donors (SN-BD), seropositive blood donors (SP-BD), and diagnosed Chagas cardiomyopathy (CC patients)] according to their target capture real time polymerase chain reaction (PCR) status**

| Variables                                    | SN-BD          | SP-BD          |                | CC patients   |               |
|--|----------------|----------------|----------------|---------------|---------------|
|  | PCR- (n = 483) | PCR- (n = 188) | PCR+ (n = 257) | PCR- (n = 19) | PCR+ (n = 82) |
| Clinical, mean ( $\pm$ SD)                   |                |                |                |               |               |
| Age  | 49.5 (10.1)    | 48.4 (10.1)    | 49.1 (10.6)    | 49.2 (6.3)    | 47.8 (7.1)    |
| Diastolic blood pressure, mmHg               | 77.3 (14.6)    | 77.1 (13.5)    | 77.4 (13.6)    | 84.1 (12.3)   | 79.3 (12.4)   |
| Systolic blood pressure, mmHg                | 126.7 (18.7)   | 126.2 (17.6)   | 127.1 (18.9)   | 125.9 (16.5)  | 117.7 (16.8)  |
| Total cholesterol, mg/dL                     | 204.5 (51.3)   | 198.0 (43.6)   | 200.6 (39.7)   | 183.3 (39.5)  | 178.5 (41.8)  |
| Low-density lipoprotein cholesterol, mg/dL   | 126.0 (38.7)   | 122.5 (38.3)   | 121.9 (35.8)   | 112.0 (36.1)  | 107.3 (33.3)  |
| High-density lipoprotein cholesterol, mg/dL  | 48.3 (13.0)    | 49.6 (13.0)    | 50.8 (13.6)    | 47.3 (11.0)   | 50.1 (18.9)   |
| Triglycerides, mg/dL                         | 150.2 (99.0)   | 133.1 (81.0)   | 142.0 (98.1)   | 122.3 (72.6)  | 113.2 (56.7)  |
| Glycaemia, mg/dL                             | 92.7 (26.9)    | 89.0 (20.3)    | 90.9 (23.0)    | 87.5 (14.1)   | 85.0 (11.9)   |
| Body mass index, kg/m <sup>2</sup>           | 27.7 (4.5)     | 26.6 (3.6)     | 26.7 (4.1)     | 27.8 (4.6)    | 25.2 (4.2)    |
| Male, n (%)                                  | 236 (48.9)     | 94 (50)        | 139 (54.1)     | 12 (63.2)     | 48 (58.5)     |
| Sao Paulo site, n (%)                        | 239 (49.5)     | 105 (55.9)     | 146 (56.8)     | 19 (100)      | 79 (96.3)     |
| NYHA functional class > I, n (%)             | 10 (2.1)       | 4 (2.2)        | 8 (3.2)        | 2 (13.3)      | 17 (26.2)     |
| ECG, n (%)                                   |                |                |                |               |               |
| Complete right bundle branch block, n (%)    | 4 (0.8)        | 13 (7)         | 59 (23.2)      | 6 (31.6)      | 38 (46.3)     |
| Pathological q waves, n (%)                  | 8 (1.7)        | 1 (0.5)        | 11 (4.3)       | 3 (15.8)      | 18 (22)       |
| Major isolated ST-T abnormalities, n (%)     | 11 (2.3)       | 8 (4.3)        | 29 (11.4)      | 5 (26.3)      | 16 (19.5)     |
| Frequent ventricular premature beats, n (%)  | 5 (1)          | 3 (1.6)        | 8 (3.1)        | 3 (15.8)      | 10 (12.2)     |
| Atrial fibrillation, n (%)                   | 1 (0.2)        | 0 (0)          | 2 (0.8)        | 0 (0)         | 3 (3.7)       |
| Pacemaker rhythm, n (%)                      | 0 (0)          | 3 (1.6)        | 2 (0.8)        | 4 (21.1)      | 17 (20.7)     |
| Left anterior hemi-block, n (%)              | 11 (2.3)       | 19 (10.2)      | 46 (18.1)      | 6 (31.6)      | 35 (42.7)     |
| Echocardiogram                               |                |                |                |               |               |
| Abnormal wall motion score index (>1), n (%) | 16 (3.4)       | 14 (7.6)       | 33 (13.1)      | 17 (100)      | 69 (95.8)     |
| Abnormal EE' ratio (>15), n (%)              | 62 (12.8)      | 33 (17.6)      | 47 (18.3)      | 8 (42.1)      | 40 (48.8)     |
| Reduced LVEF (<50%), n (%)                   | 18 (3.8)       | 12 (6.4)       | 30 (11.7)      | 17 (100)      | 68 (90.7)     |
| LVED (mean $\pm$ SD), n (%)                  | 45.1 (5.7)     | 45.8 (5.4)     | 46.6 (6.1)     | 61.5 (5.2)    | 61.8 (8.2)    |

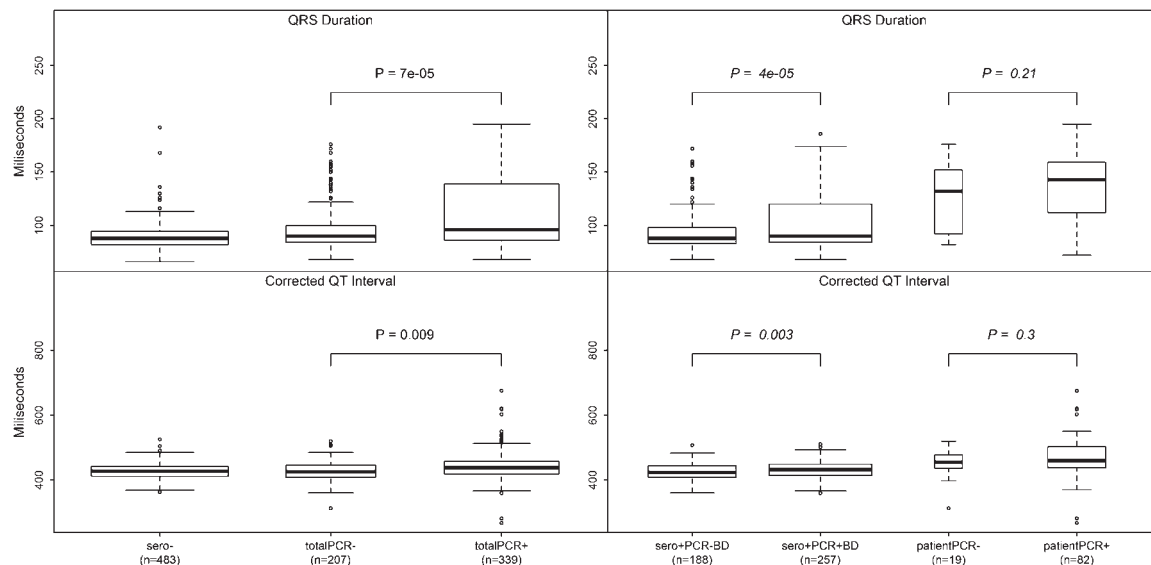
ECG, electrocardiogram; LVEF, left ventricular ejection fraction; LVED, left ventricular end diastolic dimension; NYHA, New York Heart Association.

**Table 2 Results of target-capture real-time PCR (PCR) and cardiac biomarkers according to disease classifications based on expert panel review**

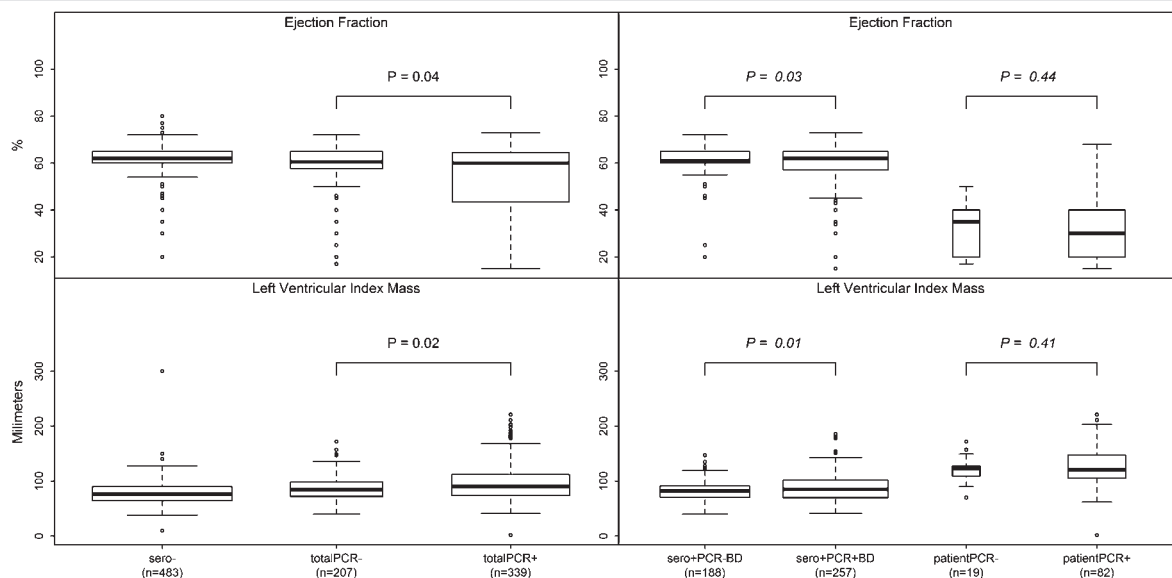
| Group                           | N   | PCR+, n (%) | Parasite concentration, Log/mL: median (interquartile range) | NT-proBNP, pg/mL: median (interquartile range) | Troponin, pg/mL: median (interquartile range) | CKMB, ng/mL: median (interquartile range) |
|---------------------------------|-----|-------------|--|--|---|---|
| CC patients                     | 101 | 82 (81.2)   | 1.77 (0.16–5)  | 746 (357–2223)                                 | 0.022 (0.013–0.040)                           | 1.06 (0.64–1.79)                          |
| SP-BD with CC                   | 105 | 79 (75.2)   | 0.31 (0–2.32)  | 75 (39–186)                                    | <0.012 (<0.012–0.021)                         | 0.85 (0.54–1.27)                          |
| SP-BD inconclusive for CC       | 61  | 35 (57.4)   | 0.08 (0–1.77)  | 56 (40–103)                                    | <0.012 (<0.012–<0.012)                        | 0.78 (0.51–1.44)                          |
| SP BD without-CC                | 279 | 143 (51.3)  | 0.05 (0–1.63)  | 39 (23–65)                                     | <0.012 (<0.012–<0.012)                        | 0.80 (0.48–1.26)                          |
| SP-BD treated with benznidazole | 48  | 13 (27.1)   | 0 (0–0.01)   | 46 (23–76)                                     | <0.012 (<0.012–<0.012)                        | 0.77 (0.44–1.02)                          |
| SN-BD                           | 483 | 0 (0.0)     | NA   | 38 (23–65)                                     | <0.012 (<0.012–<0.012)                        | 0.68 (0.42–1.17)                          |

SP, seropositive; SN, seronegative; BD, blood donors; CC, Chagas cardiomyopathy.

Statistical significance of comparisons of SP-BD with CC vs. SP-BD without CC: PCR positivity  $P = 0.003$ ; parasite concentration  $P = 0.08$ ; NT-proBNP  $P < 0.0001$ ; troponin  $P = 0.003$ ; CKMB  $P = 0.48$ .



**Figure 1** Box plots of electrocardiogram (ECG) variables (QRS and corrected QT intervals). Left: all seropositive subjects were combined according to polymerase chain reaction (PCR) status. Right: each case group [seropositive blood donors (SP-BD) and Chagas cardiomyopathy (CC) patients] were analysed separately.



**Figure 2** Box plots of echocardiogram variables (ejection fraction and left ventricular index mass). Left: all seropositive subjects were combined according to polymerase chain reaction (PCR) status. Right: each case group [seropositive blood donors (SP-BD) and Chagas cardiomyopathy (CC) patients] were analysed separately.

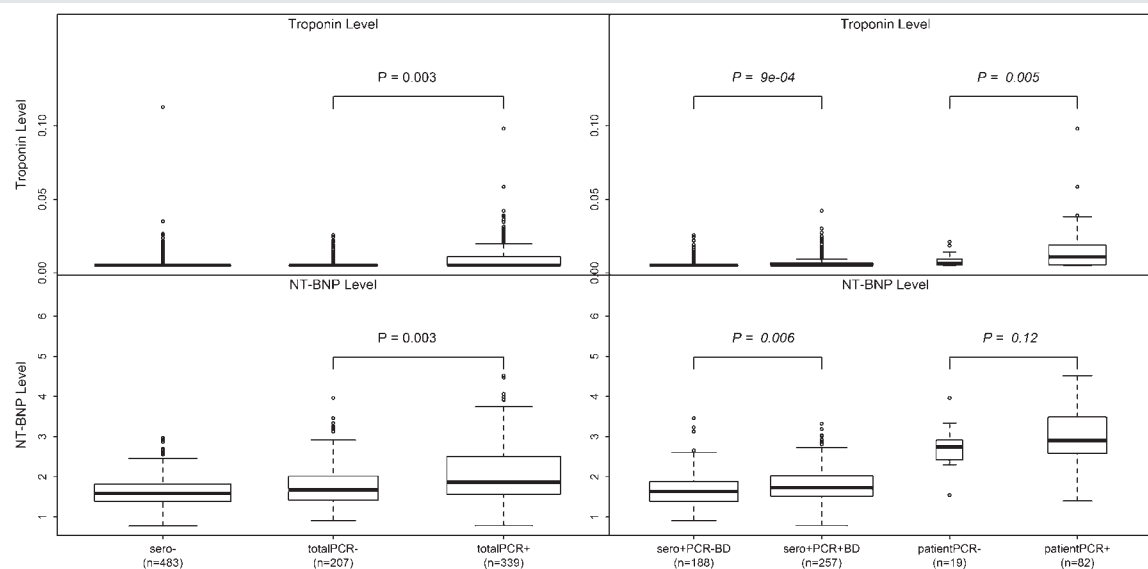
significantly lower than the 51% rate among non-CC non treated SP-BD ( $P=0.003$ ).

None of the 483 samples from SN-BD yielded a positive TC-RT-PCR result on blinded testing with the seropositive samples, indicating high specificity of the TC-RT-PCR assay and absence of serosilent *T. cruzi* infections in the location-, age-, and gender-matched control donors

## Discussion

The lack of a reliable test for *T. cruzi* detection in blood has been an obstacle to investigating the importance of parasite persistence in the pathogenesis of Chagas disease.<sup>5</sup> By testing large-volume blood samples (20 mL) from a well-characterized retrospective cohort of seropositive and control blood donors and clinical CC





**Figure 3** Box plots of troponin and Pro-BNP levels. Left: all seropositive subjects were combined according to polymerase chain reaction (PCR) status. Right: each case group [seropositive blood donors (SP-BD) and Chagas cardiomyopathy (CC) patients] were analyzed separately.

patients using a sensitive and quantitative PCR assay, we found an association between the presence of *T. cruzi* parasites in blood with a diagnosis of CC as well as with parameters of disease severity.

Our data highlight the difficulty of using a cross-sectional sample to detect an association between parasitaemia: the sample size needed to be large, and it was important to include subjects representing the full spectrum of infection and disease in the analyses (SP-BD without CC, less severe cardiomyopathy represented by the SP-BD diagnosed as having CC during the study [74% with LEVF >50%], and severe cases represented by previously diagnosed CC patients [85% with LEVF <50%]). By analysing the groups separately, the association between the detection of *T. cruzi* DNA and CC was only significant among the SP-BD group, suggesting that, in the late stage of the disease, as reflected in the CC patient group, parasite persistence is no longer driving the progression of the clinical/laboratory markers.

Norman *et al.* analysed 246 seropositive Chagas patients from Instituto de Salud Carlos III (Madrid, Spain), and could not find an association between PCR positivity and cardiomyopathy.<sup>18</sup> However, only 20% of the samples tested positive. The smaller sample size and low positive rate probably precluded them from detecting the associations between parasitaemia and disease status and severity observed in our study.

There is only one small prospective cohort study of 56 patients in Argentina who had PCR results at baseline and were followed for approximately 2.5 years.<sup>19</sup> Progression to cardiomyopathy was detected in 12 patients (21.4%) and the relative risk for disease progression was 4.09-fold higher [95% confidence interval (CI) 1.60–9.85] among the patients who tested PCR positive at baseline.<sup>19</sup>

The aetiology of myocardial damage that occurs in at least 30–40% of patients infected by *T. cruzi* is not fully understood.<sup>20</sup>

The parasite is barely detected in the heart by conventional microscopy, which has led some investigators to suggest that the inflammatory process was a consequence of autoimmunity and not a direct response to the presence of the parasite in the heart.<sup>21</sup>

The importance of parasite persistence in the pathogenesis of Chagas disease was suggested by previous experimental studies and clinical studies with small sample sizes. Schijman *et al.*<sup>22</sup> demonstrated an association of parasite level and myocardial inflammation, as detected by PCR, on heart tissue from 21 Chagas patients. This finding was confirmed by Benvenuti *et al.*<sup>23</sup> who analysed heart biopsies from 29 individuals for *T. cruzi* DNA and found 25 to be positive by PCR (immunohistochemistry failed to demonstrate *T. cruzi* antigens in any of the samples, resulting in speculation by critics that the PCR positivity could represent detection of residual DNA from dead parasites). In a murine model of *T. cruzi* infection Zhang and Tarleton<sup>24</sup> used an *in situ* PCR technique to demonstrate correlations between the persistence of parasites and the presence of disease in heart muscle tissue. In a clinical trial with unblinded, non-random assignment, Viotti *et al.*<sup>25</sup> have shown reduced progression of Chagas among benznidazole-treated patients, supporting the hypothesis that parasite persistence is a key element in the pathogenesis of the chronic form of the disease.

Although our study presents data supporting the importance of parasite persistence in the pathogenesis of cardiac disease, we recognize that parasite persistence, as detected in blood, does not fully explain the aetiology of CC; neither is it highly predictive of CC. Approximately 20% of the individuals with cardiomyopathy were PCR– and 50% of seropositive donors without CC tested PCR+, and hence the predictive value of parasite detection in blood for disease progression is limited. Furthermore, the PCR load was only weakly associated with markers of disease severity.

One of the limitations of our study was that PCR was performed at only one time-point, so it is possible that the CC cases that tested PCR– may have intermittent parasitaemia and could test positive if PCR were performed on second or third sample collections.

It is important to acknowledge that our study was based in Brazil where the Tc-II genotype of *T. cruzi* is responsible for the majority of the cases, so the findings may not be generalized to individuals infected by the Tc-I genotype that is more prevalent in Central America.<sup>5</sup> Different strains of the *T. cruzi* have also been associated with distinct clinical outcomes in experimental and clinical models.<sup>20</sup> Another limitation of our study was that PCR was performed at the time of the diagnosis of cardiomyopathy, and not at the time of blood donation, so we cannot infer how well PCR results would predict subsequent disease development.

An interesting finding of our study was that individuals with previous treatment with benznidazole had a 50% lower rate of PCR positivity compared with SP-BD without CC. However, as previously reported, the rate of cardiomyopathy was similar in this group and in the non-treated group of seropositive former blood donors (11/48 vs. 105/445  $p=1.0$ ).<sup>10</sup> As we did not have data on the timing of drug treatment relative to the diagnosis of cardiomyopathy, we cannot make any inference on whether treatment decreases the incidence of CC. A large clinical trial (BENEFIT—the BENznidazole Evaluation For Interrupting Trypanosomiasis) is currently under way to clarify the role of trypanocidal therapy in preventing cardiac disease progression and death; importantly this study includes PCR analysis so will be able to further inform the predictive value of detection of blood parasitaemia for disease progression and response to therapy.<sup>5,26</sup> In the study of Norman et al.,<sup>18</sup> negative results on PCR were associated with benznidazole treatment. Recently, two studies have shown the usefulness of PCR for monitoring response to trypanocidal treatment.<sup>27–29</sup>

Finally, we did not detect any PCR+ samples from the 483 SN-BD who were matched to the SP-BD with respect to location and demographics, which was intended to enhance the risk of exposure to *T. cruzi*. Although SN-BD had a lower risk of exposure to *T. cruzi* compared with SP-BD, 51% of them had lived in a rural area and 24% had a relative with Chagas disease.<sup>10</sup> Several previous publications have reported so-called serosilent *T. cruzi* infections, raising concerns over the sensitivity of serological assays for diagnostics and donor screening.<sup>11–13</sup> Our results are reassuring as they demonstrate that contemporary donor screening and diagnostic assays are highly sensitive in detecting antibodies in most if not all infected individuals.

In conclusion, our data provide evidence supporting a direct role of parasite persistence in chronic Chagas disease pathogenesis.

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## Supplementary Information

Additional Supporting Information may be found in the online version of this article:

FigureS1. Spearman correlation of parasite level vs. ECHO variables (ejection fraction and left ventricular index mass), biomarkers (troponin and Nt-ProBNP), and ECG variables (QRS duration and corrected QT interval).

TableS1. Dichotomous classification of ECG and ECHO measurements in controls and PCR negative and positive Chagas EIA reactive subjects (excludes 48 seropositive donors who had been treated with benznidazole).

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