1 **Title:** Host derived molecules as novel Chagas disease biomarkers: hypercoagulability markers in plasma.

2

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13 **i. Running Head:** Host-derived biomarkers for Chagas disease diagnosis.

14

15 **ii. Summary/Abstract**

16 The most severe clinical symptomatology of Chagas disease affects ~30% of those chronically

17 infected with the *Trypanosoma cruzi* parasite. The pathogenic mechanisms that lead to life-

18 threatening heart and gut tissue disruptions occur "silently" for a longtime in a majority of cases. As a

19 result, despite there are several serological and molecular methods available to diagnose the infection

20 in its acute and chronic stages, diagnosis is often achieved only after the onset of clinical symptoms in

21 the chronic phase of the disease. Furthermore, although there are two drugs to treat it, the assessment

22 of their performance is impractical with current parasite-derived diagnostics and therapeutic efficacy

23 cannot be acknowledged in a timely manner.

24 In this chapter we present two procedures to measure host derived molecules as surrogates of

25 therapeutic response against chronic *T. cruzi* infection. Their outputs relate to the generation and

activity of thrombin, a major component of the blood coagulation cascade. This is due to the fact that a hypercoagulability state has been described to occur in chronic Chagas disease patients, and revert after treatment with benznidazole.

iii. Key Words: Chagas disease, diagnosis, prognosis, therapeutic response, host-derived biological markers, hypercoagulability biomarkers.

1. Hypercoagulability markers in plasma: introduction and protocols

1.1 Introduction

Chagas disease is a complex entity in terms of pathobiology, and complex is the life cycle of its etiological agent: the protozoan parasite *T. cruzi*. It involves insect and mammalian infective forms, which in the human host are capable of infecting several host cell types and tissues to propagate the infection [1]. During its acute phase there is a strong immunological response as the presence of *T. cruzi* produces an inflammatory reaction and the secretion of pro-inflammatory cytokines, as well as parasite-specific polyclonal antibodies and activation of effector T cells [2, 3]. Despite this response, the parasite is well-dotted with anti-immune mechanisms and manages to persist leading to a chronic infectious state without causing any overt symptomatology for years. This “silent” clinical feature of Chagas disease largely hinders the clinical diagnosis of the infection before the onset of more severe symptoms [4]. These will affect ~30% of those chronically infected, who will suffer from damage at the heart and/or at the esophagus and colon. It is when tissue disruption occurs that several unspecific inflammatory mediators appear altered. Before reaching this point, it would be of major relevance to have a way of ascertaining the disease prognosis, including the evaluation of the administration of benznidazole (BNZ) or nifurtimox (NFX) treatments. This would mean a huge advantage for the daily clinical management of the disease, as well as for the clinical testing of any new drug regimens or novel anti-parasitic compounds. Unfortunately, such tool is still missing.

51 At present, there are serological and molecular diagnostics based on the respective detection of
52 anti-*T. cruzi* immunoglobulins or parasite-derived DNA sequences [5, 6]. They provide valuable
53 information on the infection status, but cannot inform on the clinical prognosis of the disease; and despite
54 recent advancements [7], they cannot yet be used to address treatment efficacy (or spontaneous cure)
55 either. This is because a positive serological result takes many years to become negative making serology
56 impractical for response-to-treatment assessment. Whereas regarding highly sensitive molecular-based
57 techniques, although a positive detection certainly indicates there are circulating parasites, a negative
58 result cannot rule out a latent very low parasitemia or the presence of tissue hidden forms that could
59 relapse later on. Thereby, in view of the absence yet of markers to address disease prognosis and/or
60 therapeutic response in the parasite, host-derived markers have also become a source of interest.

61 Even though host-parasite interactions and their role in the progression of the disease are still not
62 well known, several host biomarkers of *T. cruzi* infection have been identified during the last decade [8].
63 Moreover, their use as diagnostics to evaluate therapeutic response has been investigated [8]. In this
64 regard, there are three main groups of host-derived molecules: (i) immunological markers (cytokines and
65 surface markers) elicited by the host cellular response to the infection [9, 10]; (ii) biochemical
66 biomarkers, such as hypercoagulability markers, fragments of apolipoprotein A1 (ApoA1), tumor necrosis
67 factor (TNF) or transforming growth factor beta (TGFβ) [11–13]; and (iii) inflammatory markers of
68 cardiac damage (e.g. type-B natriuretic peptide (BNP) or highly-sensitive protein C), which have been
69 perhaps the most studied, and shown to be not very good to follow disease progression [14].

70 It has been previously described that a hypercoagulability state can appear in people with *T. cruzi*
71 infection [11, 15, 16]. In the context of an infectious disease, this hypercoagulability could be due to three
72 processes: (a) dysregulation of immunothrombosis [17]; (b) platelet adhesion events driven by a chronic
73 inflammation state [18]; and (c) vasculitis caused by the chronic infection [19]. Altogether they can lead
74 towards increased levels of pro-inflammatory cytokines, and therefore perpetuate the risk of thrombotic
75 accidents, which is one of the main causes of pathophysiology in Chagas disease.

76 Amongst the different blood coagulation markers involved in the aforementioned processes, two
77 were significantly elevated in Chagas disease patients in comparison to controls and could thereof be used
78 as biomarkers of *T. cruzi* infection diagnosis and response-to-treatment assessment [16] (Figure 1). The
79 hypercoagulability markers prothrombin fragment 1+2 (F1+2) and endogenous thrombin potential (ETP)
80 were abnormally expressed in a high percentage of patients with chronic *T.cruzi* infection before
81 treatment (77% and 50%, respectively). Shortly after BNZ treatment both returned to, and remained at
82 normal levels in 76% and 96% of patients, respectively [16]. The performance of F1+2 and ETP fulfilled
83 the Target Product Profile (TPP) defined for chronic Chagas disease response-to-treatment biomarkers [8,
84 16].

85 It cannot be disregarded that both hypercoagulability biomarkers (F1+2 and ETP) presented with
86 normal values in a percentage of *T. cruzi*-infected people, which may limit their usefulness as universal
87 biomarkers [16]. Another big limitation is that current procedures to measure them share a common
88 requirement for high-technological equipment and highly-trained technical personnel to run it. This is a
89 setback for their application in many ill-equipped laboratories from Chagas disease endemic regions at the
90 moment. Indeed there is yet a lot of work ahead, and more resources and attention should be placed on the
91 matter.

92 In this chapter, we describe the rational and protocol procedures to implement the detection of
93 presently most promising host-derived biomarkers for Chagas disease response-to-treatment assessment.
94 These are the hypercoagulability biomarkers F1+2 Enzyme-Linked Immunosorbent Assay (***Protocol 1)***,
95 and Endogenous Thrombin Potential (ETP) Kinetics Assay (***Protocol 2)*** [16].
96

97 **1.2 Protocol 1: F1+2 Enzyme-Linked ImmunoSorbent Assay**

98 The proteolytic cleavage of prothrombin into active thrombin is a key step in the blood
99 coagulation cascade. Thrombin activation is accompanied by formation of fragment F1+2. Thus,
100 quantification of F1+2 allows tracking the thrombin formation process. By measuring the concentration

101 of F1+2 in plasma samples it is possible to diagnose prothrombotic states in comparison to plasma control
102 samples. Levels of F1+2 can be detected with an immunochemical assay, such as the Enzygnost F1+2
103 that is commercialized by Siemens Healthcare (reference number OPBD03; Marburg, Germany). This is
104 an enzyme-linked immunosorbent assay (ELISA) based on an F1+2-specific monoclonal antibody (mAb).
105 It is a sandwich ELISA because the antigen to detect (F1+2) is first bound by mAb that come attached to
106 the assay wells. In a second incubation step, another antibody, specific against another antigenic
107 determinant of F1+2 is added in to close the sandwich. This latter antibody is conjugated to a peroxidase
108 (POD) enzyme and thus the presence of sandwich complexes can be evaluated with the addition of a
109 chromogenic substrate of the POD reaction. The optical density (OD) value is proportional to the
110 concentration of F1+2, the higher the absorption the higher is the concentration of F1+2 in the plasma
111 sample (Figure 2). The concentration of F1+2 in the samples is calculated by comparing the OD of the
112 samples to the calibration curve made with standard specimens.
113

114 **1.3 Protocol 2: Endogenous Thrombin Potential (ETP) Kinetics**

115 The Endogenous Thrombin Potential (ETP) is a test that reflects the capacity of a sample to
116 generate thrombin considering both formation and inhibition. ETP can be determined measuring the
117 conversion kinetics of a synthetic thrombin substrate. ETP test is performed by means of a commercial
118 assay (i.e. ETP Innovance by Siemens Healthcare) and the required corresponding piece of equipment
119 (i.e. Siemens BCS System). These resources are available in several hospital Hemostasis units.

120 The synthetic thrombin substrate included in the kit contains a chromophore that is released upon
121 its thrombin-mediated catalysis, therefore providing a chromogenic readout of thrombin activity in the
122 plasma samples. The BCS System records absorbance at 405 nm in a continuum to evaluate the
123 enzymatic kinetics of thrombin activity. BCS can also calculate the test output values. It first corrects with
124 a mathematical algorithm for the thrombin bound to α_2-macroglobulin so that the corrected curve shows
125 free thrombin kinetics and the end level of this curve correspond to the ETP value. ETP calculations are

126 automatically performed by the BCS equipment. The area under the corrected curve (AUC) and the
127 curve´s peak height (C_{max}) have been shown to be of diagnostic relevance. They can be obtained by an
128 specific additional software (provided by the manufacturer) from the thrombin generation curve, that is
129 the first derivative of the corrected curve.

130

131 **2. Materials**

132 This section is organized to list out all reagents and equipment required to perform the
133 aforementioned protocols.

134 **2.1. F1+2 ELISA *(Protocol 1)***

135 *1. Preparation of plasma sample specimens.*

136 a. Venous blood samples (see Notes 1 to 3 in *Protocol 1*).

137 b. Sodium citrate 3.2% solution (0.11 g/L).

138 c. Table top centrifuge and rotor capable of 2,500 g centrifugation.

139 *2. Materials provided by the ELISA kit* (ref. OPBD03; Siemens Healthcare) (see Note 4 in *Protocol*
140 *1*).

141 a. Enzygnost anti-F1+2 mouse mAb coated wells in strips format. Concentration of the mAbs
142 may range from 10 to 100 μg per well depending on the lot.

143 b. Microtiter assay plates to mount the strips in to perform the test.

144 c. Anti-human prothrombin-POD conjugated antibody. This is also a mouse mAb which
145 concentration can range from 2 to 20 mg/L depending on the lot.

146 d. Conjugate buffer: Tris-HCl buffer provided by the manufacturer that contains Tween and
147 bovine serum albumin.

148 e. Human prothrombin F1+2 Standards 1 to 4 to make the standard curve with them. Their
149 concentrations respectively range from ~20 to ~1,200 pmol/L (depending on the specific lot)
150 in a Z-fold increase/decrease manner.

151 f. Control plasma: lyophilized human plasma with F1+2 value as assigned in the
152 corresponding lot.
153 g. Sample buffer to dilute samples: Tris-HCl buffer that contains Tween and sodium chloride
154 (NaCl).
155 h. Washing solution: phosphate buffer containing Tween provided by the manufacturer.
156 i. Buffer solution for dilution of TMB substrate containing hydrogen peroxide (H_2O_2) in
157 acetate buffer.
158 j. Tetramethyl benzidine dihydrochloride (TMB) chromogenic substrate.
159 k. Stopping solution to stop the POD reaction: sulfuric acid (H_2SO_4) at 0.25 mol/L.
160 *3. Materials not provided by the kit.*
161 a. Micro-pipettes of variable volume dispensing capacities (from 50 µL to 1 mL), and multi-
162 channel pipette suitable to dispense from 50 µL up to 300 µL.
163 b. Water bath for 37 ºC incubation steps.
164 c. Microplate spectrophotometer reader to capture absorbance at 450 and 650 nm.
165 d. Microplate washer and aspiration system for easier performance of washing steps
166 (optional).
167
168 **2.2. Endogenous Thrombin Potential (ETP) Kinetics *(Protocol 2)***
169 *1. Preparation of plasma sample specimens:* it is the same as that referred for Protocol 1 (see section
170 2.1) (see Notes 1 to 3 in *Protocol 2*).
171 *2. Reagents included in the ETP kit* (see Note 4 in *Protocol 2*).
172 a. ETP Reagent containing fibrin aggregator inhibitor, chromogenic substrate, salts and
173 stabilizer.
174 b. Calcium chloride ($CaCl_2$) solution at 250 mM.
175 c. ETP Buffer consisting of Tris-HCl 50 mM at pH 7.4.

176 *3. Reagents not included in the kit.* (See Note 5 in *Protocol 2*).

177 a. Innovin Reagent (Innovin is a recombinant thromboplastin reagent).

178 b. ETP Standard (ETP Standard can be purchased to the same manufacturer than ETP).

179 *4. Equipment:* the hemostasis analyzer for the ETP assay by Siemens Healthcare is the BCS System

180 (see Note 6 in *Protocol 2*).

181

182 **3. Methods**

183 **3.1. F1+2 ELISA *(Protocol 1)***

184 1. Obtain venous blood mixing 9 parts of blood with 1 part of sodium citrate solution 3.2% (0.11

185 mol/L).

186 2. Centrifuge the mix for 15 minutes at 2,500 g.

187 3. Get the upper supernatant plasma, taking care not to carry over platelets as well.

188 4. Fresh plasma samples can be stored at room temperature (15 to 25 ºC) for 4 hours or in the

189 fridge (2 to 8 ºC) for 8 hours until use. If longer storage is required, samples can stay frozen

190 below -60 ºC in cap sealed tubes for up to 6 months (see Notes 2 and 5 in *Protocol 1*).

191 5. Following the manufacturer instructions prepare the following solutions: “Washing solution”,

192 “Conjugate solution”, and “Chromogen working solution”. All reagents and samples must be at

193 room temperature before starting the procedure.

194 6. Pick up the number of strips required considering that all determinations (standards 1 to 4),

195 control samples and patients samples) must be done in duplicate (ensuring the coefficient of

196 variation (CV) is ≤ 15%) (see Note 6 in *Protocol 1*).

197 7. Add 50 µL of Sample buffer into each well. Then add 50 µL of standards, controls and samples

198 where corresponding. Agitate the plate thoroughly (e.g. by aspirating and dispensing in well

199 contains a few times with a multi-channel pipette) to mix well the reagents.

200 8. Cover the plate with an adhesive foil and incubate for 30 minutes at 37 ºC in a water bath (see
201 Note 7 in *Protocol 1*).
202 9. Remove the foil, aspirate the content of the wells and wash 2X by addition of 300 µL of
203 "Washing solution" per well. Remove any remaining "Washing solution" by tapping the inverted
204 plate on tissue paper.
205 10. Add in 100 µL per well of "Conjugate solution", not dispensing it onto the edge of the well.
206 11. Cover with foil and incubate for 15 minutes at 37 ºC.
207 12. Remove the foil, aspirate the wells contain and wash 3X as described earlier. Tap the inverted
208 plate a few times against tissue paper to get rid of any "Conjugate solution" remainders.
209 13. Add in 100 µL per well of "Chromogen solution".
210 14. Cover with foil and incubate for 15 minutes at room temperature (15 to 25 ºC) keeping the
211 plate light-protected.
212 15. Remove the foil and add in 100 µL per well of Stopping solution.
213 16. Measure Absorbance within an hour time reading the plate with a spectrophotometer at 450
214 nm and 650 nm wavelengths.
215 17. To evaluate results calculate mean absorbance values of duplicates. Construct a reference
216 curve with the Standards values (*X*-axis F1+2 concentration range from 20 to 1200 pmol/L; *Y*-
217 axis reflecting Absorbance at 450 nm ranging from 0.01 to 3). F1+2 concentrations of controls
218 and samples can be directly extracted from the reference curve using their calculated mean
219 absorbance values. Healthy donor control samples concentrations are expected to fall within an
220 interval of reference values (see Note 8 in *Protocol 1*). F1+2 values above that reference range
221 can indicate a hypercoagulable state (see Notes 9 and 10 in *Protocol 1*); whereas values below it
222 can indicate hypocoagulable state. Please, be aware that a series of interfering substances in the
223 samples may interfere with the test output (see Note 11 in *Protocol 1*).
224

225 **3.2. Endogenous Thrombin Potential (ETP) Kinetics *(Protocol 2)***

226 1. Obtain venous blood mixing 9 parts of blood with 1 part of sodium citrate solution 3.2% (0.11
227 mol/L).

228 2. Centrifuge the mix for 15 minutes at a 2,500 g.

229 3. Get the upper supernatant plasma, taking care not to carry over platelets as well.

230 4. Fresh plasma samples can be stored at room temperature (15 to 25 ºC) for 4 hours until use. If
231 longer storage is required, freeze them below -60 ºC in cap sealed tubes.

232 5. Plasma controls for normal ETP range (control negative pool) and pathological ETP range (control
233 positive pool) must be made in advance and stored aliquoted in cap sealed tubes below -60 ºC until
234 needed. Confidence intervals of these controls should be calculated and established at ±2.5 standard
235 deviations from their median value (see Note 7 in *Protocol 2*). Controls will be treated as samples
236 and included in each run. Please, be aware that a series of interfering substances in the samples may
237 interfere with the test output (see Note 8 in *Protocol 2*).

238 6. Thrombin formation in the samples and controls is driven by addition of Innovin Reagent
239 (Siemens Healthcare) and calcium chloride.

240 7. The assay runs over 20 minutes during which the thrombin continuous kinetics is displayed by the
241 BCS System (Note 9 in *Protocol 2*).

242 8. The test output is the end value of ETP conversion or the AUC of the kinetics corrected curve
243 since both are proportional. Both parameters are automatically provided by the BCS System installed
244 software (Note 10 in *Protocol 2*).

245

246 **4. Notes**

247 Please be aware that Notes are enumerated per *Protocol* in the corresponding text above and
248 provided per *Protocol* below.

249

F1+2 ELISA. ***Protocol 1***

1. Improper collection of blood samples or insufficient mixing with citrate solution may lead to falsely elevated F1+2 values. A clean venipuncture is essential. The required minimum draw volume is 2.7 mL blood in a 3 mL tube. Do not use the first 2 mL of blood collected. Mix thoroughly the tube by gentle inversion immediately after venipuncture.
2. Do not refrigerate the whole blood tubes. Prothrombin F1+2 is unstable at room temperature and tubes must be processed and frozen within four hours of sample collection.
3. Sample rejection causes are clotted sample, overfilled or underfilled tube, mislabeled or unlabeled sample.
4. Good performance of the test is guaranteed only if reagents have the same lot number or the required combination of lot numbers is used.
5. It is not possible to measure F1+2 with Enzygnost monoclonal antibody out of samples that have been frozen and thawed repeatedly.
6. Each experiment and each plate used within must include its own standard curve using the provided standard samples 1 to 4. Besides, the “Control plasma” supplied with the kit has to be tested along with each series of samples. To validate the test, “Control plasma” sample F1+2 concentration must fall within the expected range as determined in the kit lot instructions.
7. Be aware that plate filling must be completed within ten minutes and the incubation time starts when the plate is placed in the water bath at 37 ºC.
8. Median value of F1+2 from healthy adults´ citrated plasma (n=137) was reported to be 115 pmol/L (with 5th to 95th reference range percentile of 69 to 229 pmol/L). This reference interval may vary from lab to lab and that is why each laboratory should establish its reference working interval. If any Absorbance values exceed that of the highest standard, such samples will have to be re-tested diluted (maximum 1:20) in Sample buffer. Be aware of the dilution factor to correct results obtained.

275 9. Plasma F1+2 levels are increased in patients with renal failure.

276 10. Since Enzygnost F1+2 detection procedure is based on a F1+2 specific monoclonal antibodies, be
277 aware that patients who have received preparations of mouse monoclonal antibodies may yield
278 falsely elevated results.

279 11. In relation to interfering substances present in the plasma samples, it has been reported that
280 Enzygnost F1+2 can work with levels of: bilirubin up to 60 mg/dL, free hemoglobin up to 600
281 mg/dL, lipids up to 3000 mg/dL, and rheumatoid factors up to 197 IU/mL.

282

283 **Endogenous Thrombin Potential (ETP) Kinetics. *Protocol 2***

284 1. Improper collection of blood samples or insufficient mixing with citrate solution may lead to
285 erroneous ETP values. A clean venipuncture is essential. The required minimum draw volume is
286 2.7 mL blood in a 3 mL tube. Do not use the first 2 mL of blood collected. Mix thoroughly the
287 tube by gentle inversion immediately after venipuncture.

288 2. Do not refrigerate the whole blood tubes. Tubes for ETP determination must be processed and
289 frozen within four hours of specimen collection.

290 3. Sample rejection causes are clotted sample, overfilled or underfilled tube, mislabeled or unlabeled
291 sample.

292 4. We have previously used an ETP kit provided by Dade Behring (reference number OPDS05;
293 Marburg, Germany). Siemens Healthcare now provides a product called ETP Innovance, which is
294 CE-marked and thus useful for research as well as for diagnostic purposes.

295 5. Innovin Reagent is purchased from Siemens (reference number B4212). Similarly occurs with the
296 ETP Standard (reference number OPDR05).

297 6. ETP kit can only be used in the BCS System.

298 7. Repetitive freeze-thaw cycles will damage control samples integrity. Therefore it is encouraged to
299 prepare pools and store them frozen below -60 ºC in aliquots containing the required operational

300 volumes. If the values of the control samples included in the run are outside their predetermined
301 confidence intervals, the controls, the test reagents, and the BCS analyzer must be checked.

302 8. Icteric, hemolytic and lipemic samples may interfere with the procedure.

303 9. BCS System rotor cuvette can run 20 samples in each run. Since measurement takes relatively
304 long time (20 minutes), ETP should be performed in batches since no other test can be made in
305 the BCS whilst ETP is running.

306 10. Current BCS System does have checking algorithms for the ETP assay. If visual inspection of
307 thrombin kinetics curves by operator, plausible kinetics must follow an initial linear rapid rise
308 that then reaches a lag (plateau) phase with a much slower linear rise. ETP Standard Plasma curve
309 can be used for comparison (see Figure 3). Invalid kinetics may show multiple rising phases, or
310 an uneven linear growth at the end of the reaction time. Curves that have falling sections are not
311 valid either and should be repeated.

312

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375 **Figure captions.**

376

377 **Figure 1.** Scheme of the blood coagulation cascade. Hypercoagulability biomarkers of the present chapter
378 are highlighted in bold.

379

380 **Figure 2.** Graph illustrating a typical calibration curve of F1+2.

381

382 **Figure 3.** Plot of the measured total conversion and the calculated thrombin kinetics of a plasma sample.
383 *Y*-axis shows the level of conversion of the synthetic substrate, whereas time of reaction is represented in
384 the *X*-axis.

385