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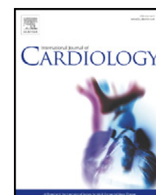

Inflammatory and cardiac biomarkers are differentially expressed in clinical stages of Chagas disease

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A B S T R A C T

Background: Chagas disease has a long clinically silent period following *Trypanosoma cruzi* infection and before development of overt clinical pathology; detectable biomarkers of infection and pathogenesis are urgently needed. We tested 22 biomarkers known to be associated with cardiomyopathy to evaluate if a biomarker signature could successfully classify *T. cruzi* seropositive subjects into clinical Chagas disease stage groups.
Methods: This cross-sectional retrospective case–control study enrolled *T. cruzi* seropositive blood donors (BD) who were further characterized as having chronic Chagas cardiomyopathy (CC-BD) or not (nonCC-BD) and seronegative (SN) control donors; we also included clinically diagnosed Chagas cardiomyopathy patients (CC-P). All subjects underwent a health history questionnaire, medical examination, electro- and echocardiograms (ECG and Echo) and phlebotomy. Biomarkers were measured on blinded samples by luminex bead array and Ortho VITROS.
Results: A clear biomarker pattern was observed only in more severe cardiac disease; this pattern included significantly elevated levels of inflammatory cytokines IFN-γ, IL-6, IL-10 and TNF-α and soluble cardiovascular disease biomarkers CK-MB, troponin, myoglobin, VCAM and NTproBNP while there were lower levels of MPO, PAI-1, and MCP-1. The markers determined to be the most predictive of disease by ROC curve analysis were NTproBNP and *T. cruzi* PCR status.
Conclusions: Although many biomarkers demonstrated increased or decreased concentrations among the clinical forms of Chagas disease, NTproBNP and *T. cruzi* PCR were the only tests that would independently be of clinical value for disease staging, in concert with ECG, Echo and clinical assessments.
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1. Introduction

Chagas disease is caused by *Trypanasoma cruzi*, a parasite which is naturally transmitted through several species of haematophagous reduviid bugs (*e.g.*, *Triatoma infestans*). It causes considerable morbidity and mortality in *T. cruzi* endemic regions of South and Central Americas and is becoming an increasing problem in non-endemic regions due to migration of infected individuals. Of particular concern is the susceptibility to acquisition of the parasite by non-traditional routes. Infections with *T. cruzi* can also occur *via* congenital transmission, organ transplantation, and blood transfusion [1]. The great majority of acute *T. cruzi* infections are unapparent and most symptomatic patients present with minor clinical manifestations. Most untreated acute cases evolve into the indeterminate stage of chronic Chagas disease (seropositive but no sign of the cardiac or digestive forms of the disease as evaluated by ECG and X-ray) [1]. However some individuals will develop cardiomyopathy (CC) and/or the mega-syndromes approximately 10 to 20 years after infection in a slow but progressive fashion. Previous studies from our group have suggested an annual incidence rate of 1.85% for the cardiac form of the disease [2].

Chronic Chagas cardiomyopathy is the most important clinical presentation of Chagas disease. It comprises a wide range of manifestations including heart failure, arrhythmias, heart-blocks, sudden death,

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thromboembolism and stroke [3]. Clinical presentation typically varies widely according to the degree of myocardial damage. Some patients present a mild form of heart disease, frequently characterized only by the presence of asymptomatic abnormalities on the ECG or in other complimentary exams as was the case of the CC-BD in our study [4]. However some patients will develop heart failure and/or severe arrhythmias, with high mortality rates, typically in adult male patients [3], and also in the elderly [5].

Biomarkers are urgently needed in the case of *T. cruzi* infection since there is a long clinically silent period from acute infection to development of clinical signs and symptoms of chronic Chagas which occur in ~30% of infected individuals [6]. During the early phase, vector-derived and blood-form parasite trypomastigotes actively infect macrophages and tissue, forming intracytoplasmic cyst-like collections. Although early immunological responses play a role in parasite control, many infected individuals continue onto chronic infection with parasite- and inflammation-related pathology. As the disease progresses to chronic infection, intracellular parasite numbers diminish and the parasites almost disappear from the peripheral blood. It can also be difficult to detect parasites in diseased tissue from individuals who died from clinical Chagas disease manifestations. Low parasite numbers and the lack of a good marker of active infection or incipient disease hinder the investigation of the etiology of parasite-induced pathogenesis and prevent evaluation of new treatments for Chagas disease [7].

The pathogenesis of chronic Chagas cardiomyopathy (CC) is not completely understood. There is now good evidence to indicate that parasitemia is important for the development of the disease [8,9]. However, parasite PCR positivity alone is not a highly informative marker of disease progression; for example, in our previous study we found that approximately 20% of the CC patients were PCR negative whereas 50% of asymptomatic *T. cruzi* seropositive individuals were PCR positive. For the past five years, our group has searched for sensitive and specific markers of parasite replication and disease progression [8,10–14]. Although some markers have shown promising correlations with disease status, most studies were performed on relatively small sets of samples. Another problem is that many markers have a high degree of overlap between groups. These findings suggest that the use of one single clinical test or laboratory analyte may not be optimal as an effective biomarker, whereas a composite set of biomarkers and an algorithm approach that integrates clinical and biomarker data may be more appropriate to achieve maximal predictive value for both prognostic and therapeutic monitoring applications.

The hallmark of CC histopathology is the presence of a mononuclear infiltrate, composed of macrophages (50%), T cells (40%), and B cells (10% or less) with virtually no NK cells. These activated lymphocytes initiate and maintain activity in cardiac tissue, resulting in the local production of inflammatory cytokines such as IFN-γ and TNF-α [15–17]. Our hypothesis is that individuals with more advanced disease would present with higher circulating levels of inflammatory cytokines and cardiac markers underlying or mediating cardiac pathogenesis.

We describe here the results obtained by testing 4 plasma markers of cardiac damage and 18 serum markers of inflammation in samples collected from a well-characterized cohort of *T. cruzi* seropositive blood donors that were further classified as having cardiomyopathy (CC-BD) or not (nonCC-BD), as well as matched seronegative (SN) control donors. This blood donor cohort is supplemented with patients presenting with more advanced Chagas disease recruited from a large Cardiology Hospital (CC-P). This study is based on analyzing repository samples collected from *T. cruzi*-infected individuals. It is one of the largest retrospective analyses to date where the primary aim of this study is to characterize biomarkers of inflammation and cardiac disease that correlate with parasitemia and clinical outcomes of cardiomyopathy. Our secondary objective is to identify a composite set of biomarkers associated with the early development of Chagas cardiomyopathy (CC) and severity of cardiac disease.

2. Methods

2.1. Study design

This retrospective cohort study, developed as part of the National Heart, Lung and Blood Institute (NHLBI) Retrovirus Epidemiological Donor Study-II (REDS-II), enrolled 499 *T. cruzi* seropositive (SP) blood donors (cases) 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 seronegative (SN) control donors frequency matched by site, donation date (year), age and gender. This blood donor cohort was supplemented by parallel enrollment and evaluation of 101 previously diagnosed cases of CC from the Heart Institute of University of São Paulo Medical School; inclusion criteria included a physician diagnosis of CC, confirmed *T. cruzi* seropositivity, no previous treatment with benznidazole (BZN), and no co-morbidities such as diabetes, hypertension or renal failure. Study subjects were recruited by letter and telephone call using the blood center and hospital databases. Recruited patients gave informed consent to participate in this study. From July 2008 to October 2010, recruited individuals (blood donors 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, *T. cruzi* PCR and other biomarkers in the United States (US; see below). All blood samples were collected in EDTA and serum tubes, processed for parasite detection (described below) or spun and aliquoted. All specimens were frozen in Brazil at −20 °C until shipped to the US REDS-II Central Laboratory (BSRI) on dry ice and maintained at −70 °C.

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 an expert panel composed of three Brazilian cardiologists to review cardiac findings blinded to the subject's serostatus. The expert panel was asked to reach a consensus regarding the following question: "If this patient was seropositive for *T. cruzi* how would you classify them: definite CC, probably CC, possible CC, or no CC". Further details of the cohort procedures, rates and clinical correlates of CC have been previously reported [2]. The local physician also received all test results and counseled the participants when necessary. For the purpose of this study we excluded all seropositive blood donors with a history of treatment with BZN, because we had no data concerning their drug regimen or treatment duration and timing.

2.2. Ethics statement

This study is approved by the UCSF CHR, Comissão de Ética para Análise de Projetos de Pesquisa (CAPPesq), Comitê de Ética em Pesquisa da Fundação Hemominas (CEP Hemominas) and National IRB — Brasília: A Comissão Nacional de Ética em Pesquisa (CONEP). Informed written consent was given by the patients for their information to be stored in the hospital database and used for research.

2.3. PCR procedures

At the time of interviews and medical examinations 20 mL of EDTA-anti-coagulated 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) *T. cruzi* real-time (RT) PCR assay used in this study [18] was developed based on the PCR method described by Virreira M et al.[19] targeting kinetoplast minicircle *T. cruzi* DNA.

2.4. Clinical cardiac 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) using standardized procedures. All ECGs were processed blindly by the central ECG laboratory (Epidemiological Cardiology Research Center, Wake Forest University, Winston-Salem, NC). ECGs were analyzed electronically, with manual over-reading by trained cardiologists to ensure quality control. ECGs were classified by Minnesota code criteria using variables that were derived from the median complex of the Marquette measurement matrix [20,21].

Echo studies were performed using a Sequoia 512 ultrasound instrument (Acuson, Mountain View, CA, USA) at the Sao Paulo site and GE Vivid3 (GE Healthcare, Waukesha, WI) at the Montes Claros site. Cardiac measurements were performed according to the guidelines of the American Society of Echocardiography [22,23]. 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) at the Cardiovascular Branch, Echocardiography Laboratory, National Heart, Lung, and Blood Institute, Bethesda, Maryland, US. Left ventricle (LV) ejection fraction was calculated based on a modified form of Simpson's biplane method [22].

2.5. Plasma and serum biomarkers

Biomarkers associated with *T. cruzi* infection, Chagas and cardiovascular disease were identified and selected for assessment in this study. Blinded serum samples were tested

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