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Alteración de los factores de hipercoagulabilidad en pacientes con enfermedad de Chagas crónica: ¿Pueden ser considerados marcadores de respuesta terapéutica?

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Tesis doctoral

Alteración de los factores de hipercoagulabilidad en pacientes con enfermedad de Chagas crónica: ¿Pueden ser considerados marcadores de respuesta terapéutica?

Altered hypercoagulability factors in patients with chronic Chagas disease: Can they be considered as markers of therapeutic response?

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[...]Sábete, Sancho, que no es un hombre más que otro,
si no hace más que otro[...]

"El ingenioso hidalgo Don Quijote de la Mancha"
Miguel de Cervantes Saavedra

Papá, esta tesis doctoral está dedicada a ti.

Tesis depositada por **María-Jesús Pinazo Delgado**, licenciada en Medicina y Cirugía, para optar al grado de Doctora en Medicina por la Universidad de Barcelona, bajo la dirección del Dr. Joaquim Gascón y el Dr. Joan Carles Reverter.

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Dr. Joaquim Gascón

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Índice

Abreviaciones y acrónimos

	Definición
ADN	Ácido desoxirribonucleico
A&T CL-ELISA	ELISA quimioluminiscente con mucinas de tripomastigote de *T. cruzi*
BZD	Benznidazol
CID	Coagulación intravascular diseminada
EC	Enfermedad de Chagas
EID	Enfermedades tropicales infecciosas desatendidas
ELISA	Ensayo por inmunoadsorción ligado a enzimas, del inglés /*Enzyme-Linked ImmunoSorbent Assay*/
ETP	Potencial endógeno de la trombina
F_{1+2}	Fragmento 1 +2 de la protrombina
FVIIa	Factor VII activado
ISGlobal	Instituto de Salud Global de Barcelona
I+D	Investigación y desarrollo
MMP9	Metaloproteinasa 9
NET	Trampas o mallas extracelulares de los neutrófilos, del inglés /*neutrophil extracellular traps*/
NHEPACHA	Nuevas Herramientas para el Diagnóstico y la Evaluación del Paciente con Enfermedad de Chagas
NFX	Nifurtimox
OMS	Organización Mundial de la Salud
PAP	Complejos plasmina-antiplasmina
PCR	Reacción en cadena de la polimerasa
PGLYRP1	Proteína de reconocimiento del péptidoglicano 1
P-Sel	P selectina soluble
qRT-PCR	Reacción en cadena de la polimerasa en tiempo real cuantitativa
RIC	Rango intercuartíllico
SSI	Servicio de Salud Internacional
T. cruzi	*Trypanosoma cruzi*

Resumen

Antecedentes y justificación

La eficacia del tratamiento de la infección crónica por *Trypanosoma cruzi* (*T. cruzi*) no está bien definida debido a la carencia de marcadores precoces de respuesta terapéutica. En este escenario, no existía un consenso en la definición de las características exigidas a un potencial biomarcador de respuesta terapéutica precoz al tratamiento de la infección por *T. cruzi*/enfermedad de Chagas (EC) crónica.

Por otro lado, existen pacientes con infección crónica por *T.cruzi* y sin miocardiopatía que han presentado fenómenos tromboembólicos, que podrían ser explicados por la alteración de algunos factores de hipercoagulabilidad.

Con las dos premisas anteriores, se plantea un trabajo de tesis doctoral cuya hipótesis es que existe un estado de hipercoagulabilidad en pacientes con infección crónica por *T.cruzi* que se normaliza precozmente tras el tratamiento de la enfermedad con benznidazol (BZD).

Para confirmar esta hipótesis, se establecen como objetivos por un lado, definir las características que debe de cumplir un biomarcador de respuesta terapéutica precoz; y por otro, detectar si existe un estado de hipercoagulbilidad en pacientes con infección crónica por *T.cruzi,* cuáles son los factores concretos que

definen el estado de hipercoagulabilidad y evaluar su utilidad como marcadores de respuesta terapéutica.

Métodos

Para definir las características que debe de cumplir un biomarcador de respuesta terapéutica precoz se realizó una revisión sistemática de la evidencia acumulada acerca de los biomarcadores de respuesta terapéutica en el contexto de tratamiento de pacientes con EC. En base a los resultados de la revisión, se definieron los criterios de biomarcador ideal o aceptable de respuesta terapéutica precoz de la infección por *T. cruzi*/EC al tratamiento etiológico mediante una tabla de perfil de producto (TPP).

Para establecer si existía un estado de hipercoagulabilidad en pacientes con infección crónica por *T. cruzi*/ EC y evaluar los factores que la definen como marcadores de respuesta terapéutica se diseñó un estudio prospectivo observacional en el que se definieron:

- Grupo 1 (G1): pacientes con infección crónica por *T.cruzi*
- Grupo 2 (G2): pacientes del mismo origen geográfico sin infección por *T.cruzi*.

Se realizó un ensayo piloto (subestudio 2.1) con 25 pacientes del G1 y 18 pacientes del G2, en los que se utilizó una batería de factores de hipercoagulabilidad antes del tratamiento y seis meses tras finalizar el mismo (G1). Se realizó una evaluación de la presencia del parásito en el seguimiento de los pacientes mediante técnicas de PCR.

En un segundo tiempo se aumentó el número de pacientes de cada grupo (56 en G1 y 43 en G2) (subestudio 2.2). Utilizando la misma metodología se realizó determinación de los factores de hipercoagulabilidad de forma semestral durante 36 meses desde la finalización del tratamiento. Adicionalmente, se realizó evaluación serológica de los pacientes mediante ELISA quimioluminiscente con mucinas de tripomastigote de *T. cruzi* (A&T CL-ELISA), y detección de ácidos nucleicos del parásito circulante mediante técnicas cuantitativas de reacción en cadena de la polimerasa en tiempo real (qRT-PCR).

Resultados clave

En cuanto a los resultados del **subproyecto 1**, en la revisión sistemática se detectaron varios grupos de moléculas que demostraron capacidad de detectar respuesta al tratamiento, en diferentes estadios de desarrollo, y ninguna por sí sola tiene la capacidad de evaluar la respuesta a corto plazo al tratamiento etiológico en pacientes con infección por *T. cruzi*/EC en diferentes situaciones clínicas. Por el momento, destacan para el uso en el seguimiento de pacientes después del tratamiento las técnicas de amplificación de ácidos nucleicos del parásito.

Como resultado del análisis de los diferentes biomarcadores y en el contexto de un trabajo colaborativo con un grupo de expertos en Chagas, se definió un perfil de producto ideal/ aceptable para los biomarcadores de respuesta terapéutica en la infección crónica por *T. cruzi*, aplicable en diferentes escenarios clínicos y epidemiológicos.

Los resultados del **subproyecto 2** mostraron inicialmente diferencias en los valores de ETP (p 0.0001) y el F1+2 (p<0.0001) en los individuos de G1 y G2, siendo los valores de los integrantes de G1 superiores al rango de la normalidad. Ambos factores se normalizaron seis meses después del tratamiento en pacientes del G1 (ETP p<0.0008 y F1+2 p<0.004). Este hecho se confirmó con una mayor potencia en con los resultados del subestudio 2.2, en el que se evidenció una alteración de los valores de F1+2 (70% de pacientes de G1) y ETP (50% de pacientes de G1) por encima de rangos normales, y diferentes estadísticamente de los valores de estos factores en los pacientes del G2. Adicionalmente, después del tratamiento, el 76% de los pacientes con alteración del F1+2, el 98% de los pacientes con alteración del ETP normalizaron los valores en nueve (rango intercuartílico (RIC) 8) y seis meses (RIC 3) de mediana respectivamente. Los valores se mantuvieron en rango normal sin cambios estadísticamente significativos durante todo el periodo de seguimiento.

En cuanto a los valores de A&T CL-ELISA, si bien no se negativizaron durante el seguimiento, se observó una reducción de los mismos a partir del mes 12, que fue progresiva hasta el final del seguimiento y estadísticamente significativa desde el mes 18 (p = 0,0052).

Conclusiones y recomendaciones

- Existen diversos biomarcadores en diferentes fases de estudio, que han demostrado ser útiles para evaluar la respuesta al tratamiento en pacientes con infección por *T. cruzi* en diferentes estadios, entre los que destacan las técnicas de amplificación de ácidos nucleicos.

- La definición de los criterios de un biomarcador ideal o aceptable para la evaluación del tratamiento específico de la infección por *T. cruzi* es indispensable para estandarizar el desarrollo de estas moléculas y su uso en ensayos clínicos con nuevos medicamentos.

- Existe un estado de hipercoagulabilidad en un porcentaje de pacientes con infección por *T. cruzi* definido por la alteración del F1+2, del ETP.

- Los factores F1+2, ETP, y en menor medida el PAP, tienen un importante valor como indicadores de respuesta precoz al tratamiento.

- De cara al futuro, se perfila el uso de una batería de biomarcadores que incluyan moléculas dependientes del parásito, del hospedador y técnicas de amplificación de ácidos nucleicos, para una evaluación precisa y temprana de la respuesta terapéutica a la infección por *T. cruzi*/EC.

Summary

Background and rationale

The efficacy of specific treatment in people with chronic *T. cruzi*-infection has not been established up to now, due to the lack of early markers of therapeutic response. In this scenario, there was no consensus on the characteristics that a potential early therapeutic response biomarker must or should fulfill in order to evaluate specific treatment in people with *T. cruzi-infection* / Chagas disease (CD).

There are patients with chronic *T. cruzi* infection without cardiomyopathy that presented thromboembolic events, which could be explained by the alteration of several hypercoagulability factors.

Given the above two premises, the doctoral thesis hypothesis is that there is a hypercoagulable state in patients chronically infected with *T. cruzi* which is normalized soon after treatment of the disease with benznidazole (BZD).

In order to confirm the thesis hypothesis, the first objective was to define the characteristics that a biomarker for early therapeutic response must fulfill (ideally or acceptably); and the second objective was to identify if there is a hypercoagulable state in patients chronically infected with *T. cruzi,* which specific factors define the hypercoagulable state and to evaluate their usefulness as markers of therapeutic response.

Methods

To define the characteristics required for a biomarker to asses early response to treatment, a systematic review of the accumulated evidence on biomarkers of

therapeutic response in the context of treatment of patients with *T. cruzi*-infection/CD was performed. On the basis of the review results, a Target Product Profile (TPP) was developed to define an "ideal" or "acceptable" biomarker for early therapeutic response of T. cruzi infection / CD to etiological treatment.

To establish whether there was a hypercoagulable state in patients with chronic *T. cruzi* / EC and to assess the hypercoagulability factors that define this state, and the usefulness as a biomarkers of therapeutic response, an observational, prospective study was designed. In the study, there were two patients groups:

- Group 1 (G1): patients with chronic *T. cruzi* infection
- Group 2 (G2): patients from the same geographical origin without *T. cruzi* infection.

A pilot trial (substudy 2.1) with 25 patients and 18 patients G1 and G2 respectively was performed. A battery of tests for hypercoagulable factors before treatment in both groups, and six months after treatment in G1 was done. To evaluate the presence of the parasite, PCR techniques were included during the follow-up.

After substudy 2.1 results, the number of patients in each group was increased (56 in G1 and 43 in G2) in order to increase the statistical power of the results. Using the same methodology, test for hypercoagulability factors were performed every six months for 36 months from the end of treatment. Additionally, serological evaluation of patients was performed by ELISA with chemiluminescent trypomastigote mucins of *T. cruzi* (A & T CL-ELISA), and detection of parasite nucleic acids by quantitative techniques of real time polymerase chain reaction (qRT-PCR).

Key results

Subproject 1 systematic review showed that several groups of molecules demonstrated an ability to detect response to treatment, but none of them had the enough power to assess, by themselves, if a response to etiological treatment was achieved in patients infected with T. cruzi / EC in different stages of the disease. To date, only nucleic acid amplification techniques have shown their usefulness to evaluate treatment failure and monitoring patients after specific treatment.

On the basis of review results, a Target Product Profile (TPP) was developed to define an "ideal" or "acceptable" biomarker for early therapeutic response of *T. cruzi* infection / CD to etiological treatment in the context of ongoing collaborative work with a group of experts in Chagas, applicable in different epidemiological and clinical scenarios.

The results of the **subproject 2** initially showed differences in ETP (p 0.0001) and F1 + 2 (p <0.0001) comparing individuals of G1 and G2, and G1 F1+2 and ETP values were over the normal ranges. Both factors values normalized six months after treatment in G1 patients of G1 (ETP p <0.0008 and F1 + 2 p <0.004). These results were confirmed with the substudy 2.2 results. An alteration of F1 + 2 values (70% G1 patients) and ETP values (50% G1 patients) were over normal range., and statistically different from G2 patients values. Additionally, 76% of patients with impaired F1+2, and 98% of patients with impaired ETP normalized values in nine (interquartile range (IQR) 8) and six months (IQR 3) medium respectively after treatment. After achieving normal ranges, values remained with no statistically significant changes during the follow-up period.

A & T CL-ELISA levels remained positive during the whole follow-up, but a progressive reduction was observed until the follow-up end, being statistically significant from month 18 ($p = 0.0052$) on.

Conclusions and recommendations

- There are different biomarkers in different development phases that are potentially useful in assessing response to treatment in patients with *T. cruzi*-infection in different stages, especially nucleic acid amplification techniques.
- The criteria for an "ideal" or "acceptable" biomarker for the evaluation of the specific treatment of *T. cruzi* infection biomarker is imperative to standardize the development of these molecules and their use in clinical trials with new drugs.
- There is a hypercoagulable state in a percentage of patients with *T. cruzi*-infection, defined by altering F1 + 2 and ETP.
- The F1 + 2, ETP (and PAP) factors have significant value as indicators of early response to treatment.
- The use of a battery of biomarkers that include parasite molecules, host molecules and nucleic acid amplification techniques, could be the future strategy for an accurate assessment of therapeutic response to *T. cruzi*-infection /CD.

I. Introducción

I.a. Enfermedad de Chagas

La enfermedad de Chagas (EC), descrita por Carlos Chagas en 1909, es una de las 17 enfermedades tropicales infecciosas desatendidas (EID), y afecta principalmente a poblaciones que viven en condiciones socioeconómicas pobres y con difícil acceso a servicios básicos y de salud. (1)

El agente etiológico responsable de esta enfermedad es *Trypanosoma cruzi* (*T. cruzi*), (2) parásito flagelado hematófago endémico de 21 países en América. Según datos de la Organización Mundial de la Salud (OMS), 100 millones de personas están en riesgo de adquirir la infección, de las cuales unos 6 millones la presentan actualmente.(3) Con una incidencia anual de 56.000 casos nuevos, la enfermedad de Chagas se considera la principal causa de muerte en 12.000 personas cada año.

En área endémica, la infección por *T. cruzi* se adquiere principalmente a través de la vía vectorial. (2) La transmisión vertical es la segunda en frecuencia, siendo la vía transfusional la tercera más común.(4) Ocasionalmente se han descrito infecciones a través de manipulación en laboratorio de sangre contaminada con el parásito, y trasplante de órganos. (5,6) Recientemente se han descrito varios brotes de transmisión oral. (7, 8)

T. cruzi tiene un ciclo de vida complejo con varias etapas de desarrollo, tanto en los mamíferos hospedadores como en los insectos vectores.(9) Los insectos vectores toman tripomastigotes circulantes de sangre infectada de mamíferos

como zarigüeyas, gatos, perros, especies de murinos y humanos. Dentro de los vectores, los tripomastigotes se transforman en epimastigotes flagelados con la capacidad de multiplicación extracelular y migración al intestino posterior del vector, convirtiéndose en tripomastigotes metacíclicos. Estos tripomastigotes metacíclicos, con capacidad infecciosa, se pueden transmitir a los humanos y otros mamíferos a través de las deyecciones del vector, en las cuáles se encuentran. Si las heces son depositadas por parte del insecto en alguna zona del futuro hospedador donde exista la posibilidad de penetración (solución de continuidad en la piel, contacto con mucosas externas o mucosa gástrica), invaden células locales. Dentro de las células locales que invaden, pierden el flagelo y se convierten en formas amastigotes que se multiplican intracelularmente, rompiendo la célula huésped, y se diseminan por vía hematógena como tripomastigote. (9) El paso del parásito por la sangre periférica se llama parasitemia, y permite que el parásito pueda llegar a órganos distantes y tejidos en los que pueden continuar en proliferación, causando como resultado daño a los tejidos en los cuáles anida.

Tras la transmisión del parásito, en el 95% de las personas no existen síntomas o estos pasan desapercibidos al ser síntomas inespecíficos. (2) Si no existe un diagnóstico y tratamiento precoz, la infección se cronifica en la mayor parte de los casos sin la aparición de síntomas durante décadas.(2) Sin embargo, y generalmente de forma insidiosa, entre el 25-30% de las personas que padecen la infección crónica por *T. cruzi* desarrollarán a lo largo de su vida miocardiopatía chagásica, y de estas, el 50% se manifestará en formas severas (arritmias severas, miocardiopatía dilatada y/o enfermedades tromboembólicas).(2,10) Las alteraciones digestivas de la enfermedad, debidas a las alteraciones en los plexos nerviosos del

tracto digestivo, también son características de la forma crónica. Se observan en el 15-20% de las personas afectadas,(2) y clínicamente se manifiestan como alteraciones de la motilidad, secreción y absorción de esófago, intestino delgado y colon, llegando en ocasiones a provocar megaesófago o megacolon graves.(11,12) En el caso de los pacientes inmunodeprimidos, el curso de la enfermedad puede ser más severo, debido a la posibilidad de replicación masiva del parásito, y suelen presentar por ello parasitemias más elevadas. (13)

El diagnóstico de la infección por *T. cruzi* en fase aguda o en pacientes inmunodeprimidos que están en fase crónica, consiste en el aislamiento del parásito en sangre mediante el examen directo o el cultivo. Las pruebas parasitológicas en la fase crónica son de baja sensibilidad, por lo que el diagnóstico está basado en pruebas serológicas, que son altamente sensibles, aunque no suficientemente específicas debido a la presencia de reacciones cruzadas. Actualmente se considera que para el diagnóstico serológico de *T. cruzi* se requieren dos pruebas positivas que determinen diferentes grupos antigénicos. Hasta ahora, los antígenos utilizados son de formas no infectivas de *T. cruzi* (epimastigote).(14)

En la actualidad, se dispone de dos fármacos para el tratamiento etiológico de la infección por *T. cruzi*: benznidazol (BZD) y nifurtimox (NFX). Ambos medicamentos presentan un perfil de toxicidad elevada,(15, 16) y los estudios de eficacia, diseñados para la evaluación de estos fármacos, han mostrado resultados muy variables dependiendo de la fase de la enfermedad, la dosis empleada, la edad y la procedencia geográfica de los pacientes.

La enfermedad de Chagas: una enfermedad globalizada

Históricamente, la migración ha sido un factor clave en la diseminación de la EC.(17) Este hecho continúa siendo vigente: los procesos migratorios desde las zonas rurales a las zonas urbanas en áreas endémicas, en primer lugar, y más tarde de América Latina a Norteamérica, Europa y el resto del mundo, han cambiado la epidemiología de la infección por *T. cruzi*. (18) Se estima que en Europa hay entre 68.000 y 123.000 personas con la infección por *T. cruzi*, y la mayoría de ellos viven en España. Sin embargo, hasta el año 2009 se ha reportado sólo 4.290 casos. (19, 20) En estas regiones, donde la EC es una enfermedad emergente además de olvidada, la transmisión vertical es la vía de transmisión más frecuentes, y la transmisión vía transfusional también se ha documentado. (20, 21)

Por estas razones, ante la presencia de la infección en un nuevo escenario y la posibilidad de transmisión en el mismo, la EC se considera actualmente un problema de salud pública en áreas en las que previamente no existía.(17, 18)

En este contexto y considerando la EC como una enfermedad emergente globalizada, se estima que la carga económica global de la EC es de unos 7 - 19 mil millones de dólares al año, similar o superior a la de otras enfermedades como la infección por rotavirus, o el cáncer de cuello uterino. (22) Sin embargo, la cantidad de fondos para la investigación de la enfermedad de Chagas es de alrededor de 1% de la financiación global de investigación y desarrollo (I + D). (23)

Limitaciones actuales para el manejo de una enfermedad olvidada.

Existe escaso conocimiento en cuanto a algunos aspectos fundamentales de la infección por *T. cruzi*, como la interacción huésped-parásito y los mecanismos por los cuáles la infección progresa a enfermedad. Este hecho, sumado a la falta de interés de los decisores en salud e investigación de los países principalmente afectados, ha dado como resultado que existan paradigmas antiguos en base a los cuáles se ha limitado el acceso a tratamiento de las personas que padecen la EC.

La persistencia de las malas condiciones sociales, la transmisión vectorial y el dominio de la teoría autoinmune, originaron durante décadas un descuido en el tratamiento de pacientes crónicos y en la investigación de nuevas herramientas de diagnóstico y seguimiento dirigidas al control de la progresión de esta enfermedad. (24)

En cuanto al manejo de la EC, sobre todo en pacientes que se encuentran en el estadio crónico, existen actualmente dos áreas en las que se hacen más evidentes las lagunas en el conocimiento, ambas relacionadas con el tratamiento etiológico de la infección. Este hecho repercute directamente en el acceso equitativo a la salud de la población afectada, tanto en áreas endémicas como no endémicas.

De un lado, no ha habido desarrollo de nuevos medicamentos para el tratamiento de la EC desde los años 70. Los fármacos actualmente usados para el tratamiento de la infección por *T. cruzi* (BZD y NFX) tienen una alta incidencia de reacciones adversas,(25-27) que si bien la mayoría son leves y pueden controlarse

satisfactoriamente con tratamiento sintomático, algunas de ellas progresan, y en consecuencia los pacientes no finalizan la dosificación completa. (25,26) En este sentido, en la última década se han diseñado ensayos clínicos con nuevos medicamentos, y con nuevos regímenes de dosis y tiempo de prescripción en el caso de los fármacos antiguos.(28, 29) La gran limitación para la evaluación de estos nuevos medicamentos y/o dosificación de los antiguos, es el déficit de un parámetro objetivable de respuesta precoz al fármaco por parte de los individuos que lo reciben. Actualmente, la OMS establece como patrón de oro de respuesta al tratamiento con BZD o NFX la seronegativización o seroconvesión, lo que ocurre transcurridos entre ocho y diez años en personas con infección crónica por *T. cruzi*. (30, 31)

Por otro lado, no existía un consenso en la definición de cuáles son las características que un biomarcador de respuesta terapéutica precoz debe de cumplir, en un contexto ambiguo que es la propia definición de respuesta terapéutica.(30, 31)

I.b. Coagulación y enfermedades infecciosas

La hemostasia es el proceso biológico que previene el sangrado después de un daño vascular, ya sea arterial o venoso. Implica la función de dos principales componentes que actúan de forma coordinada: las plaquetas y el sistema de coagulación.

El esquema tradicional de activación de la coagulación, basado en dos vías separadas (vía intrínseca y extrínseca) que confluyen en una vía común y ésta en la formación de trombina, actualmente no se considera ajustado a la realidad. En la actualidad se considera un modelo de activación más complejo (32,33) que consta de una fase inicial, que implica a los elementos celulares con capacidad de expresar factor tisular (como los monocitos), y los factores VII, X, V y II. Esta fase inicial lleva a la formación de una pequeña cantidad de trombina, la cual inicia la activación 'explosiva' de la coagulación (fase de amplificación) mediante la activación de las plaquetas, que se convierten en el soporte necesario para la acción de los factores IX, VIII, X, V y II dando lugar a la formación de gran cantidad de trombina. Finalmente este proceso acaba en una fase en la que se impone el efecto de los mecanismos inhibidores de la hemostasia. Los elementos celulares se acumulan en el coágulo en formación y al mismo tiempo se incrementa en la misma zona la concentración de factores procoagulantes, entre ellos la trombina recién formada, y de algunos factores anticoagulantes o de la fibrinolisis. En el coágulo en formación, los elementos celulares, especialmente las plaquetas, interaccionan con la fibrina activándose y aportando los fosfolípidos necesarios para sustentar la actividad de

los factores de la coagulación. (34) Las plaquetas requieren del correcto funcionamiento de sus glicoproteínas de membrana IIb-IIIa y Ib-alfa para dar soporte a la generación de trombina, (35) al contrario de lo que sucede en la fase de suspensión en la que únicamente se precisa de la acción glicoproteína IIb-IIIa. (36) En los últimos años se ha demostrado la gran importancia de las micropartículas circulantes en la iniciación y la amplificación de la coagulación. (37, 38) Su origen está en los monocitos y en las células endoteliales que una vez activados o lesionados pueden liberar fragmentos de membranas (microvesículas) de tamaño inferior a 1-1,5 µm que llevan en su superficie proteínas de membrana de las células de origen. En la hemostasia, el factor tisular es la principal de estas proteínas expresadas por las micropartículas, siendo el presente en las micropartículas circulantes la principal fuente del mismo en la formación de un coágulo normal. A este esquema se ha venido a sumar más recientemente la activación de la coagulación por la vía de la acción de los neutrófilos, como se verá en el siguiente apartado.

Interacción del agente infeccioso-sistema de coagulación

La trombosis se considera una desviación de la hemostasia fisiológica, y es actualmente la principal causa de mortalidad a nivel mundial (> 2.000 personas/ día).(39) Pese a esto, recientes hallazgos sugieren que bajo ciertas circunstancias la microtrombosis es un proceso fisiológico, que constituye un mecanismo efector intrínseco de la inmunidad innata, y a este proceso se le denomina "inmunotrombosis". La inmunotrombosis se define como la respuesta inmunitaria inducida por la formación de un trombo venoso, especialmente en la

microvasculatura. (40-42) Se considera un proceso crucial de la inmunidad intravascular. (43)

Los agentes infecciosos desencadenan a menudo los procesos de inmunotrombosis, mediante los cuales, se reconoce al patógeno en cuestión y a las células dañadas por el mismo, y se inhibe la difusión del microorganismo mediante su eliminación.(43) Diferentes parásitos estimulan diferentes mecanismos de respuesta inmune, que pueden implicar tanto las respuestas humorales y celulares. La eficacia de la respuesta inmune depende del agente infeccioso y la fase de la infección en la que se encuentre. La resistencia contra la infección podría ser regulada por las características genéticas del huésped, pero la persistencia de la infección es producida por una suma de factores en función de ambos, el parásito y el huésped.(44)

La coagulación tiene un papel fundamental en la respuesta a la exposición a patógenos infecciosos.(38,45) La fibrina puede ejercer acción antimicrobiana directa.(46) La fibrina y/o su precursor, el fibrinógeno, se unen y activan las células inmunitarias innatas, tales como neutrófilos, en los lugares donde se produce la infección.(47,48) Este hecho sugiere que además de la función hemostática, la coagulación juega un papel directo en la respuesta del hospedador al agente infeccioso.

En el lugar donde se produce el daño, se forman microtrombos que actúan como matrices antimicrobianas, mediando la respuesta inmunológica del hospedador frente a agente infeccioso.(49) La inmunotrombosis se activa y

mantiene gracias a la acumulación local de células de respuesta inmune innata, especialmente monocitos y neutrófilos . (50)

En este punto, es importante destacar la descripción durante los últimos años de fibras o redes extracelulares generadas por los neutrófilos llamadas neutrophil extracellular traps (NET), las cuales están compuestas de un esqueleto de ADN sobre el que se encuentran diversos componentes citoplásmicos –entre ellos diversas enzimas– y nucleares. Las NET son una barrera física que en muchos casos evita la diseminación de los microorganismos, e incluso facilita su muerte al favorecer una alta concentración local de moléculas antimicrobianas, incluyendo mieloperoxidasas, elastasas, lactoferina, metaloproteinasa 9 (MMP9) y la proteína de reconcimiento del péptidoglicano 1 (PGLYRP1).(51-54) Por otro lado, su estructura fibrosa limita el daño al tejido donde se generan, al restringir el radio de acción de las moléculas que son liberadas por el neutrófilo.

Los NETS inducen adicionalmente una intensa respuesta procoagulante, de un lado, atrapando las plaquetas activadas, y de otro, mediante la actividad proteolítica de la neutrófilo elastasa.

No obstante, una alteración de la immunotrombosis puede constituir un evento clave en el desarrollo de trastornos trombóticos, incluyendo infarto de miocardio, accidente cerebrovascular y la coagulación intravascular diseminada (CID).

En resumen, la presencia de microorganismos dentro de los vasos sanguíneos es una gran amenaza para el organismo huésped, ya que la circulación de patógenos puede infectar simultáneamente varios órganos que son cruciales

para la supervivencia. La immunotrombosis en este escenario es un mecanismo de defensa antimicrobiana intravascular que evita la difusión e invasión del agente infeccioso en los tejidos diana. La implicación de mediadores celulares y moleculares de la inmunotrombosis (por ejemplo, neutrófilos y factor tisular respectivamente) conlleva el riesgo de que un desequilibrio en la activación de los mismos puede dar lugar a trastornos trombóticos y activación aberrante de la coagulación, por ejemplo, por una exposición mantenida y no controlada al agente infeccioso.

En este punto, el conocimiento en profundidad de los procesos fisiopatogénicos de los diferentes agentes infecciosos y la forma en la que los mismos pueden alterar el equilibrio de la coagulación, facilitará el desarrollo de nuevas herramientas para su diagnóstico y tratamiento precoz de la enfermedad en sí, previniendo eventos trombóticos.

Alteración de la hemostasia en la infección por *T. cruzi*

La enfermedad tromboembólica asociada a la enfermedad de Chagas es bien conocida,(55) e históricamente se ha relacionado con la cardiopatía chagásica y la presencia de dilatación de las cavidades cardíacas, aneurismas ventriculares y trombosis intracavitaria,(56, 57) ya que estos condicionarían factores reológicos que favorecen la formación de trombos intraluminales con capacidad embolígena. Sin embargo, en los últimos años se ha postulado que otros factores podrían influir en la génesis de la enfermedad tromboembólica, ya que se han descritos eventos en personas con infección por *T. cruzi* pero sin miocardiopatía chagásica u otros factores de riesgo vascular.(58,59)

Adicionalmente a lo expuesto en el epígrafe anterior, el proceso inflamatorio asociado a la infección puede causar vasculitis generalizada con aumento de los niveles de factores endoteliales mediante la expresión de citocinas proinflamatorias.(60) Al adquirir la infección por *T. cruzi*, existe una respuesta inflamatoria inicial, en la cual macrófagos, linfocitos y células porlimorfonucleares son reclutadas como resultado de la ruptura de células infectadas y hay una gran cantidad de sustancias proinflamatorias secretadas.(61) En respuesta a la inflamación se producen, además, fenómenos de adhesión plaquetaria. El aumento de la viscosidad sanguínea, las microembolias plaquetarias y la activación leucocitaria pueden producir disminución del flujo circulatorio provocando enlentecimiento del flujo sanguíneo y con ello, infarto de los tejidos en los que se encuentran. Por todo lo anterior, pacientes en un estado de inflamación crónica, como el que ocurre en la infección crónica por *T. cruzi*, presentan un mayor riesgo de padecer fenómenos trombóticos, tanto por el estado inflamatorio crónico al que se ven sometidos, como por los fenómenos de inmunotrombosis que desencadena la inflamación crónica descritos anteriormente.

En estudios experimentales de infección por *T. cruzi* en modelos murinos se observan cambios en la viscosidad sanguínea secundarios a la respuesta inmunológica del huésped,(55) tanto por la presencia del parásito como por el daño que éste produce en el endotelio (55) mediante la secreción de neuraminidasas que afectan al ácido siálico de las células infectadas.(56) Sin embargo, estudios en seres humanos muestran resultados controvertidos en cuanto a la existencia de un estado de hipercoagulabilidad en personas con infección por *T. cruzi*, y si existe, si este estado puede ser calificado como factor de riesgo vascular.(62-64)

En estudios preliminares a esta tesis doctoral se describieron alteraciones de algunos marcadores biológicos de hipercoagulabilidad (F1+2 de la protrombina, complejos trombina-antitrombina, fibrinógeno / fibrina productos de degradación y el dímero D) en pacientes de infección por *T. cruzi*, incluso en las etapas tempranas de la enfermedad.(63,64) Resultados contrarios fueron obtenidos en el estudio de Carod-Artal y cols, en el que no se halló diferencia entre los factores trombofílicos estudiados en pacientes con y sin infección por *T. cruzi* (proteína S, antitrombina, proteína C activada, factor V Leiden, anticoagulante lúpico y anticuerpos anticardiolipina) en individuos infectados por *T. cruzi* y no infectadas, y considera que el estado procoagulante no es un factor de riesgo isquémico en estos pacientes.(62)

En este punto, en el estado de conocimiento actual de los factores que pueden producir un desequilibrio en el sistema de coagulación, y su relación con la respuesta inmunológica innata que se desencadena ante la presencia de un agente infeccioso, la hipótesis planteada para el desarrollo de esta tesis doctoral toma mayor sentido.

I.c. Biomarcadores de respuesta terapéutica en la infección por *T. cruzi*

Un biomarcador se define como un signo que se puede medir con precisión y que es reproducible, y refleja el estado puntual de un proceso de la enfermedad. Los biomarcadores se correlacionan, ya sea inversa o directamente con la progresión de la enfermedad en sí. Un marcador o biomarcador subrogado se define como el signo que se utiliza en los ensayos terapéuticos para medir de forma

directa una función biológica concreta, cómo responde clínicamente el paciente, su supervivencia y/o como predictor de la respuesta al tratamiento en sí. (65,66)

En el caso de la EC crónica, la identificación de biomarcadores precoces de respuesta terapéutica es indispensable para mejorar la atención integral de los pacientes.

Como se indicaba en el apartado I.a, la carencia de marcadores de eficacia terapéutica limita el tratamiento en dos aspectos fundamentales. En primer lugar, en el contexto del manejo del paciente, dado que actualmente no existen herramientas mediante las cuáles podamos predecir la progresión de la enfermedad o evaluar la respuesta óptima al tratamiento a corto plazo. La eficacia de un fármaco no puede ser actualmente evaluada en un corto período de tiempo después del tratamiento en pacientes con infección crónica por *T. cruzi*, ya que las técnicas de biología molecular no dan hasta el momento una respuesta absoluta a si existió eliminación del parásito y cuál es el significado real de esta eliminación en caso de haberse dado.(67-70)

En segundo lugar, el desarrollo o identificación de biomarcadores de respuesta terapéutica en el contexto de la EC es indispensable como herramienta para el diseño e implementación de ensayos clínicos con fármacos más seguros y eficaces.

Es evidente que existe la necesidad de desarrollo de marcadores de progresión y pronóstico de la EC, sobre todo en su fase crónica. Debido a ello, durante las últimas décadas se han identificado biomarcadores que están actualmente en diferentes puntos del proceso de validación.

En este contexto, los biomarcadores detectados en la EC pueden ser moléculas del parásito o del hospedador. En relación a las moléculas parasitarias, se han identificado fundamentalmente proteínas (antígenos) y se han desarrollado técnicas de amplificación de ácidos nucleicos. Con respecto a los biomarcadores del hospedador, existen dos grandes grupos de biomarcadores: por un lado, los relacionados con la respuesta inmunológica del hospedador (citocinas y marcadores celulares de superficie); por otro lado, los relacionados con el proceso inflamatorio y las alteraciones metabólicas que produce una inflamación crónica en el organismo. Las referencias bibliográficas de cada uno de los biomarcadores constan en el primer artículo que compone esta tesis.

Pese a que existe una amplia propuesta de moléculas, no se ha llegado, hasta el momento, a una definición consensuada de las características que debe de cumplir un biomarcador de respuesta terapéutica en pacientes con infección crónica por *T. cruzi.* Este hecho ha sido la causa de que muchos de los ensayos llevados a cabo con las moléculas seleccionadas no hayan sido homogéneos ni respondan, en muchos de los casos, a unos criterios básicos.

Durante el presente trabajo de tesis, esta limitación se hizo evidente tras completar el subestudio piloto (2.1), ya que inicialmente la investigación consistía en probar algunos de los factores de hipercoagulabilidad como biomarcadores de respuesta terapéutica en pacientes con infección crónica por *T. cruzi* o EC crónica.

En este punto, y en el marco del trabajo colaborativo en una red de investigación internacional en nuevas herramientas de manejo para la enfermedad de Chagas (Red NHEPACHA), se planteó el primero de los subproyectos de que consta este trabajo de tesis, sin el cual no se hubiera podido avanzar en las

conclusiones del subestudio 2.2. Se realizó una revisión sistemática de la evidencia actual con el fin de evaluar moléculas como posibles biomarcadores de respuesta terapéutica y sus características de una etapa específica de la enfermedad, y se estableció la definición de biomarcador de respuesta terapéutica al tratamiento específico de pacientes con infección crónica por *T. cruzi*.

II. Hipótesis

Hipótesis

La hipótesis de este proyecto es que existe un estado de hipercoagulabilidad en pacientes con infección crónica por *T. cruzi* que se normaliza de forma precoz tras el tratamiento etiológico de la enfermedad con benznidazol.

Si esta hipótesis se confirma, tales marcadores de hipercoagulabilidad podrían ser propuestos como biomarcadores de respuesta precoz al tratamiento antiparasitario en pacientes con enfermedad de Chagas crónica.

Para dar respuesta a esta hipótesis, se diseñaron dos subproyectos. El primero de ellos consistió en realizar una revisión sistemática para definir las cuáles son los biomarcadores de respuesta al tratamiento etiológico de pacientes con enfermedad de Chagas crónica que se han desarrollado hasta el momento, y definir las características que debe de cumplir un biomarcador para ser utilizado con este fin.

El segundo subproyecto consiste en la detección de marcadores de hipercoagulabilidad en una cohorte prospectiva de pacientes, comparado con individuos sanos procedentes de áreas endémicas para la enfermedad. Este segundo subproyecto se dividió en dos partes: a) un estudio piloto con seguimiento a corto plazo (seis meses) de las personas con infección por *T. cruzi* que habían realizado tratamiento; y b) una cohorte que ampliaba el estudio piloto en cuanto a número de participantes y tiempo de seguimiento (36 meses).

III. Objetivos

III.a. Subproyecto 1

1.- Evaluar los biomarcadores que existen de respuesta terapéutica a benznidazol en pacientes con enfermedad de Chagas crónica.

2.- Establecer un perfil de producto ideal/ aceptable como marcador de respuesta terapéutica en la infección crónica por *T. cruzi*, aplicable en diferentes escenarios clínicos y epidemiológicos.

III.b. Subproyecto 2

III.b.1. Subestudio 2.1

1.- Determinar si existe un estado de hipercoagulabilidad en pacientes con infección crónica por *T. cruzi*, y definir cuáles son los parámetros de coagulación que lo determinan.

2.- Determinar si existe normalización de los parámetros que marcan este estado a los seis meses de finalizar el tratamiento con benznidazol.

III.b.2.Subestudio 2.2

1.- Determinar si existe un estado de hipercoagulabilidad en pacientes con infección crónica por *T. cruzi*, y definir cuáles son los parámetros de coagulación que lo definen.

2.- Determinar si existe normalización de los parámetros que marcan este estado entre los seis y doce meses tras finalizar el tratamiento con benznidazol.

3.- Determinar si la normalización precoz de estos parámetros se mantiene en el tiempo y establecer su utilidad como marcadores precoces de respuesta terapéutica.

4.- Establecer la utilidad del ELISA quimioluminiscente ELISA con mucinas de tripomastigote de *T. cruzi* (A&T CL-ELISA) como marcador de evolución de la enfermedad para valorar respuesta a tratamiento etiológico, y ver la coherencia entre la evolución de los niveles de los títulos de este ELISA con los niveles de los parámetros de hipercoagulabilidad estudiados a lo largo del tiempo después del tratamiento, y con los análisis de qRT-PCR como parámetro de respuesta al tratamiento sostenida en tiempo.

IV. Material y métodos

IV. Material y métodos

Los trabajos incluidos en esta tesis doctoral han sido coordinados desde el Servicio de Salud Internacional (SSI) del Hospital Clínic de Barcelona. El SSI tiene una amplia experiencia en el manejo de patología importada. Es un centro de referencia nacional y con reconocimiento a nivel internacional en el manejo e investigación de enfermedades desatendidas. La investigación del SSI se canaliza a través del Instituto de Salud Global de Barcelona (ISGlobal), dónde se ha creado la Iniciativa sobre la enfermedad de Chagas (http://www.isglobal.org/web/guest/chagas).

El primero de los subproyectos se ha realizado en el contexto de una red de investigación internacional mediante la cual se establecen trabajos colaborativos para el diseño y la validación de nuevas herramientas para el diagnóstico y manejo de la enfermedad de Chagas (Red NHEPACHA).

IV.a. Población de estudio

El subproyecto 1 se basó en una revisión sistemática de la literatura.

En el caso del subproyecto 2, la población de estudio fueron pacientes procedentes de áreas en que la infección por *T. cruzi* es endémica, que acudieron al SSI del Hospital Clínic de Barcelona para cualquier tipo de consulta en el periodo de reclutamiento de cada uno de los subestudios 2.1 y 2.2, y que no presentaron ningún criterio de exclusión. Se excluyeron pacientes que presentaban comorbilidad importante: diagnóstico previo de cardiopatía de otra etiología (isquémica, alcohólica o hipertensiva), enfermedades inflamatorias o inmunológicas sistémicas

o infecciones activas por otro agente causal. Se excluyeron pacientes con infección por *T. cruzi* que habían recibido previamente tratamiento antiparasitario para la infección por *T. cruzi* (BZD y/o NFX).

Se definieron dos grupos:

- Grupo 1 (G1): pacientes con infección crónica por *T. cruzi*/ enfermedad de Chagas en cualquiera de sus fases (crónica indeterminada o crónica sintomática).
- Grupo 2 (G2): pacientes sin infección por *T. cruzi* de zona endémica para esta enfermedad.

En el subestudio 2.1, la población de estudio fue de un total de 25 pacientes del G1 y 18 pacientes del G2. En base a los resultados del subestudio 2.1, se aumentó el tamaño de muestra a 56 pacientes en G1 y de 43 pacientes en G2.

Todos estos pacientes estuvieron de acuerdo en participar en los estudios, y firmaron un consentimiento informado específico tras la explicación en profundidad de los objetivos y procedimientos de los estudios a los que aceptaban entrar.

IV.b. Metodología de los estudios

La tesis está estructurada en base a dos subproyectos.

El **subproyecto 1** es una revisión sistemática de la evidencia acumulada acerca de los biomarcadores de respuesta terapéutica en el contexto de tratamiento de pacientes con enfermedad de Chagas. Para ello, se definieron tres áreas de análisis en las que se dividieron las publicaciones incluidas: biomarcadores inmunológicos, biomarcadores bioquímicos y técnicas de amplificación de ácidos

nucleicos. En base a los resultados de la misma, se definieron los criterios de biomarcador ideal o aceptable de respuesta terapéutica precoz de la infección por *T. cruzi* al tratamiento etiológico.

El **subproyecto 2** fue un estudio prospectivo observacional en el que se definieron dos grupos: Grupo 1 (G1), pacientes con infección crónica por *T. cruzi*/ enfermedad de Chagas en cualquiera de sus fases (crónica indeterminada o crónica sintomática); Grupo 2 (G2), pacientes sin infección por *T. cruzi* de zona endémica para esta enfermedad. Una vez establecidos los grupos, a los pacientes del G1 se les ofreció tratamiento con BZD (cinco mg/ kg/ día por 60 días). Durante el tratamiento se realizó seguimiento quincenal clínico y analítico (hemograma completo, y bioquímica con perfil hepático y renal) de estos pacientes para el monitoreo y control de efectos adversos secundarios a este fármaco.

Este estudio se realizó en dos tiempos:

El subestudio 2.1 fue un ensayo piloto con 25 pacientes del G1 y 18 pacientes del G2. Se realizó una evaluación de los pacientes del G1 a los seis meses post-tratamiento, en la que se incluyeron pruebas serológicas convencionales y PCR para *T. cruzi* y la determinación de los factores de hemostasia e hipercoagulabilidad.

En base a los resultados del proyecto piloto, se realizó el subestudio 2.2, mediante el cual se aumentó el tamaño de muestra y se alargó el periodo de seguimiento de los pacientes, obteniéndose una muestra final de 56 pacientes en G1 y de 43 pacientes en G2. En los pacientes del G1 se realizó un seguimiento semestral de 36 meses post-tratamiento con la finalidad de establecer si las modificaciones de los factores de hipercoagulabilidad observadas eran mantenidas

en el tiempo. Durante el seguimiento de la cohorte completa, además de las pruebas realizadas al grupo de pacientes del proyecto piloto, se realizó ELISA quimioluminiscente con mucinas de tripomastigote de *T. cruzi* (A&T CL-ELISA).

La metodología específica de cada uno de los subproyectos está detallada en los artículos publicados e incorporados a la presente tesis.

V. Resultados

ARTÍCULO 1

Biological markers for evaluating therapeutic efficacy in Chagas disease, a systematic review

María-Jesús Pinazo, Maria Carmen Thomas, Jacqueline Bua, Alina Perrone, Alejandro-Gabriel Schijman, Rodolfo-Jorge Viotti, Janine-M Ramsey, Isabela Ribeiro, Sergio Sosa-Estani, Manuel-Carlos López, Joaquim Gascon

Expert Rev Anti Infect Ther. **2014;** 12(4), 479–496

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EXPERT REVIEWS

Biological markers for evaluating therapeutic efficacy in Chagas disease, a systematic review

Expert Rev. Anti Infect. Ther. 12(4), 479–496 (2014)

Maria-Jesús Pinazo*, M Carmen Thomas, Jacqueline Bua, Alina Perrone, Alejandro-Gabriel Schijman, Rodolfo-Jorge Viotti, Janine-M Ramsey, Isabela Ribeiro, Sergio Sosa-Estani, Manuel-Carlos López and Joaquim Gascon

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The most neglected aspects of Chagas disease (CD) have been patient care and treatment. Despite recent progress in the development of potentially improved drugs, there is no consensus among different research groups on the lack of therapeutic response markers to evaluate efficacy of newly proposed drugs early after treatment. A systematic review of current evidence regarding molecules which are potential biomarkers for therapeutic response has been conducted using quality assessment and target responses as primary criteria. The review provides a panorama of the cumulative evidence and specific needs for development of a battery of complementary biomarkers which together fulfill ideal or acceptable criteria to evaluate early responses to treatment for chronic CD. There are several marker candidates which together may fulfill acceptable criteria to indicate the efficacy of a trypanocidal treatment. Data from ongoing studies are considered essential to improve assessment of existing markers and to identify those for early follow-up of treated patients.

KEYWORDS: biological marker • biomarker • Chagas disease • cure marker • humoral and cellular immune response • PCR • treatment • *Trypanosoma cruzi*

A lack of appropriate clinical and biomarker tools limits the direct measurement of treatment impact for any infectious disease. This is a common limitation for many of the 17 internationally recognized neglected diseases. One striking example is Chagas disease (CD), an endemic zoonosis caused by the protozoan parasite *Trypanosoma cruzi*, which affects 7–8 million people currently, not only in Latin America where the disease is autochthonous [1] but also is worldwide due to population migrations [2,3].

Despite the burden of CD morbidity and lower cost of timely diagnosed and treated patients [4], only two drugs, benznidazole and nifurtimox, are currently available for treatment. Both drugs have variable efficacy depending on the disease stage, drug dose, patient age and geographical origin. *T. cruzi* infection treatment is currently strongly recommended in both acute and chronic stages of the infection [5–7]. The association of *T. cruzi* infection and disease progression, and therefore its clinical cure, remains unclear mainly because physiopathological changes develop slowly and symptoms may appear several years after infection. Disease symptoms may appear when there is an imbalance between the host immune response and parasite proliferation in tissues. Along with tissue damage caused by the presence and persistence of the parasite [8], there are inflammatory processes and cross-reactivity with host molecules [9]. Currently, the association of parasite persistence and symptom development is a recurring controversy.

In the chronic stage, drug therapy efficacy is variable and is difficult to compare since most studies use different treatment regimens and response assessment methods (variable assays, frequency and duration of follow-up) [10], in addition to the lack of class I studies [11,12]. Even in successful treatment, the gold standard for evaluating efficacy (seroconversion using conventional serological tests) may take years to decades to assess [13,14]. Hence, long-term

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Table 1. Keywords used for literature searches using the NLM gateway.

Category	MeSH term	MeSH subheading
Response to treatment by biochemical biomarkers	CD OR *Trypanosoma cruzi*	AND biological markers AND treatment
		AND cure markers AND treatment
		AND biomarker AND treatment
Response to treatment by immunological biomarkers	CD OR *T. cruzi*	AND biological markers
		AND biomarkers
		AND immunological markers
		AND biological markers AND treatment
		AND biomarkers AND treatment
		AND immunological markers AND treatment
		AND biological markers AND immunology
		AND biomarkers AND immunology
		AND immunological markers AND immunology
PCR for the evaluation of treatment	CD	AND polymerase chain reaction

CD: Chagas disease.

treatment of chronic CD cases has been neglected in two primary areas:

- A lack of interest since the 1970s to develop new drugs specifically targeting intracellular parasites in general and for *T. cruzi* in particular. Fortunately, in the last 5 years, new drugs or new schemes of current drugs have been proposed as potential alternatives and are being tested in different phases of development for safety and efficacy against *T. cruzi* in humans.
- A lack of consensus regarding therapeutic response markers for early assessment of antitrypanocidal drug efficacy for disease management and of clinical trials with new drugs for *T. cruzi* treatment.

Several prognosis and progression markers for *T. cruzi* infection have been proposed during the last 20 years. However, only a few of these have been evaluated using appropriately designed studies for therapeutic response. In order to assess molecules as potential biomarkers for therapeutic response and their disease stage-specific characteristics, a systematic review of current evidence was conducted focusing on the quality of biomarker studies in both aspects. Only studies assessing biological markers (immunological, biochemical and molecular biomarkers or nucleic acid-based biomarkers) during treatment follow-up of chronic *T. cruzi* patients have been included. On the basis of review of current evidence, a target product profile (TPP) was developed for an 'ideal' and/or an 'acceptable' biomarker for anti-*T. cruzi* treatment response in different epidemiological and clinical scenarios.

Methods

The reviewed publications addressing CD-specific treatment response markers were organized into three areas of analysis: immunological markers, biochemical biomarkers and nucleic acid amplification strategies.

Acquisition of evidence

The literature was reviewed based on electronic searches in The Cochrane Central Register of Controlled Trials on The Cochrane Library, NLM GATEWAY (PubMed/MEDLINE, Clinicaltrials.gov, Bookshelf and Meeting Abstracts), WHOLIS, BVS (BIREME and LILACS), SCIELO (1990–2012). The indexing terms used in the searches are presented in Table 1.

A secondary search was performed using the first or last article's author or the marker under analysis as keywords AND 'CD' OR 'Chagas' AND 'treatment', AND 'human', AND 'patient', OR 'patients'.

We considered reports only of original research, mainly but not exclusively intervention trials, specificity of diagnostic methods and observational studies, with scope targeted at biomarkers of treatment response for patients with chronic CD. Articles published in Spanish, English and Portuguese from 1990 through 31 December 2012 were reviewed according to the following inclusion criteria:

- Studies concerning development, standardization and/or validation of:
  - Biochemical biomarkers
  - Immunological biomarkers
  - Nucleic acid amplification strategies
- The aim of the study was to:
  - Monitor antiparasitic treatment
  - Test the response to antiparasitic treatment or to testing antiparasitic treatment outcomes
- Studies performed in humans

Articles that had the following characteristics were excluded:

- Studies previous to 1990
- Studies performed in experimental models or PCR studies using vector samples
- Studies not specifically designed to evaluate treatment impact by specific biomarkers and/or PCR techniques
- Studies that evaluated response to antiparasitic treatment with image techniques (x-ray, echocardiography, scintygraphy, etc.)
- Nucleic acid amplification studies, if the purpose of the technique was genotyping, DNA cloning or genomics and retrotranscription–PCR assays for gene expression.

Systematizing results

A matrix was constructed with the following information categories for each biomarker and article: which test was evaluated, distribution by sex, age of study population, geographical area, times of study patients follow-up, sample size, missing data, study design, stages of the disease included in the study, treatment (drug, dose, length of treatment), reference test performed, values of the reference standardized test, dispersion values of the standardized test, sensitivity and specificity of the biomarker, biomarker efficacy evaluation and study biases (Table 2). The main characteristics and limitations of each biomarker are highlighted.

Results of the searches

The results of the searches are summarized in Figure 1 and a review of the titles, abstracts and in some cases the full texts were examined to select relevant papers for the review. A more detailed review and data collection from each study were conducted after reviewing title and/or abstracts, in order to evaluate its relevance.

Evidence synthesis

The results of the searching have been summarized in Table 2.

Analysis of the results by type of molecules & technique

Immunological molecules

Four categories of 25 markers have been used to measure therapeutic efficacy for CD. The first group includes four markers that detected specific antibodies for host antigens. The second group (14 markers) involves methods to detect antibodies generated against parasite antigens. The third group includes those that measure the cytokine level and/or cytokine pattern in a patient's serum (three markers), and a fourth group includes four markers to quantify cellular immune response populations or populations expressing specific cytokines.

Host antigens

Several human antigens have been proposed as biomarkers of treatment response in CD. Increased levels of sP-selectin and soluble vascular cell adhesion molecule '1' (sVCAM-1) have been observed in 41 asymptomatic chronic CD pediatric patients. Before treatment, 83 and 71% of these exceeded the cut-off control value for sP-selectin and sVCAM-1, respectively. There was a significantly greater decrease in the titers of sP-selectin (66.7%) and sVCAM-1 (41.0%) in those children who received benznidazole therapy compared with a control group receiving placebo [15].

Levels of anti-R3 antibodies, a peptide encoded in the human autoantigen 'Cha', increased with the progression of clinical manifestations of chronic CD. Anti-R3 antibody titers decreased in 19 patients treated with antiparasitic drugs (benznidazole or nifurtimox), despite the fact that all had higher titers than those observed in healthy donors [16].

The production of anti-M2 muscarinic receptor autoantibodies (anti-M2R Ab) and IFN-γ profiles was characterized in 30 *T. cruzi*-infected children in the early stage of chronic CD, before and after trypanocidal benznidazole chemotherapy [17]. Before treatment, anti-M2 receptor autoantibodies were detected in 56% of *T. cruzi*-infected patients and none of the 19 uninfected control subjects. Infected children also exhibited a significantly higher serum IFN-γ level than that observed in healthy controls. At 6 months post-treatment with benznidazole, there was a significant decrease in anti-M2R Ab and IFN-γ levels in all patients, throughout follow-up, with a 29.7–88.1% decrease in anti-M2R Ab and 10–100% decrease of IFN-γ.

Parasite antigens

A complement-mediated lysis test (CoML) using living trypomastigotes was compared with conventional serological methods at different times following treatment [18]. Seroconversion of the CoML occurred in 8 out of 21 patients (38%) between 6 and 24 months following treatment, in 4 out of 21 patients (19%) between 24 and 36 months and in one patient within 4 years post-treatment. The use of the CoML test has, however, several limitations, in particular the need for living infective trypomastigotes. A possible substitute for the CoML test, an ELISA technique based on a low-molecular weight-recombinant protein of *T. cruzi*, rTc24 was also developed [19]. All patients with active infection (positive CoML) recognized rTc24 using ELISA and western blot, while 80% of seropositive patients with negative CoML were seronegative to rTc24. There was a decrease in anti-rTc24 antibodies in 38% of patients using ELISA between 6 and 24 months post-treatment and in 19% of patients at 36 months post-treatment.

Three groups of *T. cruzi*-infected patients, untreated cases, patients with treatment failure and successfully treated patients, were tested for antiparasite antibodies using an immunofloures-cence assay of fixed trypomastigotes (referred as ISIFA) [20]. A successfully treated patient was defined as a case with undetectable parasitemia using xenodiagnosis at 6 years post-treatment. ISIFA was able to differentiate successfully treated cases from untreated or those with treatment failure [21,22]. Treatment efficacy was monitored by using disappearance of antibodies by serological methods (complement fixation, indirect immunofluorescence, indirect hemagglutination and ELISA using total *T. cruzi* protein as antigens). Only 8% of 113 patients in the

Table 2. Review results.

Study (year)	Test	Sex distribution	Age (rank/ mean–SD)	Geographical region (country)	Follow-up (months: rank/ mean–SD)	Missing (%)	N	Study design	Chagas disease stage[†]	Treatment regime
Biochemical and metabolic biomarkers										
Pinazo *et al.* (2011)	ETP, F 1+2	YES	20–45	Latin America	6	23	43	Prospective	ALL	BZD, 5 mg/kg/day, 60 days
PCR techniques										
Britto *et al.* (1995)	PCR	NO	ND	Brasil	48	0	34	Prospective	C/ I	BZD, 50–600 mg/ day, 45–180 days
Lauria-pires (2000)	PCR	NO	31–60	Brasil	120	15.50	45	Prospective	ND	BZD/NFX, ND, 60/30/ 20 days
Braga MS (2000)	PCR	YES	ND	Brasil	120	ND	17	Prospective	ND	BZD/NFX,10 mg/kg/ day, at least 30 days
Britto *et al.* (2001)	PCR	ND	ND	Brasil	84–420/240	0	100	Retrospective	ND	BZD, 5–6 mg/kg/day, 30–60 days; NFX, 7–8 mg/kg/day, 60–90 days
Solari *et al.* (2001)	PCR	ND	0–10/6	Chile	36	15	66	Prospective	ASYMTOMATIC	NFX, 7–10 mg/kg/ day, 60 days
Galvao *et al.* (2003)	PCR	NO	7 a 12	Brazil	36	13	127	Prospective	ND	BZD 7.5 mg/kg/day, 60 days
Schijman (2003)	PCR	YES	0–17/4.2	Argentina	24/36	0	40	Prospective	ND	BZD,10–16 mg/kg/ day, 60 days; NFX,10–15 mg/kg / day, 60 days
Sánchez *et al.* (2005)	PCR and FC-ALTA	YES	22 (MEAN)	Chile	120	0	54	ND	ND	ALLO 8.5 mg/kg, 60 days; ITRA 6 mg/kg, 120 days
Lacunza *et al.* (2006)	PCR	YES	18–30	Argentina	6	17	23	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Meira *et al.* 2006	HEMOCULTURE, LMCo, PCR	ND	ND	ND	6–48/27.7	ND	31	ND	ND	BZD
Fernandes *et al.* (2009)	PCR	YES	17–42/30	Brasil	36	0	130	Prospective	ASYMTOMATIC	BZD, 10 mg/kg/day, 60 days
Lana *et al.* (2009)	PCR, SEMIQUANTITATIVE ELISA, FC-ALTA IgG	YES	6–37/27–8	Brasil	108	0	28	Retrospective	C/D/I	BZD, 5–10 mg/kg/ day, 40–60 days
Murcia *et al.* (2010)	PCR	NO	ND/33–11	Bolivia	14	68	181	Prospective	ALL	BZD, 300 mg/day (adults), 60 days 5–7 mg/kg/day (pediatrics), 60 days
Perez-ayala *et al.* (2010)	PCR	YES	29–44/36	Bolivia	12	41	195	Prospective	ALL	BZD, 5 mg/kg/ day,60 days; NFX, 8–10 mg/kg/ day, 90 days
Aguiar *et al.* (2012)	PCR	YES	8–56/ 36–7.24	Brasil	12–348	0	39	Retrospective	ND	BZD, 5–7 mg/kg/day, 60 day
Machado de assis *et al.* (2012)	PCR, Hemoculture, REC-ELISA, TESA-BLOT	YES	2–60/ 32,9–10,9	Brasil	120–432/ 16.9–6.8	0	94	Retrospective	ALL	BZD, 5 mg/kg/day, 60 days
Ramos (2012)	PCR	YES	0–72/35	Bolivia	ND	25	76	Retrospective	ALL	BZD, 5 mg/kg/day, 40–60 days

[†]Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ND	ND	ND	ND	ND	No	Yes	Yes	No	No	ETP: 73,3 F 1 +2: 80	ETP: 100 F 1 + 2: 73.3	[69]
Serology	Yes	No	ND	ND	No	Yes	No	No	No	ND	71.8	[71]
Serology	No	No	ND	ND	No	No	No	No	No	ND	3.15	
Serology	No	No	ND	ND	No	No	Yes	No	No	ND	0	
Serology	No	No	ND	ND	No	No	No	No	No	ND	65	[72]
Serology	Yes	No	ND	ND	No	No	No	No	No	100	100	[85]
Serology	Yes	ND	ND	ND	No	No	No	No	No	85.9	60.4	[81]
Serology	Yes	No	ND	ND	No	Yes	No	No	No	77.5	100	
Serology	Yes	No	ND	ND	No	No	No	No	No	ND	14.8	[76]
Serology	Yes	No	ND	ND	No	No	No	No	No	100	85.7	[78]
Serology	No	No	ND	ND	ND	ND	ND	ND	ND	77.4	50	[27]
Serology	Yes	No	ND	ND	No	ND	No	No	No	100	11.3	[73]
Serology	Yes	No	ND	ND	No	No	No	No	No	ND	14.8	[74]
Serology	Yes	No	ND	ND	ND	ND	No	No	No	68	90	[79]
Serology	No	No	ND	ND	No	Yes	No	No	No	63	100	[80]
Serology	No	No	ND	ND	Yes	Yes	No	No	No	ND	41.4	[83]
Serology	No	No	ND	ND	No	ND	No	No	No	ND	47.2	[84]
Serology	No	No	ND	ND	No	ND	No	No	Yes	65.8	100	

Table 2. Review results (cont.).

Study (year)	Test	Sex distribution	Age (rank/ mean–SD)	Geographical region (country)	Follow-up (months: rank/ mean–SD)	Missing (%)	N	Study design	Chagas disease stage[†]	Treatment regime
Immunological biomarkers										
Vitelli avelar *et al.* (2008)	IL-12, IL-10, IL-13, TNF-α	YES	43–70	Brasil	12	ND	29	Prospective	I/C	BZD
Laucella *et al.* (2009)	INF-γ	NO	23–55	Argentina	36	ND	43	Prospective	I/C	BZD, 5 mg/kg/day, 30 days
Gironés *et al.* (2001)	R3-CHA	NO	ND	Venezuela, Argentina	ND	ND	19	Prospective	I	BZD, NFX
Cancado Jr *et al.* (2002, 1999)	Parasite total protein (STc)	YES	69–9	Brasil	72–216	ND	113	Prospective	ALL	BZD (18 GR)
Viotti *et al.* (2011)	Parasite-recombinant proteins (14)	YES	8–41.4	Argentina	36	ND	142	Prospective	I/C	BZD, 5 mg/kg/day, 30 days
Galvao *et al.* (1993)	CoML	NO	ND	Brasil	120	ND	82	Prospective	ND	BZD, 5–7 mg/kg/DÍA, 30–60 days; NFX, 8–10 mg/kg/ day, 30–60 days
Meira *et al.* (2004)	recombinant complement regulatory protein	NO	ND	Brasil	37–12	13 (12 m), 48 (24 m), 32 (36 m), 80 (> 37 m)	31	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Levy *et al.* (1996)	ISIFA	YES	25–50	Brasil	ND	ND	26	Retrospective	ND	BZD, NFX
Moretti *et al.* (1998)	IV Fraction, EXO	NO	4–53	Argentina	24–240	ND	44	Prospective	I	BZD, NFX
Sanchez-negrete *et al.* (2008)	13 Antigen	YES	19–41	ND	36	28 (36 m), 17 (42–60 m), 1.5 (66 m)	18	Prospective	I/C	BZD
Andrade *et al.* (2004)	AT	YES	7–12	Brasil	72	9.4	53	Prospective	I	BZD, 7.5 mg/kg/day, 60 days
De Andrade *et al.* (1996)	AT	YES	7–12	Brasil	36	25.0	64	Prospective	I	BZD, 7.5 mg/kg/day, 60 days
Sosa estani *et al.* (1998)	F29	NO	6–12	Argentina	48	13.7	51	Prospective	I	BZD, 5 mg/kg/day, 60 days
Krautz *et al.* (1995)	rTc24	NO	ND	Brasil	ND	ND	72	Retrospective	I/C	BZD, NFX
Fernandez-villegas *et al.* (2011)	KMP11, HSP70, PAR2, Tgp63	YES	18–68	Latin America	24	24.0	46	Prospective	I/C/D	BZD, 5 mg/kg/day, 60 days
Cooley *et al.* (2008)	16 *T. cruzi* recombinant proteins	NO	29–61	Argentina	36	ND	38	Prospective	ND	BZD, 5 mg/kg/day, 60 days
Fabbro *et al.* (2011)	P2β	YES	29–35	Argentina	240–300	ND	78	Retrospective	I/C	BZD, 5 mg/kg/day, 30 days; NFX, 8–10 mg/kg/ day, 45–60 days

[†]Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ND	NO	ND	ND	ND	No	No	No	No	No	ND	ND	[36]
Serology	Yes	ND	ND	ND	No	No	No	No	No	71.0	IFN-γ decrease: 34.6% (12 m), 46.8% (24 m), 71.8% (36 m)	[41]
Serology (R3 and Shed Acute Phase Antigen Ag, total extract)	Yes	ND	92.4	100	No	No	No	No	No	92.4	100.0	[16]
ELISA, IFI, HAI, Xenidiagnosis. Hemoculture	ND	ND	ND	ND	No	No	No	No	No	100.0	8.0	[21,22]
HAI, IFI	Yes	ND	ND	ND	No	No	Yes	No	No	ND	40.0	[33]
IFI, Hemoculture	Yes	ND	ND	ND	No	No	No	No	No	ND	38% (6 m), 38% (24 m), 19% 36 (m)	[18]
CoML, Hemoculture, PCR, serology	Yes	ND	ND	ND	No	No	No	No	No	100.0	29.7% (12 m), 37.5% (24 m), 28.6% (36 m), 66.6% (48 m)	[27]
CoML, MbIFA, XENODIAGNOSIS	Yes	Yes	80–98.6	98	No	No	Yes	No	No	ND	ISIFA: 84%	[20]
IFI, HAI, SEROLOGY	Yes	ND	ND	ND	No	Yes	Yes	Yes	No	ND	F IV: 36% EXO: 44%	[26]
HAI, CMA ELISA, IFI	Yes	Yes	72.2	ND	No	Yes	Yes	No	No	72.2	66.6%	[31]
ND	No	ND	ND	ND	No	No	No	No	No	100.0	88.7%	[24]
HAI, ELISA, IFI	Yes	Yes	ND	ND	No	No	No	No	No	100.0	57.8%	[23]
HAI, ELISA, SEROLOGY	Yes	Yes	ND	ND	No	No	No	No	No	100.0	35.7% (6 m), 62.1% (48 m)	[25]
GST ELISA	Yes	ND	ND	ND	Yes	No	No	No	No	ND	ND	[19]
ELISA	Yes	ND	KMP11:90 HSP70: 90 PFR2: 75 Tgp63: 30	KMP11:85 HSP70: 90 PFR2: 92 Tgp63: 70	No	No	No	No	No	100.0	KMP11: 67% HSP70: 50% PFR2:34% K11-HP70-PFR2-Tgp63: 80%	[34]
ELISA, IFI, HAI	No	ND	ND	100	No	No	No	Yes	No	ND	ND	[42]
Xenodiagnosis	No	ND	ND	ND	No	Yes	Yes	No	Yes	ND	75.4% in I and 45% in C (23 years)	[35]

Table 2. Review results (cont.).

Study (year)	Test	Sex distribution	Age (rank/ mean–SD)	Geographical region (country)	Follow-up (months: rank/ mean–SD)	Missing (%)	N	Study design	Chagas disease stage†	Treatment regime
Cutrullis *et al.* (2011)	M2	YES	8–17	Argentina	6	33.3%	30	Prospective	I	BZD, 5–8 mg/kg/day, 60 days
Dutra *et al.* (1996)	CD3+ y CD5+/ CD19+, CD3+HLAR+	NO	29–66	Brasil	60	ND	ND	Prospective	ND	BZD, 5–7 mg/kg/day, 30–60 days; NFX, 8–10 mg/kg/ day, 30–60 days
Argüello *et al.* (2012)	$CD4^+$/LIR-1	NO	36–68	Argentina	12–50	ND	87	Prospective and retrospective	I/C	BZD, 5 mg/kg/day, 30 days
Laucella *et al.* (1999)	sVCAM y sPLectina	NO	6–12	Argentina	48	ND	23	Prospective	I	BZD, 5 mg/kg/day, 60 days
Sathler-avelar *et al.* (2012)	IL12, IL10 e IFN-γ	YES	33–56	Brasil	84	ND	14	Prospective	I	BZD, 5 mg/kg/day, 60 days
Sathler-Avelar *et al.* (2008)	Monocytes: CD16/ CD14 NK: CD3/CD16/CD56	NO	9–14	Brasil	12	ND	6	Prospective	I	BZD, 8 mg/kg/day, 60 days
Guedes *et al.* (2012)	IL17, IL10, TNF-α e IFN-γ	NO	ND	Brasil	ND	ND	8	Retrospective	I/C	BZD, 5 mg/kg/day, 60 days

†Chagas disease stage.
ALL: Acute, indeterminate, chronic cardiological, chronic digestive; C: Chronic cardiological; D: Chronic digestive; I: Indeterminate; ND: No data.

chronic stage of the disease were considered cured after 6–18 years following treatment. Unfortunately, potential reinfection for patients living outside the transmission area could not be evaluated.

Therapeutic efficacy in children with early chronic CD has also been studied. Sera from 130 children treated with benznidazole or placebo were analyzed using a purified trypomastigote glyconjugate in a chemiluminescent ELISA [23]. At 6 months post-treatment, 37 of 64 treated patients (56%) compared with 3 of 65 (5%) placebo cases were negative using this test [24]. However, although at 6 years post-treatment, 47 out of 53 patients (88.7%) and only 12 of 46 placebo subjects (26.1%) were negative in the same test. Short-term monitoring using a recombinant *T. cruzi* flagelar calcium-binding protein (F29) in an ELISA was used in a study of Argentinean chronic stage children [25]. Results from the latter study indicate that 35.2 and 62.1% among 44 benznidazole-treated children were seronegative for F29, after 6 and 48 months post-treatment, respectively.

Antibody levels against fractions obtained from *T. cruzi* extracts (FI–FV) and against exo-antigens obtained from trypomastigote-infected mice (EXO) were studied in 42 treated patients (between 2 and 20 years after treatment with benznidazole or nifurtimox) and in 42 untreated controls [26]. Negative serology using FI-V antigen in an ELISA was observed in 64% of treated versus 33% untreated patients. In addition, an ELISA using EXO as antigen was negative in 44% treated versus 8% of untreated patients.

Several researchers have used recombinant antigens for monitoring post-therapeutic cure. Antibody levels for recombinant complement regulatory protein (rCRP) were evaluated using an ELISA in 31 patients before and after treatment, monitoring an average 27.7 months after treatment [27]. There was an inverse relationship between rCRP ELISA positivity and period of follow-up, decreasing from 100% at treatment to 70.3, 62.5, 71.4 and 33.4% in the first, second, third and fourth years after treatment, respectively. Additionally, antibody levels against antigens 1, 2, 13, 30, 36 and Shed Acute Phase Antigen (SAPA) [28–30] were assessed in sera from 18 CD patients before and after 3 years follow-up post-treatment. Antigen 13 was shown to be a good marker of treatment efficacy using ELISA, since negative conversion occurred in 67% (6 of 9) of patients ($p = 0.002$) [31].

Sera had a distinctive but highly consistent reactivity pattern using a panel of 16 *T. cruzi* proteins (recognized by sera from CD patients living in endemic areas) in a multiplex system [32]. There was a decreased response to the panel in six patients followed by 36 months after treatment. Two treatment failures did not have a change in antibody response pattern over time. Seronegative conversion, as well as a decrease in antibody titers, was measured serially in 53 benznidazole-treated and 89-untreated chronic patients, with a median follow-up of 36 months using conventional serological assays (IA, IFI and ELISA) and the above-mentioned multiplex assay [33]. Remarkably, there was a strong correlation between results from both conventional serological tests and the multiplex assay [33]. A decrease in conventional serology titers against *T. cruzi* was measured in 64% of treated patients versus 21% of untreated patients, while there was negative seroconversion in 40% treated versus 7% of untreated patients.

Standardized reference test	Standardized reference test values defined	Dispersion measures of the test	S (%)	E (%)	Review bias	Verification bias	Spectrum bias	Representation bias	Detection bias	Patients with basal value of the test altered (%)	Patients with normal test value after treatment (%)	Ref.
ELISA, HAI	Yes	YES	ND	56.70	No	No	No	No	No	56.7	antiMR2: 29.7–88.1% IFN-γ: 10–100% (6 m)	[17]
Hemoculture, CoML ELISA	Yes	ND	ND	ND	No	No	Yes	No	No	ND	ND	[39]
ND	ND	ND	ND	ND	No	No	Yes	No	No	60.0	60.0%	[43]
ND	ND	ND	ND		No	No	No	No	No	sPselectin: 83 sVCAM: 71	sPselectin: 66.7% sVCAM: 41%	[15]
Cytokine measure in stimulation culture	ND	ND	ND	sPselectin: 83 sVCAM: 71	No	Yes	No	No	No	ND	ND	[37]
ND	ND	ND	ND	ND	No	No	No	No	No	ND	ND	[40]
ND	ND	ND	ND	ND	No	Yes	Yes	No	No	ND	ND	[38]

A serological test using the KMP11, HSP70, PFR2 and Tgp63 recombinant proteins was evaluated in 35 treated patients before and after benznidazole administration [34]. A statistically significant decrease in reactivity against KMP11 occurred 6 months post-treatment in 26 out of 35 of patients (74%), at 9 months post-treatment against PFR2 in 26 out of 35 (74%) and against HSP70 in 25 out of 35 (71%) CD patients. When the response against only two of these antigens was evaluated, the decrease in specific antibody titer occurred in 80% of patients at 9 months post-treatment and continued during the 2-year post-treatment follow-up period. The overall decrease in titers against KMP11, HSP70 and PFR2 24 months post-treatment was 67, 50 and 34% of patients, respectively.

Antibody levels against *T. cruzi* ribosomal acidic protein P2β (Tc P P2β) were analyzed in: 30 asymptomatic CD patients having received specific treatment with clinical follow-up for more than 20 years (group A); 37 asymptomatic CD patients not having been treated (group B); and 11 untreated chronic CD patients (group C) [35]. Antibody levels against TcP2β were significantly lower only in patients from group A.

Cell markers: cytokines

Active parasite-specific B and T cell responses and specific cytokine profiles in patient sera are associated with development of CD pathology and have been proposed as immune markers for disease progression and for treatment assessment and follow-up. Circulating leukocytes from asymptomatic *T. cruzi*-infected patients (without stimuli) secreted a predominant regulatory cytokine profile, whereas symptomatic cardiac patients had a predominant inflammatory cytokine pattern [36]. This cytokine profile reverts after treatment, with asymptomatic patients shifting to predominant inflammatory profiles, and symptomatic cardiac patients upregulate regulatory cytokine production. A similar profile is observed following *in vitro* stimulation of leukocytes with *T. cruzi* trypomastigotes. Untreated asymptomatic patients have a type-1 regulated cytokine profile (innate immune compartment) and a predominantly type-2 adaptive profile in *ex vivo* analysis. Following treatment, they have a downregulated cytokine profile in both innate and adaptive immune compartments [37].

Patients with moderate and severe cardiomyopathy produce high levels of TNF-α and IFN-γ and low levels of IL-10 and IL-17 compared with mild cardiomyopathy or cardiomyopathy-free patients [38]. Treated patients with mild or free cardiomyopathy produced high levels of IFN-γ compared with untreated patients with mild or free cardiomyopathy. Deficient suppressor activity controlling myocardial inflammation by regulatory T cells may cause the altered immune response observed in patients with moderate and severe cardiomyopathy.

Cellular surface markers

The phenotype of T and B lymphocytes from peripheral blood mononuclear cells (PBMCs) was analyzed in untreated, treated (uncured and cured) CD patients and healthy donors. The patients were considered cured when hemoculture and the above-mentioned CoML tests were negative [39]. Untreated patients had a lower proportion of $CD3^+$ T lymphocytes and a higher proportion of $CD5^+$ B cells than healthy donors, while

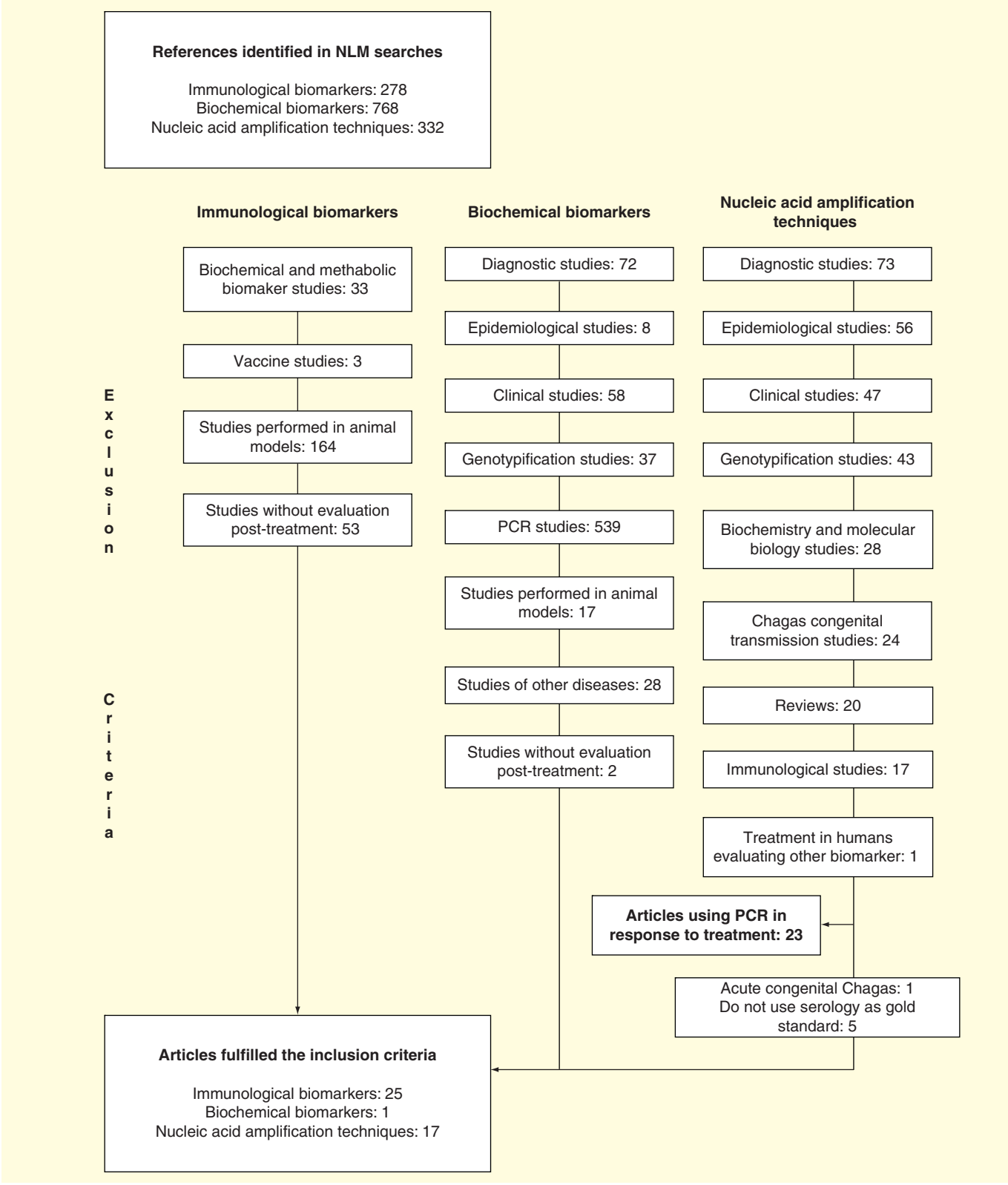

Figure 1. Flow of inclusion of studies on biological markers for evaluating.

treated patients (uncured and cured) had intermediate values. Treated or untreated patients had approximately 2.5-fold more $CD3^+/HLA\text{-}DR^+$ cells than those detected in uninfected individuals. The mean proliferative response *in vitro* of PBMC from cured patients to parasite-related stimuli was equivalent to the low levels detected in healthy donors.

Analysis of the immune response changes following etiological treatment of CD with benznidazole was analyzed in children at an early indeterminate stage [40]. Treated patients had a higher activation status of circulating monocytes, inversely associated with the level of $IL\text{-}12^+CD14^+$ cells. Moreover, benznidazole treatment triggered a high proportion of circulating $CD3^-/CD16^+/CD56^-$ NK cells associated with a type 1-modulated cytokine pattern. The benznidazole treatment induced substantial T and B cell activation associated with an overall IL-10-modulated type 1 cytokine profile.

The proportion of PBMC-expressing IFN-γ was measured in 67 indeterminate patients (treated or untreated) and in 8 treated patients with abnormal electrocardiographic findings [41]. Following treatment, there was either nil or a threefold decrease of IFN-γ expression compared with pretreatment levels, from 9 of 26, 15 of 32 and 23 of 32 patients, at 12, 24 and 36 months after treatment, respectively. The antibody response of these patients to a pool of recombinant *T. cruzi* proteins using a multiplex system also decreased over time [42]. The increase in cells not expressing IFN-γ is associated with an early increase in IFN-γ-producing T cells with effector/effector memory cell phenotype (result observed in 7 of 19 patients analyzed).

The proportion of total $CD4^+LIR^+$ T cells decreases significantly in 60% of patients (6 of 10) with indeterminate stage CD, after benznidazole treatment [43]. The decrease is observed as early as 2–6 months after treatment and is sustained at least for 2 years.

In summary, cellular markers are not direct markers of treatment as they do not pretend to detect parasite presence. However, they may be used to asses the influence of the treatment on the clinical improvement in a particular CD patient group, by comparing the presence or expression level of the marker, in other words, in symptomatic versus asymptomatic patients. Most of the data on immunological biomarkers were published before the development of nucleic acid amplification techniques.

Biochemical & metabolic molecules

CD is a chronic infection, which stimulates a continuous inflammatory immune response. Molecules that are inflammatory mediators of metabolic processes are altered in chronic states of inflammation and/or infection such as chronic CD. Even though many biochemical biomarkers are easily accessible and easy to use at a reasonable cost, we found that few of them have been considered as potential treatment response biomarkers, until recently.

Certain biomarkers of cardiac damage, troponin I and T and natriuretic peptides, have been analyzed to determine progression of the primary complication of CD and are also being proposed as diagnostic tools for *T. cruzi* infection progression [44]. Atrial natriuretic peptide, brain natriuretic peptide (BNP) and N-terminal proBNP have been assessed to discriminate myocardial involvement in early stages of CD [45–50]. Results from these studies are contradictory, indicating that serum BNP levels are similar in *T. cruzi*-infected and uninfected individuals [51]. Other natriuretic peptides (NT-proBNP [52–54], BNP [55–57] and atrial natriuretic peptide [46,49]) and troponin I and T [58] have been proposed to differentially diagnosis late stages of CD.

Clinical trials have assessed the serum enzymes glutamic oxaloacetic transaminase, glutamic–pyruvic transaminase, alkaline phosphatase, acid maltase and alpha-hydroxybutyric dehydrogenase (alpha-HBDH or LDH1) for early detection of myocardial involvement [59]. Patients with chagasic cardiomyopathy have low leptin [54], adipokynes [60] and angiotensin-converting enzyme levels [57], which suggest their potential use as progression markers. Low selenium levels have also been suggested as a progression marker for chronic digestive and cardiac CD [61]. Using animal models, caveolin-3 (Cav-3) [62], myocardial and peripheral protein-3-nitrotyrosine (3NT) and its protein carbonyl formation [63], the higher level of catalase, glutathione peroxidase, glutathione reductase and reduced function of glutathione and Mn(2+) superoxide dismutase have been reported in *T. cruzi*-infected individuals [64].

Hypercoagulability markers such as prothrombin fragments 1+2 (F 1+2), thrombin–antithrombin complex (ATM complex), fibrinogen/fibrin degradation products, D-dimer [65] and lipid bodies [66] in addition to apolipoprotein A1 (ApoA1) [67] have been measured in *T. cruzi* patients. Despite the important number of biomarkers studied from this latter group, response to treatment has not been evaluated except in two of these in a single study. These unpublished studies demonstrated the usefulness of ApoA1 and fibronectin [68].

Hemostatic biomarkers such as endogenous thrombin potential (ETP) and 1+2 prothrombin fragments 1+2 (F 1+2) have altered levels in *T. cruzi*-infected patients compared with controls (73 and 80% of patients, respectively), which decreased significantly 6 months after treatment (100 and 73%, respectively) [69]. Evaluation of ETP and F 1+2 was conducted in a nonendemic area on all stages of CD, thereby controlling for possible reinfection. Nevertheless, since the results are unpublished from a preliminary phase of a larger study, the small sample size, short follow-up period after treatment, large number of cases lost to follow-up (due to high mobility of the study population) and lack of a standardized test to compare proposed biomarkers argue for caution in data interpretation.

In summary, biochemical and metabolic molecules could be useful surrogates for response to treatment of *T. cruzi* infection due to their easy analysis and low cost. However, only four of these (ETP, F 1+2, potentially ApoA1 and fibronectin) have been evaluated after treatment of chronic CD patients, which implies that further studies will be required to assess their specificity and validate their use in diagnosis or prognosis.

Table 3. Target product profile for an 'ideal' or/and an 'acceptable' biomarker criteria for anti-*T. cruzi* treatment response.

	Acceptable	Ideal
Indications and usage	Chronic CD in the symptomatic and asymptomatic form	Acute and chronic CD in all the stages
Samples (Sample collection, conservation)	Peripheral blood (cubital puncture) Collection in stabilizing buffer for conservation and transportation at room temperature or at 4°C	Peripheral blood (digital puncture) Urine Umbilical cord blood for congenital CD Collection in stabilizing buffer for conservation and transportation at room temperature
Number of samples and volume	Three samples • One pretreatment • Two post-treatment Maximum volume: 5 ml in adults; 2 ml in children, 1 ml neonates and newborns	Two samples • One pretreatment • One post-treatment Maximum volume: 2 ml in adults, 1 ml in children, 0.5 ml umbilical cord blood in newborns
Storage conditions technology • Equipment required • High technology required • Human resources	Storage at room temperature, 4°C or -20°C • Laboratory equipment • No high technology required • Specialized human resources (second/third-level center)	Storage at room temperature or 4°C • Point of care • No high technology required • Nonspecialized human resources (primary care center)
Time to processing	48–72 h	<24 h
Methods	Qualitative Semi quantitative	Qualitative Quantitative
Sensitivity and specificity	≥95%, ≥95%	100%, ≥98%
Time of response	12–24 months	3 months
Percentage of expression of altered BMK values (nontreated *T. cruzi*-infected patients)	50%	100%
Percentage of response in treated *T. cruzi*-infected patients	70%	100%
Precautions	None	None
Costs	Low	Low
Availability	In endemic countries	In all countries

CD: Chagas disease

Nucleic acid amplification techniques

Amplification of *T. cruzi* DNA has been tested and is being evaluated as a reliable marker of therapeutic response in clinical trials for efficacy of trypanocidal drugs in *T. cruzi*-infected patients. When parasite DNA amplification was compared with other parasitological diagnostic methods, such as hemoculture and xenodiagnosis, the sensitivity obtained clearly favors the PCR technique for patient treatment follow-up [70–74]. PCR alone or combined with DNA hybridizations was used to evaluate efficacy in patients treated with itraconazole or allopurinol, having a significantly higher sensitivity compared with xenodiagnosis [75–77].

Although *T. cruzi* DNA amplification in the blood of chronic *T. cruzi*-infected and treated patients proved to be in most cases a useful tool to demonstrate failure of treatment, different research groups reported variable success in complete negativization. Unsuccessful specific elimination of *T. cruzi* was reported in 85–89% of patients positive for PCR after trypanocidal treatment [70,73,74] although in other studies, a high percentage (>70%) of patients given trypanocidal treatment converted to negative when tested by PCR [71,78–82]. After an average follow-up of 20–35 years after trypanocidal treatment, 30–60% of *T. cruzi*-infected patients continued to be positive using DNA amplification [72,83,84].

Pharmacological treatment does not correlate with parasite elimination or sustained elimination of parasite DNA, which may be related to many factors such as CD stage, patient pathology at the time of treatment, drug and treatment protocols, parasite strain and load, DNA amplification technique and/or the number of patients studied. The most sensitive

method, unfortunately not used in all studies, is to test several samples from each patient at different intervals, which increases the sensitivity of the PCR to detect *T. cruzi* DNA in blood samples. Nevertheless, while PCR-negative conversion could be achieved in many studies and in a variable proportion of treated patients, a significant seroconversion has not been recorded [71,73,74,78–80,83,85]. It is important to note that obtaining a negative PCR result does not guarantee parasitological cure, since parasitemia may fluctuate, at least in the chronic phase of the infection and parasitemia may be below the PCR detection level, especially after a long period of follow-up after trypanocidal treatment [79,80,86]. In order to avoid false negatives and measure test confidence, repeated PCR of new patient samples is necessary over time.

T. cruzi nucleic acid amplification techniques offer the most sensitive parasitological diagnostic method for infection. Early detection of parasite susceptibility to drugs, and therefore, the use of PCR as a method to promptly detect failure for lack of adherence or parasite resistance to chemotherapy, far out-weighs serology as a tool for patient follow-up after trypanocidal treatment. Since *T. cruzi*-specific antibodies could persist for many years after the etiological treatment in chronic chagasic patients, parasite DNA amplification provides a more rapid, sensitive and cost-effective test to avoid very demanding long patient follow-up, which is always very difficult to accomplish.

Recommendations based on the review: what to expect from early markers of therapeutic response – a definition based on the TPP model

A biomarker is defined as a sign that can be measured accurately and reproducibly to reflect the status of a disease process. Effective markers quantitatively correlate (either directly or inversely) with disease progression. A surrogate marker could be defined as a sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint, that is a direct measure of how a patient feels, functions or survives and is expected to predict the effect of the therapy [87,88]. One of the major reasons for identifying early biomarkers of cure/progression of chronic CD is to improve patient management and has available tools to evaluate clinical trials. Currently, the efficacy of a drug cannot be evaluated in a short period of time after treatment, since parasitological clearance cannot be measured except if it correlates with a significant decrease in titers from conventional serology, which may take many years if at all.

Early biomarkers and some surrogate markers of therapeutic response for chronic CD should be molecules that fulfill specific quality criteria. 'Acceptable' and 'ideal' characteristics that are necessary for biomarkers used to evaluate response to treatment in patients with chronic CD are proposed in Table 3 according to TPP models used for development and evaluation of drugs. There are two primary and essential technical aspects to fulfill for biomarkers: the level of biomarker expression before treatment and the elapsed time in which the marker begins to decrease. There is no consensus regarding the definition of an 'early' therapeutic response for chronic CD, and there is dearth of evidence to define the optimum timing for marker assessment. Evidence suggests that the parasite genotype will have a direct influence on the treatment efficacy, but a biomarker will assess the patient's response to treatment and hence the biomarker effectiveness would not be influenced by *T. cruzi* genotype. The ideal biomarker must be expressed at high levels in chronic *T. cruzi* patients before treatment, but evidence for proportional change after treatment will depend on normal population variation and statistical difference for each individual marker.

Future strategies in order to validate early biomarkers of response to treatment

There are several key questions in order to drive future studies regarding specific response biomarkers to treatment with benznidazole and/or nifurtimox in chronic *T. cruzi*-infected patients. Validation and use criteria have also been included in the TPP model (Table 3). It is important to highlight the heterogeneity of data from the markers studied by different groups, which do not evaluate the same parameters. There are, however, several markers that do have the ability to detect treatment response and can be classified in two groups:

- Parasite biomarkers

There are two main classes of recombinant proteins that are effective at different ages and stages of the disease:

- a 16 protein group [42]
- a combination of four recombinant proteins [34]: KMP11, HSP70, PAR2 and Tgp63.

Other parasite biomarkers include the CoML marker purified from trypomastigotes [18]; and in pediatrics, the AT antigen, which is treatment response indicators still needing to be tested in adults [24] and the F29 protein, recently tested in adults [25,89]. In this field, the disaccharide Galα(1,3)Galβ as an immunodominant glycotope of a synthetic glycoarray containing nonreducing α-galactopyranosyl moieties related to mucin O-glycans, evaluated by a chemiluminescent enzyme-linked immunosorbent assay, has showed its usefulness in the diagnosis of the infection in chronic stages, but it has not been tested to assess response to treatment [90].

- DNA amplification techniques

Recently, Nagarkatti *et al.* (2014) used short RNA ligands called aptamers to detect biomarkers of *T. cruzi* infection in the plasma of infected mice [91]. Aptamers were generated against *T. cruzi* excreted/secreted antigens (TESA) purified from *in vitro* culture supernatants of infected host cells and used as specific ligands in enzyme-linked aptamer assays. TESA molecules could be detected in the blood of infected mice during both the acute and the chronic phases of the disease. Although the identity of the TESA biomarkers is currently not known, these molecules represent novel markers of *T. cruzi* infection. Their detection in clinical samples is currently being assessed (personal communication). These assays also have great

potential in drug development applications and/or to help evaluate treatment efficacy and possibly parasitological cure in human clinical trials.

- Host response/damage biomarkers

Biochemical biomarkers such as F 1+2, ETP [69], apolipoprotein 1 and fibronectin [68] (Ndao *et al.*, personal communication) detect early treatment response at different stages of chronic CD in adults. Muscarinic receptor antigen M2 is effective to detect treatment response, but it has only been studied in patients under 18 years old. Cytokines and surface markers that characterize host cellular responses need to be further assessed although the ELISPOT for IFN-γ is standardized [41].

Expert commentary & five-year view

Based on current published data, there are certain biomarkers that have shown their effectiveness assessing responses to specific treatment with benznidazole and nifurtimox in different stages of CD. Nucleic acid amplification techniques have demonstrated their effectiveness to assess therapeutic failure. Immunological and biochemical biomarkers have not been fully developed as tools to monitor treatment response, even if they are considered interesting research paths. There is heterogeneity in methodologies and scarce data evaluating specificity and sensitivity of assays using these biomarkers.

In our opinion, the availability of suitable biomarkers would open the door for new drugs with better tolerance profiles and greater efficacy in clinical trials. Standardized methods for evaluation of diagnostic tools (specificity, sensitivity, precision and reproducibility) are currently needed to improve biomarker assessment and develop new markers and to motivate research on CD diagnosis and treatment.

The present article reviews the landscape of existing evidence in biomarkers of chronic CD, and specific needs, to develop a battery of complementary biomarkers which together fulfill ideal or acceptable criteria to evaluate response to treatment for chronic CD. Currently, there is no published data to support the use of a single biomarker to monitor treatment efficacy. DNA amplification techniques and other marker candidates show promise and are currently being tested in different population groups. New studies are necessary to improve assessment of existing markers and to identify those that could be useful for early follow-up of treated patients.

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Key issues

- There is a lack of biomarkers for early therapeutic response to antitrypanocidal drugs.
- Accurate and early biomarkers that assess the effectiveness of new drugs or which are useful for management of patients with Chagas CD are needed.
- Certain biomarkers have shown their effectiveness to assess treatment response in different CD stages including DNA amplification techniques.
- There is heterogeneity of treatment response and therapeutic failure biomarkers available.
- Data suggest that no current biomarker may be sensitive enough to be used as a single tool to monitor the efficacy of a trypanocidal treatment.
- Early surrogate markers of therapeutic response in chronic CD should be defined following quality criteria. Using the target product profile model, acceptable and ideal characteristics for a biomarker are proposed to evaluate response to treatment in patients with chronic CD.

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•• **Over a 10 years follow-up period, a complement-mediated lysis test using living trypomastigotes implemented in *T. cruzi*-infected patients after specific treatment reverted negative, even in cases in which conventional serology remaining positive.**

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ARTÍCULO 2

Hypercoagulability biomarkers in *Trypanosoma cruzi*-infected patients

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Hypercoagulability biomarkers in *Trypanosoma cruzi*-infected patients

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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 (F_{1+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 F_{1+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

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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 myocardiopathy 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 presence of 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 leucocytes 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.

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 Clínic, 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 cardiopathy, inflammatory or immunological diseases (active infections of other etiology, inflammatory intestinal diseases and other autoimmune diseases such a rheumatoid arthritis, systemic lupus erythematosus) 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 –80ºC 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 **PAP ((please spell out first time used))** 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 1.

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.

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).

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

	Infection by *T. cruzi*	Control
Sex		
Men	3	4
Women	22	14
Country of origin		
Bolivia	23	10
Ecuador	1	1
Brazil	1	1
Colombia	0	3
Peru	0	1
Argentina	0	2
Atherothrombotic risk factors		
High blood pressure	0	0
Diabetes mellitus	0	0
Hyperlipidaemia	1	2
Smoking	0	1
Alcohol consumption	0	2

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 2. 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 F_{1+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 3??)). In spite of these differences, the medians of PAP, P-Sel, D-dimer and FVIIa were sometimes within the normal ranges. Results of F_{1+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 ($p=0.004$), F_{1+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; F_{1+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 Santibáñez, a rural area of Cochabamba (Bolivia): in this case, the

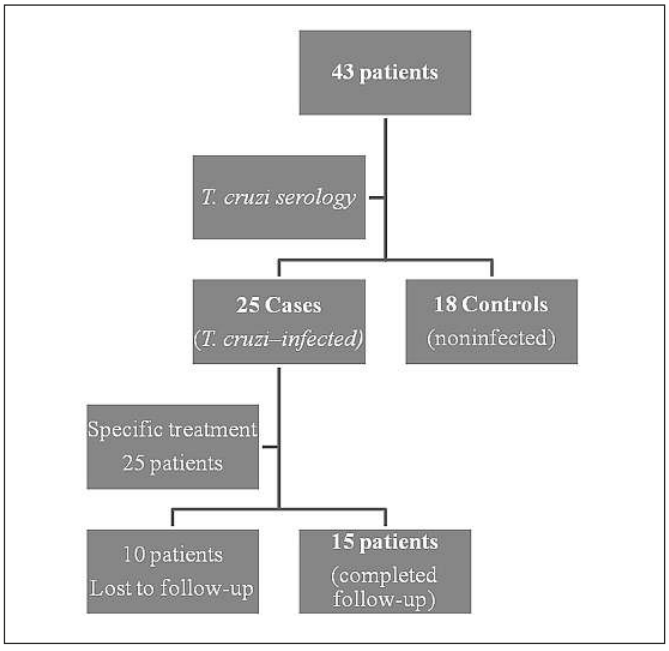

Figure 1: Recruitment and follow-up.

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

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

aPTT, activated partial thromboplastin time; F_{1+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. [a] Wilcoxon rank-sum test.

PCR before treatment was negative and RT-PCR six months after 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 F_{1+2}.

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 *T. cruzi*-infected patients, ischaemic neurological events were first associated with the presence of thromboembolic phenomena related to cardiac events such as arrhythmias, heart failure or aneurisms. However, stroke has been described in *T. cruzi*-infected patients without cardiomyopathy. Parasympathetic nervous system dysfunction has been proposed as a pathogenesis of these ischaemic white matter lesions in patients with CD and no cardiologic risk factors (24).

On the other hand, it is well established that infections can produce endothelial dysfunction and activation of inflammatory and atherogenic phenomena (25, 26). *T. cruzi* infection, like other chronic inflammatory diseases (27–29), has itself been proposed as a thromboembolic risk factor (9, 15).

Among the haemostasis parameters studied, ETP, F_{1+2}, PAP, P-Sel, D-dimer, and FVIIa showed significant differences between cases and controls, but only ETP and F_{1+2} showed values clearly outside the normal levels. F_{1+2} is one of the main markers of thrombin generation *in vivo*. An increase in F_{1+2} values indicates a hypercoagulable state that may be due to endothelial injuries in several pathologic settings (30). The role of F_{1+2} as a hypercoagulability biomarker has 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 F_{1+2} identify two different sides of potential hypercoagulability: F_{1+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).

In CD patients, both ways of measuring thrombin generation showed a marked increase in comparison with controls, sustaining

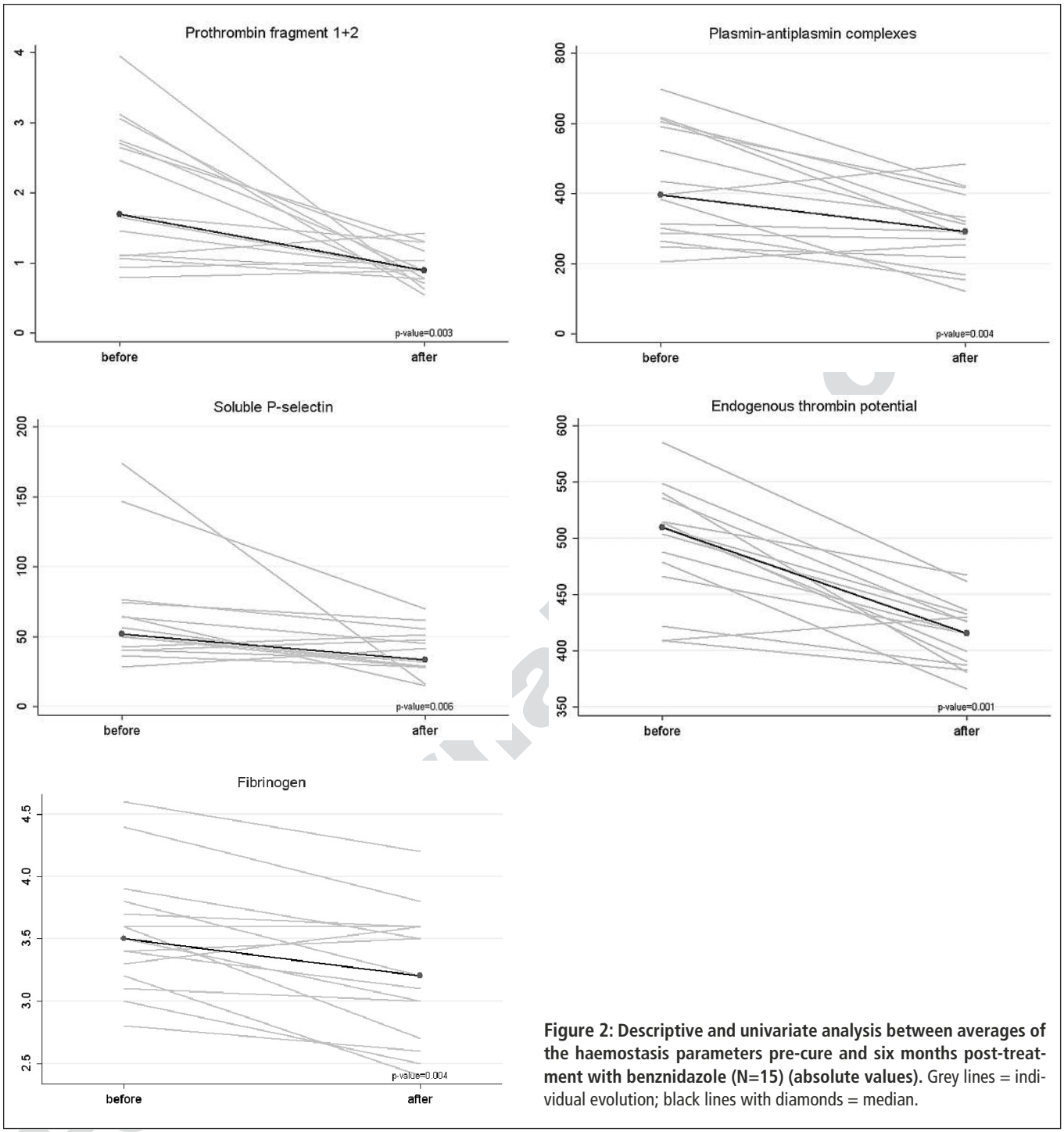

Figure 2: Descriptive and univariate analysis between averages of the haemostasis parameters pre-cure and six months post-treatment with benznidazole (N=15) (absolute values). Grey lines = individual evolution; black lines with diamonds = median.

the hypothesis of a hypercoagulable state in *T. cruzi*-infected patients. Changes in coagulation and platelet function have been well 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 significative drop in the parasite burden (38). Interestingly we observed the maintenance of the F_{1+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 discriminates 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.

What is known about this topic?

- Thromboembolic events has been observed in patients with *T. cruzi* infection
- Stroke has been described in *T. cruzi*-infected patients without myocardiopathy.
- Thromboembolic events could be related to hypercoagulable state in patients with Chagas disease.

What does this paper add?

- *T. cruzi*-infected patients present higher levels of several prothrombotic factors.
- High levels of ETP and F_{1+2} among *T. cruzi*-infected patients could be modified after treatment with Benznidazole.
- If Chagas disease is considered a thromboembolic risk factor, the antiparasitic strategy (still controversial in adults with chronic Chagas disease) is further indicated.

Abbreviations

CD: Chagas' disease; *T. cruzi*: *Trypanosoma cruzi*; F_{1+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.

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Conflict of interest

None declared.

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ARTÍCULO 3

Altered hypercoagulability factors in patients with chronic Chagas disease: potential biomarkers of therapeutic response.

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RESEARCH ARTICLE

Altered Hypercoagulability Factors in Patients with Chronic Chagas Disease: Potential Biomarkers of Therapeutic Response

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Abstract

Thromboembolic events were described in patients with Chagas disease without cardiomyopathy. We aim to confirm if there is a hypercoagulable state in these patients and to determine if there is an early normalization of hemostasis factors after antiparasitic treatment. Ninety-nine individuals from Chagas disease-endemic areas were classified in two groups: G1, with *T.cruzi* infection (n = 56); G2, healthy individuals (n = 43). Twenty-four hemostasis factors were measured at baseline. G1 patients treated with benznidazole were followed for 36 months, recording clinical parameters and performance of conventional serology, chemiluminescent enzyme-linked immunosorbent assay (trypomastigote-derived glycosylphosphatidylinositol-anchored mucins), quantitative polymerase chain reaction, and hemostasis tests every 6-month visits. Prothrombin fragment 1+2 (F1+2) and endogenous thrombin potential (ETP) were abnormally expressed in 77% and 50% of infected patients at baseline but returned to and remained at normal levels shortly after treatment in 76% and 96% of cases, respectively. Plasmin-antiplasmin complexes (PAP) were altered before treatment in 32% of G1 patients but normalized in 94% of cases several months after treatment. None of the patients with normal F1+2 values during follow-up had a positive qRT-PCR result, but 3/24 patients (13%) with normal ETP values did. In a percentage of chronic *T. cruzi* infected patients treated with benznidazole, altered coagulation markers returned into normal levels. F1+2, ETP and PAP could be useful markers for assessing sustained response to benznidazole.

OPEN ACCESS

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Author Summary

The manuscript describes the results of a study whose aim was to assess the tendency to coagulate in people suffering from a parasitic infection frequent in Latin America named *T. cruzi* infection or Chagas disease, by the study of several coagulation factors. According to the state of the art in this topic, specific treatment for Chagas disease is recommended in recent (acute) and late (chronic) stages of the infection. The effectiveness of current available drugs in the chronic stage of infection is still a topic of debate due to inconsistent results across studies and a lack of early measurable parameters of response to specific treatment. Another aim of this study was to determine if the presence of an upregulated procoagulative activity in plasma in people suffering *T. cruzi* infection could be used as potential marker that indicates therapeutic response in people at chronic stage of the disease. The results of this study suggest that measurements of alterations of procoagulative activity may be useful to indicate specific treatment for *T. cruzi* chronically infected patients and new data concerning early response to treatment biomarkers.

Introduction

Chagas disease (CD) is one of 17 neglected tropical diseases recognized by the World Health Organization. Caused by the protozoan parasite *Trypanosoma cruzi*, it mainly affects people with poor socioeconomic status and limited health care access in endemic and nonendemic countries. [1, 2]

Thrombosis is considered as a pathological deviation of haemostasis, and it is characterized by intravascular thrombus formation and vessel occlusion. Perturbation of hemostasis is an important factor in the pathogenesis of thromboembolic events, which can be caused by blood flow dysregulation, endothelial injury, and coagulation system alterations.

Recently, is has been described that under certain circumstances thrombosis is a physiological process that constitutes an intrinsic effector mechanism of innate immunity, and the process has been defined as "immunothrombosis". [3] It is activated after the recognition of pathogens and damaged cells, and inhibits pathogen dissemination and survival. Immunothrombosis can therefore be regarded as a newly identified, crucial element of intravascular immunity, which is a part of the immune system that encompasses a wide range of host strategies to detect and protect against pathogens in the vasculature. Dysregulation of immunothrombosis is likely to constitute a key event in the development of thrombotic disorders. [3]

Infectious disease can cause a hypercoagulable state through the upregulation of tissue factor in monocytes, the generation of procoagulant microparticles, the activation of the coagulation intrinsic pathway, platelet activation, and NETs (Neutrophil Extracellular Traps) release. [3] Different infectious agents may cause different responses but a final degree of hypercoagulability can be a common trait as one of the biological endpoints. Additionally, patients with chronic inflammation may also present platelet adhesion events, which are considered inflammatory processes and can be observed in patients with chronic *T. cruzi* infection, even in the asymptomatic stages. [4] Infection itself can cause vasculitis, increasing proinflammatory cytokine levels and perpetuating the risk of thrombotic events. [5] In the case of the Chagas' disease the effect of hemostasis in the bradikinin formation, through the effect of factor XII activation in the Kallikrein-Kinin system, can modify the type 1 immune response and then modulate the antiparasite immunity as suggested in a mice model of subcutaneous infection by *T.cruzi*. [6]

Thromboembolic events and dilated cardiomyopathy, ventricular aneurysms, and intracavitary thrombosis are associated with CD. [7, 8] Rheological factors can induce intraluminal thrombus

formation with the risk of embolism. [9] Alterations of molecular markers of coagulation system activation have been described in *T. cruzi* infection individuals with or without clinical thrombosis. [9–12] Other factors, such as injury to vessel walls by parasites or changes in blood viscosity due to host immune response, may influence in the development of thromboembolic events in *T. cruzi*-infected individuals without Chagas cardiomyopathy or other vascular risk factors. [13] Based on studies performed in humans with chronic *T. cruzi* infection, there are controversial results regarding the existence of a prothrombotic status in *T. cruzi*-infected patients. [13,14] There is an study in which a of higher prothrombotic status in the CD group was not found, but the control group were individuals without *T. cruzi* infection and heart failure. [14] In previous studies performed in murine models, several abnormalities of the heart microcirculation of individuals with chronic CD were pointed out, but they did not find evidence of thrombi and neither thromboembolism. [15, 16] Higher levels of the hypercoagulability markers prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complexes (TAT), fibrinogen/fibrin degradation products, plasminogen activator inhibitor type 1 (PAI-1), and D-dimer have been reported in *T. cruzi*–infected patients compared with healthy individuals. [10, 11] A pilot study performed by our group showed that endogenous thrombin potential (ETP) and F1+2 levels were outside normal ranges in 73% and 80% of *T. cruzi*–infected patients without advanced heart disease, respectively. [12] We demonstrated a 100% and 73% decrease in these levels six months after treatment with benznidazole. Thus, if they prove to remain stable in time, hypercoagulability factors could be used as biomarkers of therapeutic response in CD. Besides, although whether or not chronic Chagas disease is an independent vascular risk factor remains to be confirmed. [17,18]

While specific treatment is recommended in both acute and chronic stages of infection [19,20], there are only two drugs (i.e., benznidazole and nifurtimox) available for the treatment of CD. The mechanism of action of benznidazole relates to the nitro-reduction of components of the parasite, the binding of metabolites of the nuclear DNA and k-DNA of *T. cruzi* and the lipids and proteins of the parasite. [21] In adults, benznidazole has a high rate of adverse effects, which can be classified into three groups: (i) hypersensitivity, including dermatitis with cutaneous eruptions (usually appearing between days 7 and 10), myalgias, arthralgias, and lymphadenopathy; (ii) polyneuropathy, paresthesias, and polineuritis usually during the 4th week of treatment); and (iii) bone marrow disorders, such as thrombopenic purpura and agranulocytosis (usually after the second week of treatment). [22]Furthermore, the effectiveness of these drugs in the chronic stage of infection is still a topic of debate due to inconsistent studies' results [23–25] and a lack of early biomarkers of response to specific *T. cruzi* treatment with benznidazole. [26]

Following on from our pilot study [12], here we increased the sample size and extended follow-up to further investigate the value of hypercoagulability factors as biomarkers of treatment response in CD. We also added current treatment response parameters measured by conventional serology, serology for lytic anti-α-galactosyl (anti-α-Gal) antibodies against *T.cruzi* [27–29], and quantitative reverse transcription polymerase chain reaction (qRT-PCR). [30]

The aims of the study were to investigate alterations of hypercoagulability factors in patients chronically infected with *T. cruzi* and determine whether there is an early and sustainable improvement of the hypercoagulability factors after antiparasitic treatment.

Methods

Ethics Statement

Written informed consent was obtained from participants before being recruited (all of them were adults). Approval for the protocols and for the informed consent was obtained from the Hospital Clínic of Barcelona Ethics Review Committee.

Design and Setting

This is a descriptive study of 99 individuals (56 with *T.cruzi* infection and 43 healthy individuals) from Latin American, where CD is endemic. All the individuals were evaluated at the Centre for International Health at Hospital Clínic in Barcelona, Spain.

Recruitment and Participants

Ninety-nine individuals from CD-endemic areas living in Barcelona were invited to participate. Inclusion criteria were an age of over 18 years and provision of signed informed consent. Exclusion criteria were pregnancy, non-Chagasic cardiopathy, late chronic cardiac or digestive forms of CD, other acute or chronic infections, inflammatory or immunological diseases, and chronic systemic diseases (high blood pressure and diabetes).

Procedures

After signing the informed consent form, participants were asked for clinical and epidemiological data, including area of origin and risk factors for the CD transmission. The information recorded included vascular risk factors, toxic habits, and cardiological and/or vascular events.

Conventional serology of *T.cruzi* infection was established using two ELISA kits: a commercial kit with recombinant antigens (BioELISA Chagas, Biokit S.A.,Barcelona-Spain) and an in-house kit with whole *T.cruzi* epimastigote antigen, as described. [12, 31]. Diagnosis was confirmed by a positive result on both tests. [19] Following serological tests results, participants were divided into two groups: those with *T.cruzi* infection (Group 1 [G1]) and those without (Group 2 [G2]). All the participants underwent human immunodeficiency virus testing, basic blood and biochemical tests (including renal and liver function), and specific evaluation of hemostasis factors.

For the hemostasis studies, blood was collected in citrate-containing tubes (Becton Dickinson), samples were centrifuged, and platelet-poor plasma aliquots were frozen at –80°C until assayed. Prothrombin time, activated partial thromboplastin time, coagulation factor VIII, protein C activity, free and total protein S levels, antithrombin and plasminogen activity, F1+2, plasmin-antiplasmin complexes (PAP), factor VIIa, PAI-1, P-selectin, factor V Leiden and prothrombin gene G20210A mutation, lupus anticoagulant and anticardiolipin antibodies were measured as previously described. [12] D-dimer was measured using an automated turbidimetric test (Siemens Healthcare Diagnostics) and ETP was assessed using a continuous chromogenic thrombin generation assay and ETP Curves software (Siemens). The ETP coagulation test was initiated by using human recombinant tissue factor, phospholipids, and calcium ions. ADAMTS-13 was measured using a commercial chromogenic method (American Diagnostica). Factor XIIa was determined by a direct quantitative commercially available immunoassay (Shield Diagnostics) with a highly specific monoclonal antibody that does not recognize its zymogen factor XII.[32] Plasma tissue factor levels were determined using a commercial kit (American Diagnostica) according to the manufacturer's protocol. Plasma levels of von Willebrand factor antigen were determined by enzyme-linked immunosorbent assay (ELISA) (Corgenix). Procoagulant activity of microparticles was measured using a functional assay with the addition of factors Xa, Va, and prothrombin after microparticle capture in the solid phase using annexin V (Hyphen Biomed). Soluble CD40L was measured by ELISA (R&D Systems).

qRT-PCR [30] and a chemiluminescent ELISA assay based on a highly purified, trypomastigote-derived glycosylphosphatidylinositol-anchored mucin (tGPI-mucin) antigen for the serological detection of lytic anti-α-Gal antibodies against *T.cruzi* (AT CL-ELISA) [27–29, 33–36], were performed in G1 at month 0 (baseline), and 6, 12, 18, 24, 30, and 36 months post-treatment. For AT CL-ELISA, a serum sample was considered positive when the titer was $\geq$1.0 and

negative when it was ≤0.9. Inconclusive or equivocal results were determined by a titer between 0.9 and 1.0. [27, 35]All sera were tested in duplicate and the results were expressed as the mean of two simultaneous determinations.

G1 patients were studied using a protocol that included a 12-lead electrocardiogram, chest X-ray, and echocardiogram. They were followed up every 6 months for at least 36 months. At each visit, clinical data were collected and the following tests were performed: ELISA, AT CL-ELISA, qRT-PCR, and hemostasis tests. Other tests were performed according to individual symptoms. Specific treatment with benznidazole (5 mg/kg/day for 60 days) was offered to all *T. cruzi*–infected patients, and those treated were monitored fortnightly for clinical and analytical assessment. Treatment was considered complete when at least 80% of the total dose was reached.

A hypercoagulable state is defined as the presence, in certain individuals, of thrombotic potentialities that activate the endothelium and the formative elements of the blood (mainly, platelets) that favors plasma kinetics that lead to the formation of thrombin, which disturbs fibrinolytic activity and produces hemorheological changes with turbulence phenomena that predispose to thrombogenesis. [18]

Statistical Analysis

Quantitative variables were presented as medians and interquartile range (IQR) and were compared between groups using the Wilcoxon rank sum test. Qualitative variables were reported using absolute frequencies and percentages and between-group comparisons were made using Fisher's exact test. Hypercoagulability biomarker variation over time was assessed using a mixed-effect linear regression model with a random intercept structure. Hypercoagulability factors were used as dependent variables and follow-up time as the explanatory variable, with one category for each time point: baseline, month 6 (reference for comparisons), and months 12, 18, 24, and 36. This type of model allows for the inclusion of random effects in addition to the overall error term. Random intercept regression was also used to assess whether antibody levels measured by ELISA and AT CL-ELISA approached the negative threshold during follow-up. The response variable was the distance from this threshold (i.e., the difference between each ELISA or AT CL-ELISA value and the negative cutoff) and the explanatory variable was the follow-up time from month six (reference) to month 36. The regression coefficients express the effect estimate of follow-up on the outcome variable.

The pattern of the relationships between hypercoagulability biomarkers was assessed by multiple correspondence analysis (MCA) using the Burt matrix approach. [37, 38] The MCA represents a method for analyzing multi-way contingency table containing measure of correspondence between row (subjects) and columns (levels of variables). The interpretation is based upon proximities between levels of variables (or points) in a low-dimensional map. The firsts dimensions (usually one or two) account for meaningful amounts of variance and are those retained for the map definition and interpretation. The first dimension accounts for a maximal amount of total variance in the observed variables. Under typical conditions, this means that the first component will be correlated with at least some of the observed variables. The second dimension has two important characteristics: it accounts for a maximal amount of variance in the data set that is not accounted for by the first dimension, thus it is correlated with some of the observed variables that not display strong correlations with dimension 1; and it is uncorrelated with dimension 1. Looking at the map, the proximity between levels of different variables means that these levels tend to appear together in the observations. Since the levels of the same variable cannot occur together, the proximity between levels of the same variable means that the groups of observations associated with these levels are themselves

similar. A level far away from the origin (of the dimensions) means that is well-represented in the map, thus that level is meaningful for the interpretation of the dimension(s). All levels that are not useful for the solution are near the origin. Supplementary (passive) variables are those not used for the solution but mapped in the graph in order to help in the interpretation.

The biomarkers were classified into three categories: normalization of values throughout follow-up, non-sustained normalization during follow-up and normal values at baseline. Two additional variables were considered: qRT-PCR results during follow-up (categories: always negative and sometime positive) and level of adherence (categories: 80% and 100%). All the tests were 2-tailed and the confidence level was set at 95%. The analyses were performed using Stata 13 (Stata Corporation, College Station, TX, USA).

Results and Discussion

Ninety-nine individuals (76 women) were studied. Fifty-six of these (43 women) were *T.cruzi*–positive (G1) and 43 (33 women) were *T.cruzi*–negative. The mean ages were 34 (SD, 9) years for the overall group (range 17–56, median 33), 37 (SD, 9) years for G1, and 32 (SD, 7) years for G2. Fifty G1 patients were treated with benznidazole (six were lost to follow-up before starting treatment due to unexpected work-related changes in the migratory process). Forty-five (90%) completed treatment. Eighty-six participants (87%) (51 [91%] in G1 and 35 [81%] in G2) were from Bolivia. None of the participants traveled to their countries or other CD-endemic areas during follow-up. The clinical and demographic data are summarized in Table 1. The epidemiological and baseline clinical data were similar in both groups, making them statistically comparable.

Comparison of the 24 hypercoagulability biomarkers at baseline between (untreated) G1 and G2 individuals showed statistically significant differences for D-dimer ($P = .0262$); F1+2 (abnormal values in 43/56 G1 patients [77%], $P < .0001$), PAP (abnormal values in 17/56 G1 patients [30%], $P = .0111$), P-selectin (abnormal values in 7/56 G1 patients [13%] $P = .0177$), and ETP (abnormal values in 28/56 G1 patients [50%], $P < .0013$), and circulating

Table 1. Epidemiological data, vascular risk factors, and cardiovascular events in healthy and *T. cruzi*-infected individuals.

		Group 2: Healthy Individuals *n* (%)	Group 1: *T. cruzi*-Infected Patients (Baseline) *n* (%)
Country of origin	Bolivia	35 (81)	51(91)
	Argentina	1 (2)	2(4)
	Brazil	1 (2)	1 (2)
	Colombia	3 (7)	0
	Ecuador	2 (5)	1 (2)
	Paraguay	0	1 (2)
	Peru	1 (2)	0
Toxic habits	Smoking	1 (2)	1 (2)
	Alcohol intake	5 (12)	1 (2)
Vascular risk factors	High blood pressure	0	0
	Hyperlipidemia	4 (9)	5 (9)
	Diabetes mellitus	0	0
Cardiovascular events	Atrial fibrillation	0	0
	Valvulopathy*	0	4 (8)
	Cardiac failure	0	0
	Myocardial ischemia	0	0
	Stroke	0	0

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Table 2. Descriptive analysis and comparisons of hemostasis parameters between pretreatment Group 1 (N = 56) and Group 2 (N = 43).

VARIABLE		Group 1 Median (IQR) [n]	Group 2 Median (IQR) [n]	*P* Value [a]	Normal range (units)
D-dimer		228.5 (119.0) [56]	201.0 (125.0) [43]	0.0262	50–400 (μg/L)
Prothrombin fragment 1+2		**1.8 (1.3) [56]**	**0.8 (0.4) [43]**	**< 0.0001**	**0.40–1.1 (nM)**
PAI-1		24.6 (14.5) [56]	21.3 (14.5) [43]	0.0680	4.0–43.0 (ng/mL)
Factor VIIa		3.5 (1.4) [56]	2.9 (1.8) [43]	0.4312	1.5–4.1 (ng/mL)
PAP complexes		**360.9 (275.8) [56]**	**258.1 (225.8) [43]**	**0.0006**	**80–470 (μg/L)**
P-selectin		**41.8 (40.0) [56]**	**32.1 (21.7) [43]**	**0.0200**	**3–90 (μg/mL)**
ETP		**475.2 (99.2) [56]**	**412.4 (75.8) [43]**	**<0.0001**	**351–473 (mEq)**
Prothrombin time		98.5 (5.0) [56]	98.0 (5.0) [43]	0.4701	0.85–1.15 (ratio)/ 80–100 (%)
Attp		30.0 (3.0) [56]	30.0 (4.0) [43]	0.4849	25–35 (sec)
Fibrinogen		3.5 (0.8) [56]	3.4 (0.8) [43]	0.8540	1.5–4.5 (g/L)
Antithrombin		104.5 (16.5) [56]	101.0 (18.0) [43]	0.4518	60–140 (%)
Plasminogen		108.5 (17.5) [56]	107.0 (16.0) [43]	0.1467	60–140 (%)
Protein C		103.0 (30.5) [56]	104.0 (27.0) [43]	0.7082	60–140 (%)
Total protein S		87.0 (14.5) [56]	88.0 (12.0) [43]	0.8156	60–140 (%)
Free protein S		85.5 (11.5) [56]	88.0 (13.0) [43]	0.1125	60–140 (%)
FVIII		112.5 (51.0) [56]	103.0 (36.0) [43]	0.1391	60–140 (%)
FvWAg		136.0 (52.0) [56]	116.0 (46.0) [43]	0.0758	65–150 (U/dL)
Microparticles		21.1 (11.0) [56]	17.7 (13.5) [43]	0.0112	8–30 (nM)
CD40L		98.2 (39.3) [56]	89.0 (42.1) [43]	0.6952	30–145 (pg/mL)
Tissue factor		116.5 (50.1) [56]	124.2 (57.3) [43]	0.3737	80–280 (ng/mL)
ADAMT13		103.8 (41.9) [56]	97.9 (60.1) [43]	0.8905	50–120 (ng/mL)
Factor XIIa		3.7 (3.9) [56]	3.1 (4.6) [43]	0.3178	1.0–4.4 (ng/mL)
Factor V Leiden [b]	No mutation	55 (98%)	42 (98%)	1.0000 [c]	Mutations/no mutations
	Heterocygote	1 (2%)	1 (2%)		
G20210A[b]	No mutation	56 (100%)	43 (100%)		Mutations/no mutations

[a] Wilcoxon rank sum test *P* value

[b] Absolute frequency (column percentage)

[c] Fisher's exact test

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microparticles (*P* = .0112) (Table 2). D-dimer levels were normal in all the individuals in G1 and G2, and microparticles were within the normal range in a high percentage of patients (86% in G1 and 93% in G2, *P* = .3402). Our findings showed that a high percentage of patients with chronic *T.cruzi* infection have a hypercoagulable state regardless the clinical stage of disease, thus confirming the observations of previous studies. [11–13]

Thirty-three (76%) of the 43 patients with abnormal baseline F1+2 values achieved normal levels after a median follow-up of 9 month (IQR, 8). All but one of the 28 patients with abnormal ETP values before treatment showed normal values at 6 months (IQR, 3). These values were maintained throughout follow-up (30 months; IQR, 28) in 15 patients (60%). Fifteen of the 17 patients with abnormal baseline PAP values showed normal values 7 months (IQR, 7) after treatment and nine of these (60%) maintained these values throughout follow-up (28 months; IQR,11). However, PAP values at 12 and 48 months seemed to be higher than those at 6 months, but the confidence interval indicates a lack of precision for both time point effect estimates (Table 3). Thus, once normalized, F1+2 and ETP levels did not increase again significantly after treatment. Fig 1 shows a graphic representation of these results.

Table 3. Variations in hemostasis F1+2, ETP, and PAP during the follow-up of 50 treated patients.

Variable		F1+2[b]		ETP[b]		PAP[b]	
		Effect Estimate (95% CI)	*P* Value	Effect Estimate (95% CI)	*P* Value	Effect Estimate (95% CI)	*P* Value
TIME	Baseline [c]	0.88 (0.66; 1.10)		52.58 (33.96; 71.21)		101.26 (48.39; 154.14)	
	6 mo	0		0		0	
	12 mo	-0.08 (-0.36; 0.21)		-8.54 (-31.96; 14.88)		65.30 (-1.58; 132.18)	
	18 mo	-0.01 (-0.27; 0.25)	< 0.0001	-6.06 (-27.87; 15.75)	< 0.0001	29.03 (-33.24; 91.29)	< 0.0001
	24 mo	0.03 (-0.25; 0.30)		2.88 (-19.65; 25.41)		-3.97 (-68.14; 60.20)	
	30 mo	-0.10 (-0.39; 0.19)		13.19 (-10.58; 36.95)		49.83 (-17.91; 117.57)	
	36 mo	0.10 (-0.18; 0.37)		0.92 (-21.88; 23.73)		-27.76 (-92.97; 37.45)	

[b] F 1+2, prothrombin fragment 1+2; ETP, endogenous thrombin potential; PAP, plasmin-antiplasmin

[c] Baseline value compared to value at 6 months of follow-up.

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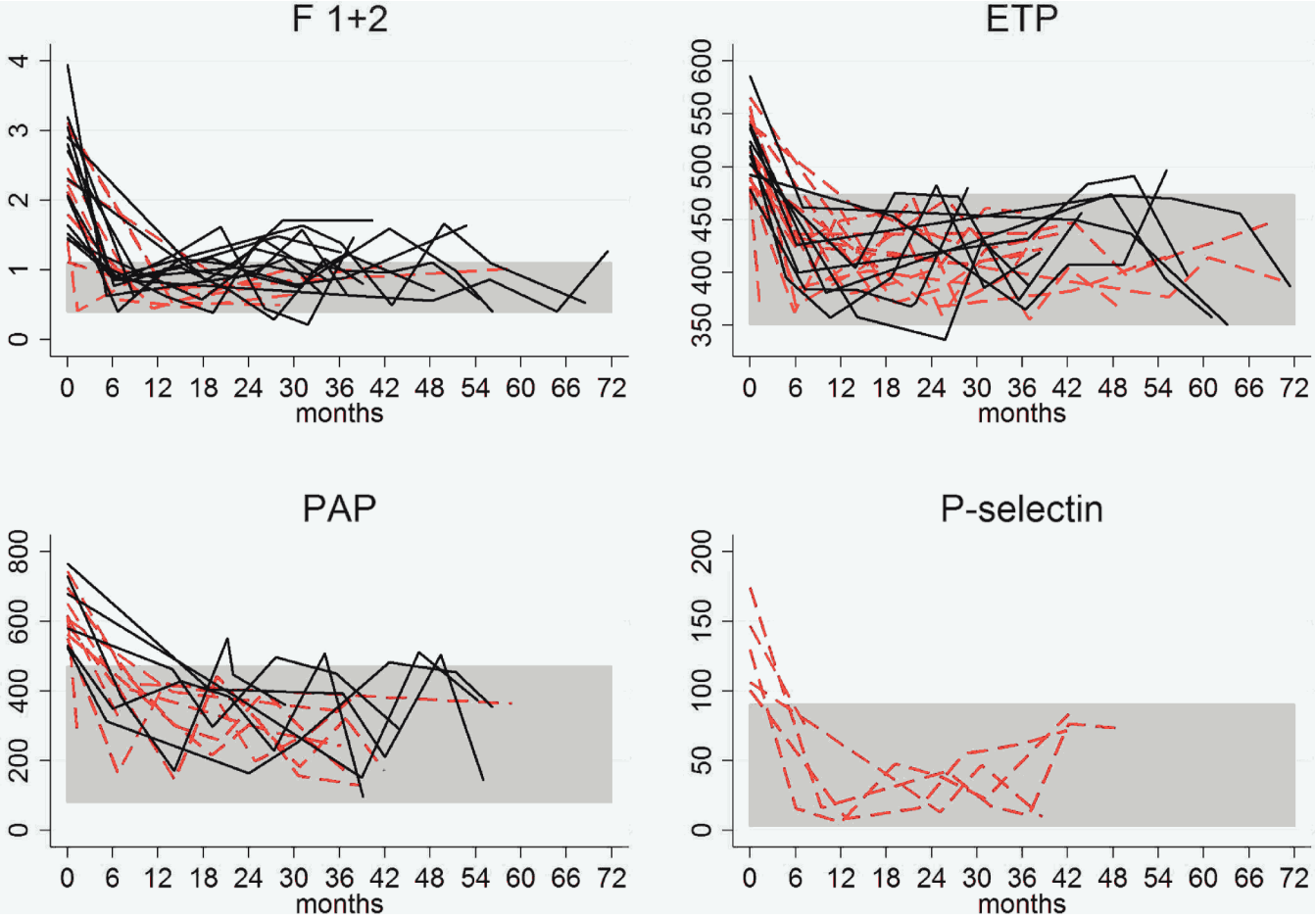

Fig 1. Hemostasis factor levels (baseline and follow-up) in patients with altered levels before treatment who achieved levels within normal ranges during follow-up. *The discontinuous red lines indicate patients who maintained normal values throughout follow-up. The continuous black lines indicate patients who experienced a return to abnormal values at some time during the follow-up. Abbreviations: ETP, endogenous thrombin potential; F 1+2, prothrombin fragment 1+2; PAP, plasmin-antiplasmin.

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F1+2 values are an indirect measure of the amount of thrombin generated in vivo (mainly due to endothelial injury, even in subclinical states) [39], and ETP levels indicate the potential amount of thrombin that can be formed when blood coagulation is activated through the addition of tissue factor. PAP complexes are markers of fibrinolysis. Upon activation, plasmin, which is primarily responsible for a controlled and regulated dissolution of the fibrin polymers into soluble fragments, is immediately inactivated by antiplasmin, forming PAP complexes. [40] Therefore, it is conceivable that the increase formation of PAP complexes stems from excessive formation of fibrin in the blood stream of untreated *T*. *cruzi* infected patients. Soluble P-selectin is considered a biomarker of in vivo platelet activation. P-selectin is contained in the α-granules of platelets; following platelet activation, the soluble form is expressed on the platelet surface and then shed by cleavage. P-selectin has been shown to act as a link between thrombosis and inflammation. [41] Additionally, the four biomarkers-F1+2, ETP, PAP complexes, and P-selectin-reflect are highly stable over time.

A hypercoagulable state is a term that pretends to denominate a condition in which there is an increased tendency toward blood clotting. There is not a universally accepted definition for this state based in biomarkers values, but an increase in several of them suggests the possibility of an increase in the person's chances of developing blood clots. The increases in F1+2, PAP and ETP are congruent with this idea: F1+2 and PAP indicate the actual amount of thrombin and plasmin formed, as markers in procoagulant and fibrinolysis pathways, respectively; and ETP indicates the potential amount of thrombin that can be formed considering globally all the activators, inhibitors and substrates of the hemostasis present in the plasma. The increase observed in these biomarkers is good enough to be an argument to point out a hypercoagulable state in patients with Chagas' disease.

Sixteen (33%) of the 56 G1 patients had a positive qRT-PCR result at baseline, but only four of these had a positive result after treatment (treatment failure rate of 25% in this subgroup). Five of the 34 patients with a negative baseline qRT-PCR result showed a positive result during follow-up. None of the patients with normal F1+2 values during follow-up had a positive qRT-PCR result, but 3(13%) of the 24 patients with normal ETP values during follow-up did. Of the patients with altered levels of F1+2, ETP, or PAP complexes at baseline, a positive qRT-PCR result during follow-up was not significantly associated with changes observed in lytic anti-α-Gal antibodies, F1+2, ETP, and/or PAP levels.

A positive qRT-PCR result after treatment in patients who achieved normalization of F1+2, ETP, and/or PAP could mean that a decrease in parasite load is sufficient to modify the hypercoagulable state or that benznidazole, which acts on the redox system, could modify these biomarkers without eliminating the parasites. This would limit the use of these factors as biomarkers for parasite elimination, although they could be valuable indicators of treatment response and add support to the theory that, by reverting the hypercoagulable state, benznidazole may also prevent clinical thrombotic events.

Conventional ELISA results were positive in all the patients in G1. Although, as expected, antibodies remained positive throughout follow-up, a slight decrease was detected by the commercial and in-house methods during this period. A statistically significant relevant decrease, was only observed with the in-house test from month 18 onwards ($P = .0006$).

Lytic anti-α-Gal antibodies were positive in 52 (96%) of the 54 patients tested before treatment, and in all patients AT CL-ELISA remained within positive levels to the end of the follow-up (Fig 2). Besides, there was no correlation between lytic anti-α-Gal antibody assay and the hemostasis factors evaluated. In relation to previous studies' results, early decreases in lytic anti-α-Gal antibodies were expected to be observed. On the contrary, a decrease in levels was evident at month 12 and this was significant since month 18 and forward ($P = .0052$). [28, 34]

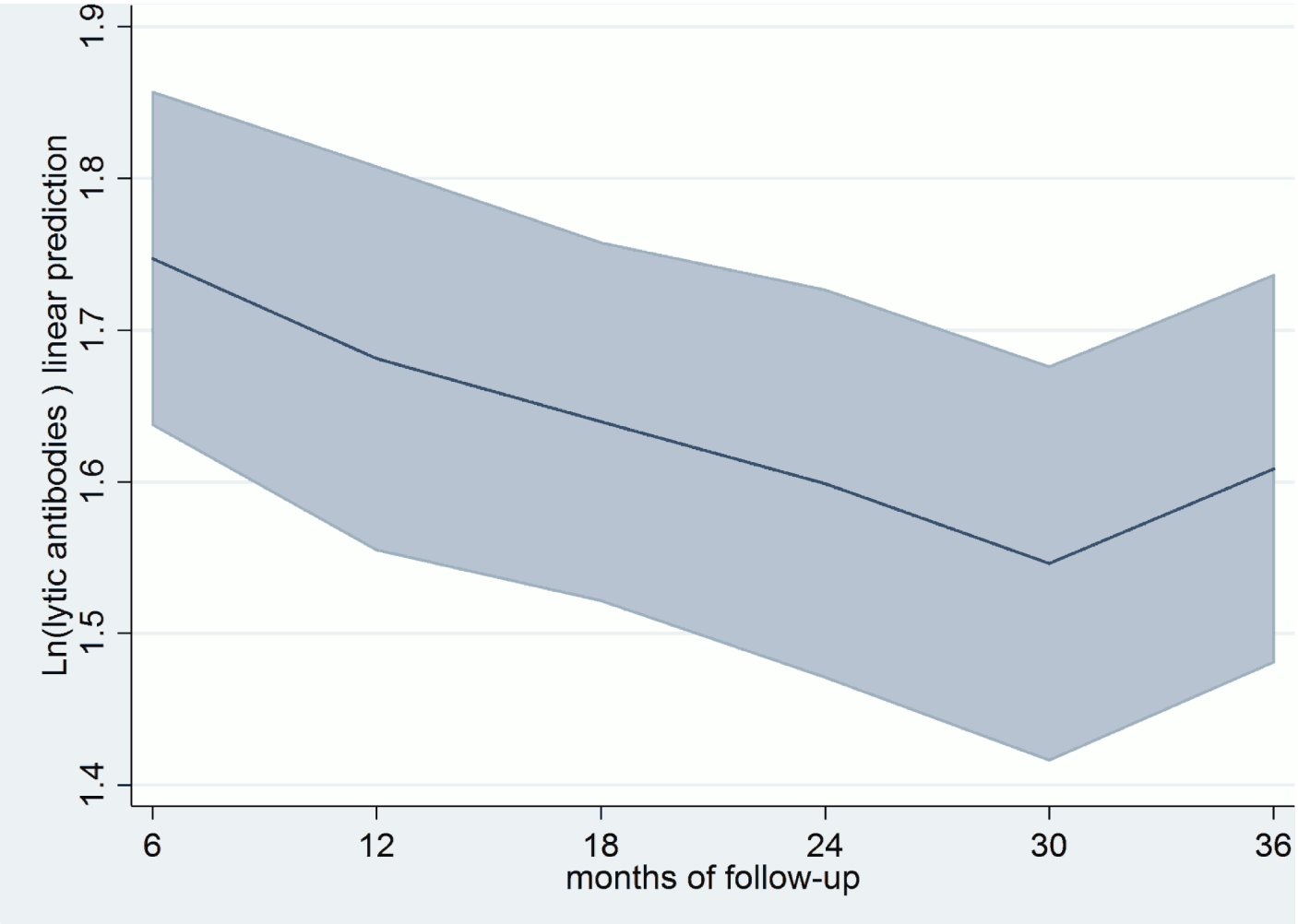

Fig 2. Variations in AT CL-ELISA levels. Months of follow-up predictive margins with 95% CI. Cutoff AT CL ELISA = 0.

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Adherence to treatment was high, with only five patients not achieving 80% of the total dose. All five patients showed abnormal F1+2 values throughout follow-up and 3 (60%) had abnormal ETP and PAP values. One of the five patients had a positive qRT-PCR result during follow-up, and all five maintained the same positive ELISA and AT CL-ELISA results throughout follow-up. A large cohort of adolescents with *T cruzi* infection treated with benznidazole showed seronegativity in lytic anti-α-Gal antibodies, as measured by AT CL-ELISA, in 58% and 85% of the patients 36 and 72 months after treatment, respectively. [28, 34] The differences between those studies and ours may be due to the nature of the cohorts (adolescents vs. adults) and the stage of the disease. Nevertheless, both studies showed a similar trend towards a reduction in lytic anti-α-Gal antibodies following treatment with benznidazole.

We studied the relationship between normalization of hypercoagulability markers F1+2, PAP, and ETP and qRT-PCR results by multiple correspondence analyses (MCA). Due to the low rate of positive qRT-PCR results, this variable was used as a supplementary variable jointly with treatment adherence. The MCA results (Fig 3) showed an association between complete normalization of PAP and ETP levels and non-sustained and marginally abnormal values in F1+2. These factors had the highest contribution and correlation in the positive part of the second

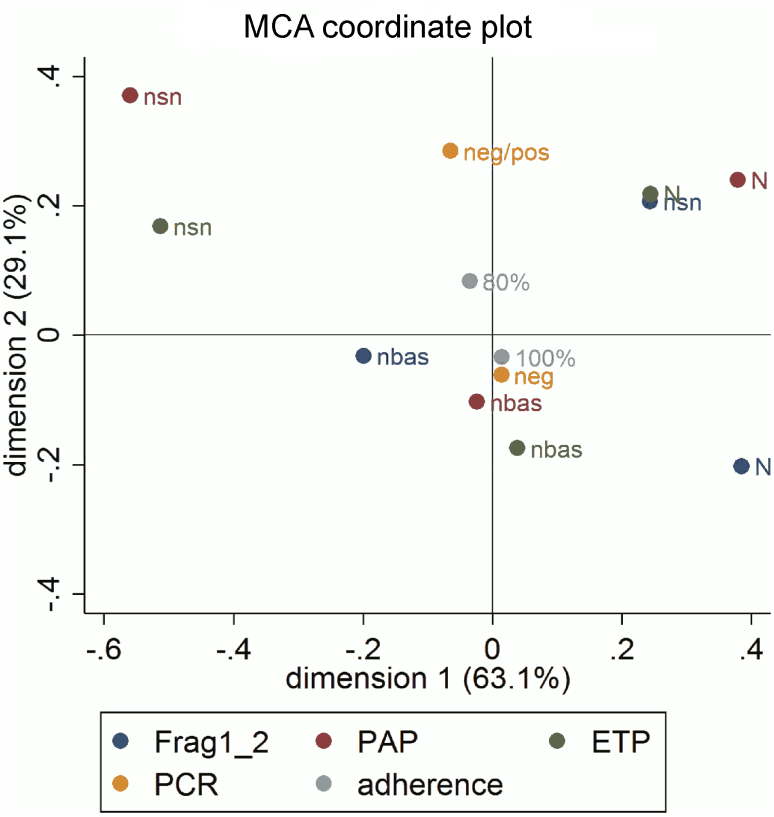

Fig 3. Multiple correspondence analysis coordinate plot. Biomarkers: N, sustained normalization of values throughout follow-up; nsn: non-sustained normalization throughout follow-up; nbas, normal value at baseline. Supplementary variables: qRT-PCR during follow-up: neg (negative); pos (positive); Level of adherence: 80%; 100%. Abbreviations: ETP, endogenous thrombin potential; F1+2, prothrombin fragment 1+2; PCR, polymerase chain reaction.

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dimension, while normal baseline ETP and PAP values had the highest contribution and correlation in the negative part. F1+2 normalization clearly characterized the positive part of the first dimension, while non-sustained normalization of PAP and ETP values clearly characterized the negative part. In other words, the sustained normalization observed post-treatment in PAP and ETP, could, despite the non-sustained normalization of F1+2 values, reflect response to antiparasitic treatment due to the strong correlation between these three variables.

The projection of qRT-PCR results and adherence to treatment in the solution space provided little additional information. Consistently negative qRT-PCR results throughout follow-up appear to be related to 100% treatment adherence.

In a recent study, the authors found that the serum samples of 37 individuals with chronic Chagas disease showed an upregulation of specific fragments of apolipoprotein A-1 (Apo A1) and one fibronectin fragment, that returned to normal levels in 43% of them three years after a treatment with nifurtimox. [38] Apo A1 and fibronectin fragment were altered in all the 37 patients with *T.cruzi* infection before treatment, but the number of patients treated with that normalized levels was lower than in our series (60% and 96% of patients who normalized F1+2 and ETP values).

This study has some limitations. Although the sample size was calculated to obtain sufficient statistical power to answer the hypothesis, a larger sample may have detected differences that would be expected to appear earlier (e.g., before 12 months). The lost to follow-up samples also

affected the estimates. Even within Spain, it is difficult to follow individuals with high migratory mobility for long periods. In addition, the fact that only 30% of patients had a positive baseline qRT-PCR result was a constraint for assessing the effect of treatment.

In conclusion, patients with chronic *T.cruzi* infection have a potential hypercoagulable state, regardless of cardiological and/or digestive involvement. The hypercoagulability markers F1+2 and ETP were abnormally expressed in a high percentage of patients with chronic *T.cruzi* infection before treatment (77% and 50%, respectively) but returned to and remained at normal levels shortly after treatment in 76% and 96% of patients, respectively. Baseline PAP values were altered in just 30% of patients before treatment, but normalized several months after treatment in 88% of these. These three hypercoagulability biomarkers could be useful for assessing short-term response to treatment. However, the fact that normal values were seen in some infected patients, including some with positive post-treatment qRT-PCR results, reduces their usefulness as universal biomarkers. The decrease in hypercoagulability factor levels could be explained by a decrease in parasitemia or by other benznidazole effect.

Author Contributions

Conceived and designed the experiments: MJP JM LI JCR JG. Performed the experiments: MJP EdJP LI DT AFM EA JM AA ST MG ICdA JCR JG. Analyzed the data: MJP EdL ICdA JCR JG. Contributed reagents/materials/analysis tools: MJP LI DT AFM AA ST MG ICdA JCR JG. Wrote the paper: MJP EdJP LI DT AFM EA JM AA ST MG ICdA JCR JG.

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VI. Resumen de resultados y discusión

Los estudios presentados en esta tesis contribuyen a profundizar en el conocimiento acerca de la fisiopatología de la infección por *T. cruzi*, y a mejorar de forma directa el manejo de la personas que la padecen, mediante a) la definición de biomarcadores de respuesta terapéutica; y b) la identificación de algunos de los factores de hipercoagulabilidad como biomarcadores de respuesta terapéutica precoz.

Subproyecto 1:

Evaluación de los biomacadores de respuesta terapéutica existentes y definición de perfil de producto

La primera de las observaciones de la revisión sistemática realizada en el contexto del primero de los subproyectos es la heterogeneicidad de datos que se extraen de los diferentes estudios y de los parámetros de evaluación de los biomarcadores seleccionados. Este hecho dificultó la comparación de la eficacia de los mismos. No obstante, existen varios grupos de moléculas que demostraron capacidad de detectar respuesta al tratamiento, y que en nuestro trabajo dividimos en dos:

a) Biomarcadores del parásito

Se detectaron dos tipos principales de proteínas recombinantes que demostraron ser útiles como marcadores de respuesta terapéutica en diferentes

estadios de la enfermedad y en poblaciones de diferentes edades, que son las siguientes:

- Grupo de 16 proteínas recombinantes. (71)
- Grupo de cuatro proteínas recombinantes: KMP11,HSP70, PFR2 y Tgp63. (72)

En la revisión destacaron otras moléculas que han demostrado utilidad práctica en estadios concretos de la enfermedad, como la CoML (purificado de tripomastigote)(73) o el antígeno F29,(74,75) o en población pediátrica, como el antígeno AT. (76)

En el grupo de biomarcadores o moléculas dependientes del parásito se incluyen las técnicas mediante las cuáles se detectan los ácidos nucleicos del mismo. Hasta el momento, la reacción en cadena de la polimerasa (PCR) es la que ofrece mayor sensibilidad en el diagnóstico parasitológico de la infección. Las técnicas de PCR se usan para evaluar la presencia de fallo terapéutico o falta de adherencia al tratamiento de forma precoz, mediante la detección del ADN del parásito en el hospedador, por lo que se establece como una de las técnicas de elección para el seguimiento después del tratamiento específico en todos los estadios de la infección/ enfermedad. (77) No obstante, el uso de estas técnicas no está exento de limitaciones: la sensibilidad del test en fases crónicas de la enfermedad no es del 100%, por lo que un resultado negativo no excluye que el parásito permanezca en el organismo después de que se haya completado el tratamiento. Por otro lado, es necesario un equipo específico para la realización de estas técnicas, y personal

altamente cualificado, lo que supone una limitación en su uso, sobre todo en áreas en que la infección por *T. cruzi* es endémica.

b) Biomarcadores del hospedador

En relación a la respuesta a la infección por parte del hospedador, se detectan dos grupos de marcadores bien definidos: aquellos que surgen como respuesta inmunológica al parásito, ya sea celular o humoral; y aquellos que dan noticia del daño que el parásito produce en el organismo del hospedador.

En cuanto a los marcadores de respuesta inmunológica, se han detectado diferentes moléculas para el diagnóstico de la infección o alguno de sus estadios, pero solo el receptor muscarínico del antígeno M2 ha sido evaluado como marcador de respuesta terapéutica en poblaciones de edad inferior a los 18 años.(78) Otros marcadores inmunológicos como citokinas, interferón gamma, o marcadores de superficie celular del hospedador se encuentran en un estado de desarrollo inicial, y no han sido hasta el momento evaluados como marcadores de respuesta terapéutica en el contexto de la infección crónica por *T. cruzi*.

En cuanto a los marcadores de daño en el hospedador, se han estudiado varias moléculas en este contexto, pero solo cuatro entre ellas (ETP, F1+2, ApoA1 y fibronectina) (artículo 2 de la presente tesis, 79) han sido evaluadas como marcadores de respuesta terapéutica en diferentes estadios de la infección. No obstante, en el momento en el que se realizó la revisión sistemática, todas ellas requerían ser evaluadas en profundidad para una mejor definición de su uso como

herramienta de diagnóstico y/o pronóstico. El objetivo del subestudio 2.2 de la presente tesis doctoral fue la evaluación en profundidad de ETP y F1+2.

El segundo de los objetivos del primer subproyecto fue, en base a la revisión sistemática de los estudios publicados acerca de biomarcadores de respuesta terapéutica, establecer un perfil de producto ideal/ aceptable como marcador de respuesta terapéutica en la infección crónica por *T. cruzi*, aplicable en diferentes escenarios clínicos y epidemiológicos, hasta el momento inexistente.

Para ello, en el artículo 1 se incluye una tabla en la que se define cuáles son los criterios de calidad ideales y/ o aceptables que debe de cumplir una molécula para ser considerado como biomarcador de respuesta terapéutica al tratamiento específico de la infección por *T. cruzi*.

En este punto, es importante destacar, que entre los criterios exigidos a las moléculas evaluadas, destacan dos aspectos esenciales:

- El porcentaje de personas con infección por *T. cruzi*/ EC que expresan el marcador antes del inicio del tratamiento específico: idealmente se debería de expresar en el 100% de las personas antes del tratamiento, pero se consideró aceptable su expresión en al menos un 50% de las personas con la infección.
- El tiempo en el que el biomarcador se modifica hacia rangos de normalidad una vez completado el tratamiento: se consideró ideal que la normalización después del tratamiento ocurriera en un plazo de tres meses después del tratamiento, pero se consideró aceptable que

ocurriera en 12-24 meses (en relación con los actuales ocho-diez años que demora la serología convencional en modificarse).

Asimismo, es importante destacar que si bien se espera que el genotipo parasitario tenga una influencia directa en la respuesta al tratamiento, la expresión y la respuesta del biomarcador no debería verse de influenciada por el mismo.

Con los resultados previamente expuestos se dio respuesta a los objetivos definidos para el subproyecto 1.

Una vez definidos los criterios ideales/ aceptables que debe de cumplir un biomarcador de respuesta al tratamiento etiológico de la infección por *T. cruzi*, pudo ser completado el segundo de los subestudios, cuyos principales resultados y discusión se describen a continuación.

Subproyecto 2:

Desarrollo de factores de hipercoagulabilidad como marcadores de respuesta terapéutica en la enfermedad de Chagas crónica.

Como resultados de este subproyecto se incluyen los datos obtenidos de los subestudios 2.1 y 2.2.

El primero de los subestudios (proyecto piloto) fue publicado en Thrombosis and Haemostasis, 2011.

Se reclutaron 43 pacientes, 25 del G1 y 18 del G2, con un rango etario entre 20 y 45 años. De los pacientes del G1, se completó seguimiento en 15. No se hallaron diferencias entre pacientes de ambos grupos en factores de riesgo aterotrombótico, y ninguno de ellos presentó antecedentes de ictus y/o fibrilación auricular. Tres de los pacientes del G1 presentaban enfermedad de Chagas en fase crónica cardiológica, ninguno de ellos avanzada (moderada o estadio Kuschnir II en dos de los tres pacientes). Se hallaron diferencias estadísticamente significativas en algunos de los factores de hipercoagulabilidad, entre los pacientes del G1 antes del tratamiento con benznidazol y los pacientes del G2. Estos factores fueron F1+2 (p<0.001), PAP (p=0.002), P-Sel (p=0.001), ETP (p=0.001), D-dímero (p=0.049) y FVIIa (p=0.03). Pese a las diferencias encontradas, las medianas del PAP, P-Sel, D-dimer y FVIIa se hallaron en ambos grupos dentro del rango de normalidad, por lo que no podemos concluir que en pacientes con infección con *T. cruzi*/ EC exista una alteración de estos factores. Los resultados del F1+2 y del ETP mostraron sin embargo que en el 84% y en el 64% de los pacientes del G1 presentaban valores alterados respectivamente, existiendo una diferencia de estos en relación a los hallado en el G2, tal y como se ha descrito. En el análisis a los seis meses después del tratamiento de los pacientes del G1 (N=15), se halló una disminución estadísticamente significativa de los niveles de fibrinógeno (p=0.004), F1+2 (p=0.003), PAP (p=0.004), P-Sel (p=0.006), y ETP (p=0.0008). Sin embargo, pese a la diferencia estadísticamente significativa del descenso observado, solo el F1+2 y el ETP presentaban una mediana de valores alterado antes de realizar el tratamiento, y se normalizaron seis meses después del mismo.

En consecuencia, si evaluamos el F1+2 y el ETP en base a los parámetros definidos en el subproyecto 1, ambos cumplen los criterios de biomarcador aceptable de respuesta terapéutica en cuanto a las características principales que se le exigen a los mismos: expresión en un alto porcentaje de las personas no tratadas con infección por *T. cruzi* y normalización de los mismos a los seis meses desde el final del tratamiento (siendo el criterio de aceptabilidad entre los 12- 24 meses).

La RT-PCR fue positiva en tres pacientes antes de iniciar tratamiento, negativizándose en dos de ellos seis meses después del tratamiento. En dos pacientes se observó una RT-PCR positiva 6 meses después de realizar tratamiento (uno de ellos había presentado PCR positiva antes del tratamiento). En estos dos pacientes los parámetros hemostáticos también se mantenían alterados seis meses después del tratamiento.

Con estos datos se responde al segundo de los objetivos definidos para el subestudio 2.1, pero queda por analizar si los valores fuera de rango del F1+2 y del ETP definen un estado de hipercoagulabilidad en aquellas personas en las que se hallan alterados, lo que pasamos a analizar después de exponer los datos del subestudio 2.2.

En base a los resultados del subestudio 2.1, y una vez definidos los criterios de biomarcador ideal/ aceptable de respuesta terapéutica al tratamiento de la infección por *T. cruzi*, se completó el diseño del subestudio 2.2. Para la realización de este estudio, se aumentó el tamaño de muestra para dar una mayor potencia a los resultados, y se realizó un seguimiento más prolongado en el tiempo con la finalidad

de evaluar si la respuesta terapéutica con la normalización de los mismos era sostenida en el tiempo.

En el subestudio 2.2. participaron 99 pacientes, 56 fueron incluidas en el G1 (con infección por *T. cruzi*) y 43 personas en el G2 (no presentaban la infección). La edad, el origen y las características clínicas del grupo ampliado de participantes fueron similares al del subestudios 2.1. En este subestudio, con un periodo de seguimiento de 36 meses, es importante destacar que ninguno de los participantes considerado en el análisis viajó a su país de origen durante el periodo del estudio, evitando así la posibilidad de reinfección después del tratamiento. Entre los pacientes del G1, 50 de los 56 realizaron tratamiento con BZD, de los cuales 46 lo completaron (90%).

En el subestudio 2.2 se evidencia una alteración de los valores de F1+2 (70% de pacientes de G1) y ETP (50% de pacientes de G1), por encima de rangos normales, y diferentes estadísticamente de los valores de estos factores en los pacientes del G2, en los que todos ellos se mantuvieron dentro de rangos normales. Los hallazgos del subestudio 2.2 refuerzan los resultados del subestudio 2.1 y de otros estudios publicados, si consideramos que la alteración de estos factores define un estado de hipercoagulabilidad.(63, 64, 80)

Después del tratamiento, el 76% de los pacientes con alteración del F1+2, el 98% de los pacientes con alteración del ETP normalizaron los valores en nueve (rango intercuartílico (RIC) 8) y seis meses (RIC 3) de mediana respectivamente. Los valores se mantuvieron en rango normal o con mínimas alteraciones durante todo el periodo de seguimiento, sin cambios estadísticamente significativos.

En G1 se detectaron nueve pacientes con qRT-PCR (reacción en cadena de la polimerasa en tiempo real cuantitativa) en algún punto del seguimiento después del tratamiento (cuatro de ellos con qRT-PCR positiva antes del tratamiento), lo que indica una tasa de fracaso del tratamiento del 18%. Es importante destacar que ninguno de los pacientes con valores de F1+2 normales durante el seguimiento positivizó a qRT-PCR durante el seguimiento. Tres (13%) pacientes con valores normales ETP durante el seguimiento positivizaron la qRT-PCR.

La lectura de los resultados del subestudio 2.2, en base a los criterios de evaluación de estos factores como marcadores de respuesta terapéutica, indica que ambos son aceptables como biomarcadores de respuesta terapéutica en pacientes con infección por *T. cruzi*, respondiendo así a los objetivos 2 y 3 de este subestudio. No obstante, y en base al análisis de la coherencia de normalización de estos factores en relación a los resultados de qRT-PCR, un resultado positivo qRT-PCR después del tratamiento en pacientes que alcanzaron la normalización de F1+2 y/o ETP, podría significar que una disminución de la parasitemia sería suficiente para modificar el estado de hipercoagulabilidad. Por otro lado, se podría interpretar que BZD, que actúa sobre el sistema oxidación-reducción, podría modificar estos biomarcadores sin eliminar la parasitemia por completo. En este caso, esto limitaría el uso de estos factores como biomarcadores que indiquen la eliminación del parásito, aunque siguen teniendo un importante valor como indicadores de respuesta al tratamiento.

En este punto retomamos la definición del estado de hipercoagulabilidad de las personas con infección por *T. cruzi* y si este puede ser definido por la alteración del F1+2 y del ETP.

El F1+2 es una medida indirecta de la cantidad real de trombina generada in vivo (principalmente debido a la lesión endotelial, incluso en estados subclínicos) (81), y los niveles de ETP indican la cantidad potencial de trombina que se puede formar cuando la coagulación sanguínea se active a través de la adición de factor tisular.(81) La alteración de ambos en rangos por encima de la normalidad se expresaría, desde el punto de vista clínico, como un estado de hipercoagulabilidad.

Otros factores en los que se hallaron alteraciones, pero que sin embargo no cumplen con los criterios de biomarcador ideal/ aceptable para la evaluación de respuesta terapéutica en la infección por *T. cruzi* fueron el PAP y la P-Sel. Los complejos PAP son marcadores de la activación de la fibrinólisis. Tras la activación, la plasmina, que es la principal responsable de la disolución controlada y regulada de los polímeros de fibrina en fragmentos solubles, se inactiva inmediatamente por antiplasmina, formando complejos inactivos de PAP.(82) Por lo tanto es de esperar un aumento en los complejos de PAP, que indica de modo indirecto la cantidad de plasmina formada in vivo, en pacientes con infección por *T. cruzi*, en los que se observa un aumento de la trombina que es activador suyo. La P-selectina soluble se considera un biomarcador de la activación plaquetaria in vivo, y se ha demostrado que actúa como un enlace entre la trombosis y la inflamación.(83) Adicionalmente, estos cuatro factores (F1+2, ETP, complejos PAP, y P-Sel) son altamente estables en el tiempo en los individuos.

En el subestudio 2.2 se definió como objetivo 4 establecer la utilidad A&T CL-ELISA como marcador de evolución de la enfermedad, y ver si existía coherencia entre la evolución de los niveles de los títulos de este ELISA con los niveles de los factores de hipercoagulabilidad estudiados a lo largo del tiempo después del tratamiento. Los resultados de A&T CL-ELISA fueron positivos en 52 (96%) de los 54 pacientes evaluados antes del tratamiento, y en todos los pacientes se mantuvieron dentro de los niveles positivos al final del seguimiento. Sin embargo, se observó una disminución en los niveles de A&T CL-ELISA a partir del mes 12, que fue progresiva hasta el final del seguimiento y estadísticamente significativa desde el mes 18 ($p = 0,0052$). Resultados similares se obtuvieron en las pruebas de ELISA convencional, con la diferencia de que el resultado esperado en la reducción de títulos serológicos en los ensayos convencionales tras 36 meses de seguimiento, era que no hubiera variación o que el descenso de títulos fuera mínimo. En el caso de los A&T CL-ELISA, y en base a lo recogido en la literatura, en una cohorte de adolescentes con infección por *T. cruzi* tratados con BZD los anticuerpos líticos anti-α-Gal, medidos por A&T CL-ELISA, mostraron seronegativización en el 58% y el 85% de los pacientes a los 36 y 72 meses después del tratamiento, respectivamente. (84,85) Las diferencias entre estos estudios y los nuestros pueden deberse a la naturaleza de las cohortes (adolescentes frente a adultos) y la etapa de la infección. Sin embargo, ambos estudios mostraron una tendencia similar hacia una reducción de los anticuerpos anti-α-Gal líticas medidos por A&T CL-ELISA después del tratamiento con BZD.

De los pacientes con niveles alterados de F1+2, ETP, o complejos PAP al inicio del estudio, un resultado positivo qRT-PCR durante el seguimiento no se asoció

significativamente con los cambios observados en A&T CL-ELISA, F1+2, ETP y / o PAP.

La adherencia al tratamiento de los pacientes de la cohorte fue elevada (sólo cinco pacientes no superaron el 80% de la dosis total de BZD prescrita). Los cinco pacientes mostraron valores anormales de F1+2 durante todo el seguimiento y tres (60%) tuvieron valores de ETP y PAP anormales. Uno de los cinco pacientes tuvo un resultado qRT-PCR positivo durante el seguimiento, y los cinco mantuvieron los valores de ELISA y A&T CL-ELISA en rango similar durante todo el seguimiento.

Por último, y en base a los resultados del subestudio 2.2, se analizó la relación entre la normalización de los factores de hipercoagulabilidad (F1+2, ETP y PAP), los resultados de qRT-PCR y la adherencia al tratamiento mediante un análisis de correspondencias múltiples. El análisis muestra que la normalización sostenida observada después del tratamiento en el PAP y ETP, podría, a pesar de la normalización intermitente de los valores de F1+2, reflejar la respuesta al tratamiento antiparasitario, debido a la fuerte correlación entre estas tres variables. El modelo también indica que resultados consistentemente negativos de qRT-PCR durante todo el seguimiento parecen estar relacionados con pacientes que completaron el 100% del tratamiento.

VII. Conclusiones

VII.a. Subproyecto 1

1.- Existen diferentes biomarcadores que han demostrado ser útiles para evaluar la respuesta al tratamiento antiparasitario en pacientes con infección por *T. cruzi* en diferentes estadios.

2.- Hasta el momento, las técnicas de amplificación de ácidos nucleicos son las de elección en la evaluación de fallo terapéutico del tratamiento específico de la infección por *T. cruzi*.

3.- Los biomarcadores bioquímicos e inmunológicos de respuesta terapéutica se encuentran en diferentes fases de desarrollo, y para estandarizar su uso se requiere de nuevos estudios que obtengan resultados más robustos.

4.- Los biomarcadores de respuesta al tratamiento en pacientes con infección crónica por *T. cruzi* han sido evaluados en base a parámetros heterogéneos, lo que dificulta la comparación de eficacia entre ellos.

5.- La definición de los criterios de un biomarcador ideal o aceptable para la evaluación del tratamiento específico de la infección por *T. cruzi* es indispensable para estandarizar el desarrollo de estas moléculas, y poder comparar la eficacia de las mismas en este uso.

6.- De cara al futuro, se perfila el uso de una batería de biomarcadores que incluyan moléculas descritas en cada uno de los grupos (dependientes del parásito, del hospedador y de técnicas de amplificación de ácidos nucleicos) para una evaluación precisa de la respuesta terapéutica a la infección por *T. cruzi*.

VII.b. Subproyecto 2

7.- En los pacientes con infección por *T. cruzi* incluidos en este subproyecto, el F1+2 y el ETP estaban elevados en rango patológico antes del inicio del tratamiento antiparasitario con BZD en un porcentaje superior al 50% de los individuos.

8.- Existe un estado de hipercoagulabilidad en un porcentaje de pacientes con infección por *T. cruzi* definido por la alteración del F1+2, del ETP, y en menor frecuencia del PAP.

9.- Los factores F1+2, ETP, y en menor medida el PAP, tienen un importante valor como indicadores de respuesta al tratamiento, pese a que tienen, sobre todo el ETP, un valor limitado como biomarcadores que indiquen la eliminación del parásito.

10.- Se observa una normalización estadísticamente significativa del F1+2 y del ETP después de la administración de tratamiento con BZD para la infección por *T. cruzi* en un tiempo inferior a 12 meses.

11.- El F1+2 y ETP cumplen con los criterios que definen un biomarcador de respuesta al tratamiento específico anti- *T. cruzi* como aceptable.

12.- Los anticuerpos líticos anti-α-Gal medidos por A&T CL-ELISA después del tratamiento con BZD mostraron una reducción tras 36 meses de seguimiento, sin observarse negativización en los mismos.

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