A preliminary study of platelet hyperactivity in the chronic indeterminate phase of Chagas' disease

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Abstract. The chronic indeterminate phase of Chagas' disease is asymptomatic despite positive test results for antibodies specific to *Trypanosoma cruzi*. CD62P-APC (P-selectin) and PAC-1 FITC (GpIIb/IIIa) may improve diagnosis as biomarkers of platelet activity. Nine asymptomatic seropositive subjects, previously untreated, were selected from a blood bank within a year of Chagas' disease detection, in addition to a control group of four. All subjects were evaluated by flow cytometry for CD62P, PAC-1 and CD41, and in a complementary study, by Tissue Doppler Echocardiography for isovolumic relaxation times (IVRT) and E/A ratios. The subjects were classified as positive or negative for CD62P and PAC-1 by a cut off obtained from their mean±2SD. For IVRT and E/A ratios, cut offs were obtained from the American Society of Echocardiography and the European Association of Cardiovascular Imaging recommendations. Fisher's exact test was used for associated findings. Pre-test and post-test probability, sensitivity, specificity, positive and negative predictive values and likelihood ratios were calculated. Abnormalities were expressed as platelet hyperactivity and ventricular dysfunction in CD62P, PAC-1, IVRT and E/A ratios. CD62P appears to have greater sensitivity (0.75) and PAC-1, more accurate specificity (0.75), which may explain thrombotic events in Chagas' disease. We recommend the use of CD62P and PAC-1 as biomarkers of platelet hyperactivity in patients in the chronic indeterminate phase of Chagas' disease.

INTRODUCTION

Around 6 to 7 million people worldwide are estimated to be infected by *Trypanosoma cruzi*, which causes Chagas' disease. (WHO, 2014) Febrile symptoms are observed at the start of acute Chagas' disease. After an initial early recovery, those infected remain in the indeterminate phase for 5-10 years. This phase is characterized by the absence of clinical symptoms, nevertheless, the subjects retain *T. cruzi* antibodies (Ribeiro & Rocha 1998).

T. cruzi is an infectious agent affecting platelet aggregation (Gazos-Lopes *et al.*, 2014), and causing endothelial cell dysfunction (Tanowitz *et al.*, 1990). Although a prothrombotic state is evident (Pinazo *et al.*, 2011), little intervention is given in terms of diagnosis and treatment (Kawano *et al.*, 2011).

No studies of platelet activity have yet been reported in clinical practice. However, molecules in *T. cruzi* are known to synthesize C18:1-lysophosphatidylcholine (LPC), with activity similar to platelet-activating factor (PAF) (Kawano *et al.*, 2011), which could explain signs of thrombosis (Bestetti *et al.*, 2011; Arteaga-Fernández *et al.*, 1989). PAF interacts with the its receptor and causes platelet aggregation (Arteaga-Fernández *et al.*, 1989; Gomes *et al.*, 2006). It may be that an active prostanoid receptor mimicking TXA2 also participates in cardiovascular disease caused by Chagas' disease (Mukherjee *et al.*, 2013).

Echocardiography is effective in evaluating ventricular function (Pereira & Markman 2014). A reduction in the LVEF, and an increase in the left ventricle end-diastolic diameter are significant diagnostic criteria in cardiopathy (Garcia-Alvarez *et al.*, 2010). There are many abnormalities in Chagas' disease cardiomyopathy, such as ventricular arrhythmias, ventricular segmental lesions, left ventricle dysfunction, sinus node dysfunction and intraventricular conduction system abnormalities (Viotti *et al.*, 2004).

We believe that the platelet markers PAC-1 (GPIIb / IIIa) and CD62P (P-selectin) contribute in the early detection of platelet hyperactivity in subjects in the chronic indeterminate phase of Chagas' disease. Nevertheless, the use of echocardiography is also effective in detecting ventricular dysfunction.

MATERIALS AND METHODS

The screening tests at blood banks were used to narrow down the general population and to detect and confirm antibodies to *T. cruzi*. Nine seropositive Chagas' disease patients, seven men and two women, and four healthy subjects were selected in a convenience sample, all from the Dr. Aurelio Valdivieso General Hospital blood bank, Oaxaca, Mexico. Age and blood pressure was recorded. No subjects with a diagnosis of diabetes mellitus, hypertension, or senility were included. In addition, subjects with a clinical history of coronary or restrictive heart disease, or dilated and hypertrophic cardiomyopathy, or those who had been given anti *T. cruzi* treatment were excluded to avoid misinterpretation of echocardiography and platelet markers.

All cases, including healthy subjects, had *T. cruzi* antibodies in two consecutive tests: the first test, using purified antigen (Accutrack Chagas ELISA, Laboratorio Lemos, Buenos Aires, Argentina), and the second, using recombinant antigen (Chagastest ELISA recombinant v.4.0 Wiener Lab, Rosario Argentina).

Each subject was informed of the nature of the study, and gave written consent before blood was drawn. The study was carried out according to the General Health Law, the Helsinki Declaration, and the Nuremberg Code. It was also approved by the CEI committee at the General Hospital Dr. Aurelio Valdivieso SS. Oaxaca, Mexico.

A MACSQuant Cytofluorometer analyzer (Miltenyl Biotec, Bergisch Gladbach, Germany) was used to identify platelets in blood based on forward and side-scatter log characteristics and CD41-FITC (Anti-Human/Mouse Ebioscience). P-selectin (Anti-Human/Mouse CD62P, APC, Ebio-science) was used for detection of silent thrombosis, and GPIIb/IIIa (PAC-1, FITC, Ebioscience), for its association with platelet activation (Hou *et al.*, 2014). Ten thousand platelet events were acquired from each sample in list mode, and a positive expression rate for CD41, CD62P, and PAC-1 was calculated. Details of this method have been described previously (Moreno-Rodríguez *et al.*, 2014).

LVEF, E/A ratio and IVRT were evaluated by echocardiography using the Teichold method with a Tissue Doppler (TDE) (Prosound α7 ALOKA. Hitachi Aloka Medical, Ltd p-42109DV model IPF-M01B. Japan). All echocardiography studies were observed by two different specialists at the Dr. Aurelio Valdivieso General Hospital, Oaxaca, Mexico. Prothrombin and partial thromboplastin time tests were performed, and glucose, haemoglobin, and glycated haemoglobin levels were measured.

Pre- and post-test probability, sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), likelihood ratios for positive and negative results were calculated using the Prism 5 program for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com, and the programme written by DJR Hutchon (Hutchon 2016).

RESULTS AND DISCUSSION

All subjects came from the central valleys of Oaxaca and had an average age of 41 years, with 13.63 years' standard deviation (SD). Also, 4 healthy men, averaging 29.8 years, with 4.06 years SD were studied as controls. This selection resulted from the different proportion of female versus male blood donors at the Dr. Aurelio Valdivieso General Hospital, Oaxaca, Mexico, and affected the selection of gender in both groups.

All nine asymptomatic seropositive subjects showed average systolic blood pressure of 106 mmHg with 9.42 SD. The healthy group showed an average of 114 mmHg with 8.8 SD. In relation to diastolic blood pressure, the seropositive group gave an average of 80 mmHg, with 8.16 SD, and the healthy group gave an average of 72 mmHg, with 7.48 SD. All subjects had normal fasting glucose, glycosylated haemoglobin, prothrombin and partial thromboplastin time tests.

Table 1 shows positive and negative results of the biomarkers. They were classified as positive when above the mean cut off point, at ± 2SD. LVEF, IVRT, and E/A ratios were used as recommended by the American Society of Echocardiography and the European Association of Cardiovascular Imaging (Lang *et al.*, 2015; Mesquita & Jorge 2013).

Echocardiography results demonstrated that, in subjects in the chronic indeterminate phase of Chagas´ disease, the Left Ventricular Ejection Fraction was similar to the control group. However, isovolumic relaxation times (IVRT) and E/A ratios increased and showed positive results, suggesting diastolic dysfunction. Echocardiography results in Chagas' disease cardiomyopathy have been studied extensively (Gazos-Lopes *et al.*, 2014; Viotti *et al.*, 2004; Valerio *et al.*, 2011), but are not discussed further in this work.

Considering that thrombotic events (Bestetti *et al.*, 2011; Arteaga-Fernández *et al.*, 1989) and cardiac abnormalities (Garcia-Alvarez *et al.*, 2010) are known to be associated with the various stages of Chagas' disease, platelet activation was evaluated in all subjects. CD41 staining was performed to identify platelet population. In the chronic indeterminate phase of Chagas' disease we found subjects responded positively to the platelet markers, CD62P (P-selectin) and GpIIb/IIIa (PAC-1), as shown in Table 1. Although it is necessary to increase the number of subjects to have greater certainty in the study, when CD62P, PAC-1, IVRT, and E/A were compared, we found CD62P had more sensitivity than PAC-1 and detected true positives more accurately. PAC-1 had better specificity than CD62P and detected true negatives more accurately (Table 2). The use of these methods is based on the function of CD62P, which is found in α-granules, and is released on the surface of the platelets when in an activated state, in addition to PAC-1 activity (van Velzen *et al.*, 2012).

Table 1. Platelet and echocardiography biomarkers in the chronic indeterminate phase of Chagas' disease

	CD62P %	PAC-1 %	LVEF %	IVRT m/s	E/A ratio
Positive	5	4	0	5	3
Negative	4	5	9	4	6

CD62P (P-selectin), PAC-1 (GPIIb/IIIa), Left ventricular ejection fraction (LVEF), isovolumic relaxation time (IVRT) and E/A ratio. These biomarkers were classified as positive when above a mean of ± 2SD.

Table 2. Diagnostic tests in platelet biomarkers in the chronic indeterminate phase of Chagas' disease

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Pre-test probability	Post-test probability
CD62P vs PAC-1	0.75	0.60	0.60	0.75	0.44	0.59
PAC-1 vs CD62P	0.60	0.75	0.75	0.60	0.55	0.75
CD62P vs IVRT	0.40	0.25	0.25	0.25	0.55	0.40
CD62P vs E/A rate	0.66	0.50	0.50	0.75	0.33	0.39
PAC-1 vs IVRT	0.40	0.50	0.50	0.40	0.55	0.50

CD62P (P-selectin), PAC-1 (GPIIb/IIIa), Left ventricular ejection fraction (LVEF), isovolumic relaxation time (IVRT) and E/A ratio.

Earlier studies have reported changes in the purinergic system of ecto-enzymes, which are present on the platelet surface. These changes could regulate, counteract or even modulate factors, such as, trans-sialidase effects from *T. cruzi* (Tribulatti *et al.*, 2005), modifying platelet activity (Souza *et al.*, 2012). Platelet activation markers or pro-thrombogenic markers are recommended for use in the chronic indeterminate phase of Chagas' disease because *T. cruzi* has a strong association with thrombosis.

This study was limited by its small sample size due to the difficulty in identifying potential subjects at this stage of the disease, and would benefit from a greater number of seropositive subjects at different stages, to have more definite conclusions. A further difficulty in the predominantly male sample, is the fact that male subjects generally have a poorer prognosis of Chagas' disease (Assunção *et al.*, 2016). Family members or friends of hospital patients donating blood with a diagnosis of Chagas' disease, were not found in great numbers. Also, subjects treated for other diseases affecting the myocardium, in particular, ventricle dysfunction, were discarded due to complications in diagnosis (Jeong & Dudley 2015). Another limitation was the lack of follow up to the treatment in monitoring CD62P and PAC-1 biomarkers. We recommend a test of *T Cruzi* antigen in urine, which may characterize this stage more accurately (Málaga-Machaca *et al.*, 2017).

In conclusion, identification of platelet activity with CD62P and PAC-1, in con-junction with other tests such as echo-cardiography, may benefit subjects in the chronic indeterminate phase of Chagas' disease. It could also improve detection in subjects with platelet hyperactivity, which can lead to thrombosis.

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