Hypercoagulability biomarkers in Trypanosoma cruzi-infected patients

Maria-Jesús Pinazo1; Dolors Tassies2; José Muñoz3; Roser Fisa1; Elizabeth de Jesús Posada1; Juan Monteagudo2; Edgar Ayala4; Montserrat Gállego2; Joan-Carles Reverter2; Joaquim Gascon3

1Barcelona Centre for International Health Research (CRESIB), Hospital Clinic / IDIBAPS, Universitat de Barcelona, CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain; 2Hemotherapy and Hemostasis Department. Hospital Clinic, Barcelona, Spain; 3Laboratori de Parasitologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain; 4Health Biostatistics, Centre for International Health Research (CRESIB), Barcelona, Spain

Introduction

Chagas disease (CD) is a neglected disease that affects 8–10 million people worldwide (1). Traditionally considered to be a rural disease of poor people in Latin America, it is currently being diagnosed in urban areas of Latin America and other continents. Thus, following the recent migration trends, CD should be considered a public health problem not only in Latin America but also in non endemic countries (2).

About 25–30% of patients in the chronic stage suffer heart disease. Chagasic cardiomyopathy can be manifested as heart failure, cardiac arrhythmias and arterial or venous thromboembolism (3). Cardiac embolism has been suggested as the most common cause of stroke in patients with heart Chagas disease up to now (4, 5). However, stroke has been evidenced in Trypanosoma cruzi-infected individuals without myocardopathy or other classic vascular risk factors (6, 7).

Thromboembolic events are related to endothelial injury and hypercoagulable states that produce circulatory stasis and alteration of blood flow. However, in the last few years other factors have been postulated to play a role in thrombosis (8). In an animal model, T. cruzi infection induces changes in blood viscosity as a result of the immune response of the host (9). The parasite itself in plasma fluid modifies its viscous properties (9). The parasite directly attacks the vascular endothelium with the secretion of a neuraminidase that allows sialic acid to be removed from the endothelial infected cells (10).

Infection increases pro-inflammatory cytokine expression, which causes generalised vasculitis with increased levels of endothelium factors, such as thromboxane A2 and endothelin-1 (11–13). Platelets are also concentrated at the vessel wall, where they can be activated by high shear stresses and can interact with endothelium factors, resulting in platelet adhesion and the initial stages of haemostasis. Subsequently, blood viscosity, platelet microemboli, and activated leukocytes may each reduce post-stenotic microcirculatory blood flow, promoting infarction (14). Such mechanisms may partly explain the increased risk of thrombotic phenomena (myocardial, cerebral and limb infarction) in CD patients.

Summary

There is a current controversy over the hypothesis that a number of thromboembolic events could be related to hypercoagulable state in patients with chronic Chagas disease. This study was designed to determine whether a prothrombotic state existed in chronic Trypanosoma cruzi-infected patients and, if so, to describe its evolution after treatment with Benznidazole. Twenty-five patients with chronic Chagas disease and 18 controls were evaluated. The markers used were prothrombin time, activated partial thromboplastin time, fibrinogen, antithrombin, plasminogen, protein C, total protein S, free protein S, factor VIII, D-dimer, activated factor VIIa, tissue-type plasminogen activator inhibitor-1, prothrombin fragment 1+2 (F1+2), plasmin-antiplasmin complexes, soluble P-selectin and endogenous thrombin potential (ETP).

Despite statistically significant differences between cases and controls in several markers, only ETP (which quantifies the ability of plasma to generate thrombin when activated through tissue factor addition) (p<0.0001) and F1+2 (a marker of thrombin generation in vivo) (p<0.0001) showed values outside the normal levels in patients compared with controls. Similar results were obtained in these markers six months after treatment in the cohort of cases (p<0.0008 and p<0.004, respectively). These results may be relevant in clinical practice. Though current treatment for Chagas disease is still controversial, if it were considered as a thromboembolic risk factor the antiparasitic treatment strategy could be reinforced. The results also support further research on haemostasis parameters as candidates for early surrogate biomarkers of cure or progression of Chagas disease.

Keywords

Chagas Disease, hypercoagulability, biomarkers, prothrombin fragment 1+2, endogenous thrombin potential

Correspondence to:
Maria-Jesús Pinazo, MD
Barcelona Centre for International Health Research (CRESIB)
Hospital Clinic / IDIBAPS, Universitat de Barcelona
CIBER Epidemiología y Salud Pública (CIBERESP)
Roselló, 132. 4th, 08032 Barcelona, Spain
Tel.: +34 932275400 ext. 2182, Fax: +34 932279853
E-mail: mpinazo@clinic.ub.es

Received: April 18, 2011
Accepted after minor revision: June 17, 2011
Prepublished online: August 25, 2011
doi:10.1160/TH11-04-0251
Thromb Haemost 2011; 106: 617–623

Thrombosis and Haemostasis 106.4/2011
There is currently controversy concerning the role of thrombophilia in thromboembolic events in CD patients. High levels of some hypercoagulability biomarkers, such as prothrombin F$_{1+2}$, ATM-complex, fibrinogen/fibrin degradation products and D-dimer have been described as high in *T. cruzi* infection patients, even in early stages of the disease (15) and have been considered as thrombotic risk factors (16). However, one study shows that there are no differences between thrombophilic factors (protein S, antithrombin, activated protein C resistance, factor V Leiden, lupus anticoagulant and anticardiolipin antibodies) in *T. cruzi*-infected and noninfected individuals and considers that the procoagulant state is not an ischaemic risk factor in these patients (17).

One of the main issues that complicate the management of CD is the lack of early markers of disease progression and severity as well as markers of cure after antiparasitic treatment. Currently, the decrease in serum IgG anti-*T. cruzi* titers, which occurs more than 10 years after treatment is completed in chronically infected patients, is the only accepted criterion of cure (18).

The objective of our study was to determine whether a prothrombotic state exists in *T. cruzi*-infected patients and, if so, to describe its evolution after treatment with Benznidazole.

**Materials and methods**

**Design and setting**

This is a descriptive study of 43 individuals (25 cases and 18 controls) coming from Latin American countries where CD is endemic and attending the Centre for International Health in the Hospital Clinic, a university hospital.

**Recruitment and participants**

Forty-three persons from *T. cruzi* endemic areas who were over 18 years old and living in Barcelona were invited to participate in the study. Exclusion criteria were pregnancy, non-chagasic cardio-pathy, inflammatory or immunological diseases (active infections of other etiology, inflammatory intestinal diseases and other autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematous) and chronic systemic diseases such as high blood pressure and diabetes.

**Procedures (including ethics)**

After signing the informed consent form, the subjects were asked for clinical and epidemiological data, including age, sex, area of origin, history of rural environments and mud houses, previous contact with the vector, and their history of blood donation or transfusion. Past history – including toxic habits and other vascular risk factors – and previous vascular events were recorded.

Serological tests for *T. cruzi* and human immunodeficiency virus (HIV) infection were conducted on all participants. Haematology, biochemistry (including renal and liver function), and a specific determination of haemostasis factors were also performed. For haemostasis studies, blood was collected in citrate-containing tubes (Becton Dickinson, San José, CA, USA), samples were centrifuged and platelet-poor plasma aliquots were frozen at ~80°C until assayed. PT and aPTT were determined in an automated BCS XP analyzer (Siemens, Marburg, Germany) using standard reagents (Thromborel and Actin FS; Siemens). Fibrinogen was measured by the Clauss technique. Coagulation factor VIII was determined using a chromogenic assay (Chromogenix IL, Milano, Italy). Protein C activity was quantified by a colorimetric assay (Chromogenix). Free and total protein S were quantified by enzyme-linked immunosorbent assay (ELISA) (Stago, Asnières, France). Antithrombin and plasminogen activity were measured using chromogenic assays (Siemens). The F$_{1+2}$ and plasmin-antiplasmin complex (PAP) were quantified by ELISA (Siemens). D-dimer was measured with a turbidimetric method (Siemens). FVIIa and PAI-1 antigen were determined by ELISA (American Diagnostica, Greenwich, CT, USA). Plasma levels of P-Sel were also measured by ELISA (R&D Systems, Abingdon, UK). ETP, calculated as percentage ETP value of the control group, was measured using a chromogenic method in a continuous thrombin generation assay and the ETP Curves software (Siemens). Factor V Leiden and prothrombin gene G20210A mutation were determined by real-time polymerase chain reaction (PCR) (Roche, Mannheim, Germany). Lupus anticoagulant was detected following the guidelines of the International Society on Thrombosis and Haemostasis, and anticardiolipin antibodies were measured by ELISA (Chesire Diagnostics, Chester, UK). Normal values of the haemostasis factors are given in Table 2.

For laboratory diagnosis of *T. cruzi* infection, three serum ELISA tests were performed: a commercial ELISA with recombinant antigens (BioELISA Chagas®, Biokit S.A., Lliçà d’Amunt, Barcelona, Spain), an in-house ELISA (whole *T. cruzi* epimastigotes antigen) (19) and a conventional ELISA (Orthoclinical Diagnostics, Johnson & Johnson Company, Rochester, NY, USA) (20). Participants were considered infected if the results from at least two serological methods were positive (18). Blood nested-PCR (21) and RT-PCR (22) were performed on seropositive participants. Nested-PCR was carried out at the beginning of the study. This was later replaced by a RT-PCR that amplifies the same DNA region as the nested-PCR but is safer and easier to perform, while giving similar results (22).

*T. cruzi*-infected patients were studied using a protocol that included a 12-lead electrocardiogram, chest X-ray and echocardiogram. Other tests were made according to the individual symptoms.

Specific treatment with Benznidazole (5 mg/kg day for 60 days) was offered to *T. cruzi*-infected patients regardless of their clinical stage, followed by fortnightly clinical and analytical follow-up during the treatment.

Thrombosis and Haemostasis 106.4/2011 © Schattauer 2011
Six months after treatment finished T. cruzi-infected patients were tested, including serology, PCR for T. cruzi and haemostasis factors.

**Statistical analysis**

Fisher’s and chi-square tests were used to compare qualitative variables. A variable t-test was used to compare normally distributed continuous variables and a Wilcoxon test for non-normally distributed variables. Crude and multivariate logistic regression models were estimated to identify factors associated with outcome. Multivariate analyses were performed by a forward stepwise procedure, using p<0.05 and p>0.10 from the likelihood ratio test to enter and remove criteria, respectively. In case of co-linearity of two variables, the model took into account only one of them.

Results from estimated models were expressed as odds ratio (OR) and 95% confidence interval (CI). The analyses were performed using Stata 10 (Stata Corp., College Station, TX, USA) (23).

**Results**

We recruited 43 patients: 25 T. cruzi-infected individuals (cases) and 18 non-infected individuals (controls), all of them 20–45 years old. The follow-up was completed in 15 of the 25 patients who were treated with benznidazole (Fig. 1), and none of them travelled to their countries or other endemic areas during the follow-up. Demographic and atherothrombotic risk factors are shown in Table 1. Twenty-two cases were in an indeterminate state of the disease, and the other three had mild to moderate cardiac involvement (two cases of Kuschnir group II and one case of Kuschnir group I). None of the cases or controls had a past history of ischaemic events (central nervous system and heart included) or atrial fibrillation.

Statistically significant differences in hypercoagulability biomarkers between untreated cases and controls were observed for F1+2 (p<0.001), PAP (p=0.002), P-Sel (p=0.001), ETP (p=0.001), D-dimer (p=0.049) and FVIIa (p=0.03). The results of all the variables are shown in Table 2. In spite of these differences, the medians of PAP, P-Sel, D-dimer and FVIIa were sometimes within the normal ranges. Results of F1+2 and ETP show that 84% and 64%, respectively, of the patients had abnormal values before treatment.

In the cohort of follow-up cases (N=15), a statistically significant decrease was observed for fibrinogen (p=0.004), F1+2 (p=0.003), PAP (p=0.004), P-Sel (p=0.006), and ETP (p=0.0008), when baseline results were compared with the results obtained six months after treatment (Fig. 2). However, fibrinogen, PAP and P-Sel baseline data were within the normal ranges in most cases.

In order to evaluate the possibility of correlation between some haemostasis parameters, we studied the correlation between all of them with the values before the treatment (cases and controls), and six months after treatment in cases. The analysis did not show high correlation values, and the values over 0.650–0.600 (free protein S and total protein S in baseline values; F1+2–Antithrombin, Fibrinogen-PAP, ETP-aPTT, TP–Free and total protein S of six months after values), were not relevant.

RT-PCR was positive in three patients before treatment and became negative in two of them after treatment. RT-PCR was positive six months after treatment in two cases. One of them was from Santibañez, a rural area of Cochabamba (Bolivia): in this case, the PCR before treatment was negative and RT-PCR six months after

© Schattauer 2011

**Table 1: Demographic and clinical data of the recruited patients.**

<table>
<thead>
<tr>
<th></th>
<th>Infection by T. cruzi</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Women</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td><strong>Country of origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolivia</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>Ecuador</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brazil</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Colombia</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Peru</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Argentina</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Atherothrombotic risk factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High blood pressure</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Smoking</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 1: Recruitment and follow-up.**
Discussion

A hundred years after its discovery, CD remains a complex disease with many unknown aspects. The cardiovascular profile of patients with CD is changing: their life-expectancy is increasing and, due to vector control programs, re-infection is less likely. However, for those who migrate some habits such as diet and physical exercise have also changed, leading to a possible increase in chronic systemic diseases such as high blood pressure and diabetes in this population.

In CD patients, both ways of measuring thrombin generation showed a marked increase in comparison with controls, sustaining the hypothesis of a hypercoagulable state in T. cruzi-infected patients. Changes in coagulation and platelet function have been well established in ischaemic cardiomyopathy, even in subclinical states (31). ETP is a functional test that quantifies the ability of plasma to generate thrombin when activated through tissue factor addition. Thus, ETP and F1+2 identify two different sides of potential hypercoagulability: F1+2 values measure indirectly the real amount of thrombin generated in vivo and ETP indicates the potential amount of thrombin that can be formed when blood coagulation is activated. In addition, these factors are proved to have stability over time. In a control group of 12 healthy Caucasian volunteers, mean differences in the quantitative haemostasis variables between two determinations performed between 30 and 45 days apart ranged from −3.0% to 3.3% (p=NS in all of them)(personal communication, data from Dr. Reverter).

Table 2: Descriptive and baseline univariate analysis between averages of haemostasis parameters in pre-treatment cases (25) and controls (18).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (18)</th>
<th>Cases (25)</th>
<th>P*</th>
<th>Normal range (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (C25; C75)</td>
<td>Median (C25; C75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein C</td>
<td>103.50 (91.00; 109.00)</td>
<td>100.00 (96.00; 108.00)</td>
<td>0.640</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>Total Protein S</td>
<td>83.50 (81.00; 88.00)</td>
<td>82.00 (79.00; 90.00)</td>
<td>0.711</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>Free Protein S</td>
<td>83.00 (81.00; 88.00)</td>
<td>81.00 (77.00; 84.00)</td>
<td>0.128</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>112.00 (92.00; 132.00)</td>
<td>106.00 (94.00; 123.00)</td>
<td>0.980</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>Antithrombin</td>
<td>104.00 (98.00; 109.00)</td>
<td>106.00 (95.00; 109.00)</td>
<td>0.666</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>aPTT</td>
<td>29.00 (27.00; 31.00)</td>
<td>30.00 (28.00; 31.00)</td>
<td>0.494</td>
<td>25–35 (sec)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>3.35 (3.10; 3.70)</td>
<td>3.60 (3.20; 3.90)</td>
<td>0.261</td>
<td>1.5–4.5 (g/l)</td>
</tr>
<tr>
<td>D-dimer</td>
<td>201.50 (118.00; 255.00)</td>
<td>229.00 (204.00; 289.00)</td>
<td>0.049</td>
<td>50–400 (μg/l)</td>
</tr>
<tr>
<td>F 1+2</td>
<td>0.75 (0.61; 0.97)</td>
<td>1.80 (1.37; 2.71)</td>
<td>0.000</td>
<td>0.40–1.1 (nM)</td>
</tr>
<tr>
<td>PAP</td>
<td>249.25 (169.60; 366.60)</td>
<td>341.20 (267.00; 591.40)</td>
<td>0.002</td>
<td>80–470 (μg/l)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>110.00 (102.00; 113.00)</td>
<td>109.00 (103.00; 115.00)</td>
<td>0.853</td>
<td>60–140 (%)</td>
</tr>
<tr>
<td>Factor Vila</td>
<td>2.44 (2.20; 3.49)</td>
<td>3.49 (2.89; 3.98)</td>
<td>0.027</td>
<td>1.5–4.1 (ng/ml)</td>
</tr>
<tr>
<td>PT</td>
<td>1.02 (0.96; 1.06)</td>
<td>1.01 (0.96; 1.05)</td>
<td>0.203</td>
<td>0.85–1.15 (ratio, no units)</td>
</tr>
<tr>
<td>P-Sel</td>
<td>31.85 (20.90; 41.20)</td>
<td>50.10 (40.00; 64.90)</td>
<td>0.001</td>
<td>3–90 (ng/ml)</td>
</tr>
<tr>
<td>ETP</td>
<td>415.35 (388.90; 436.00)</td>
<td>478.80 (413.00; 510.60)</td>
<td>0.001</td>
<td>351–473 (me)</td>
</tr>
<tr>
<td>PAI-1</td>
<td>21.40 (15.00; 34.20)</td>
<td>24.10 (19.50; 34.50)</td>
<td>0.205</td>
<td>4.0–43.0 (ng/ml)</td>
</tr>
</tbody>
</table>

aPTT, activated partial thromboplastin time; F1+2, prothrombin fragment 1+2; PAP, plasmin-antiplasmin complexes; PT, prothrombin time; P-Sel, P-selectin; ETP, endogenous thrombin potential; PAI-1, plasminogen activator inhibitor-1. *Wilcoxon rank-sum test.

treatment was positive (CT32). The other patient was from Santa Terezinha (Goias, Brazil), and showed qualitative PCR positive before treatment and RT-PCR positive after treatment (CT39). In these two individuals all the haemostatic parameters studied remained altered, included F1+2.
described in infections (12). As neurological events in older people have multiple etiologies and risk factors, the prothrombotic trend seen in CD is one of the risk factors that may play a role in infected people, and has probably remained unappreciated until now due to the other confounding factors.

These results may be relevant in clinical practice. Due to the low efficacy (32, 33) and toxicity (34–36) of current antiparasitic therapy in chronic infected patients, specific treatment of CD is still controversial. However, if CD is considered as a thromboembolic risk factor due to rheological changes and due to the presence of the parasite itself, the antiparasitic treatment strategy could be reinforced.

Currently, T. cruzi-infected people with no symptoms and no pathological changes in the electrocardiogram, echocardiogram, chest radiograph, and gastrointestinal images are considered free of disease and classified in the indeterminate form of the disease.
The present results, along with changes in these biological markers, also challenge the notion of the indeterminate form of the disease.

Six months after treatment, a significant decrease in the blood levels of several hypercoagulability biomarkers was observed. Some questions remain on this subject: a low percentage of cure after benznidazole treatment has been described in chronically infected patients (34), but decreasing parasitaemia has been postulated to be related to less progression of the disease (37). The decrease in hypercoagulability after benznidazole therapy could be explained by the eradication of T. cruzi or only by a significant drop in the parasite burden (38). Interestingly we observed the maintenance of the F1+2 levels in the two patients with treatment failure proved by RT-PCR.

One of the major reasons for seeking biomarkers of cure/progression of CD is to improve the management of these patients. Currently we do not know the results of the treatment until several years after it has finished, when conventional serology shows a significant decrease in titres. The PCR can only detect failures of treatment in some patients, but a negative result cannot prove eradication of the parasite.

The present study has three main limitations: 1) The small number of patients included, as it was a preliminary phase aimed at testing a wide panel of haemostasis parameters to discriminate potentially useful biomarkers; 2) The short period of follow-up after treatment; and 3) the high number of cases lost to follow-up, due to high mobility of the population studied. Currently this cohort is still under supervision for a larger follow-up and new patients have been included in order to strengthen this initial result.

Conclusions

In conclusion, there is a need for new drugs against T. cruzi. Early biomarkers of cure or progression of the disease are also a crucial tool for future clinical trials with new drugs. Other strategies for obtaining early biomarkers of cure of CD have been proposed, and several groups have obtained encouraging results, but all the strategies proposed have limitations (38). Due to the complexity of the T. cruzi infection, there will probably not be a single marker that can be used alone. The results obtained in this cohort support the need for continuing research on haemostasis parameters as candidates for early biomarkers of cure of CD.

Abbreviations

CD: Chagas’ disease; T. cruzi: Trypanosoma cruzi; F1+2: prothrombin fragment 1+2; ATM-complex: antithrombin-enzyme complex; PAP: plasmin-antiplasmin complexes; FVIIa: activated factor VII; PAI-1: tissue-type plasminogen activator inhibitor-1; P-Sel: soluble P-selectin; ETP: endogenous thrombin potential; aPTT: activated partial thromboplastin time; PT: prothrombin time; RT-PCR: real-time PCR.

Acknowledgements

The authors would like to thank Fundación Mundo Sano España for support of our research on Chagas Disease. This material is based upon work supported by grant 2009GR385 from the Department d’Universitats, recerca I Societat de la Informació de la Generalitat de Catalunya, Spain.

Conflict of interest

None declared.

References

23. StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP.