Trypanosoma cruzi Trypomastigote Glycosylphosphatidylinositol-Anchored Mucins And A Synthetic Alpha-Gal-Containing Neoglycoprotein As Potential Biomarkers And Vaccines For Chagas Disease

Igor Leandro Estevao
University of Texas at El Paso
Trypanosoma cruzi TRYPOMASTIGOTE
GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED MUCINS
AND A SYNTHETIC α-Gal-CONTAINING NEOGLYCOPROTEIN AS
POTENTIAL BIOMARKERS AND VACCINES FOR CHAGAS DISEASE

IGOR LEANDRO ESTÊVÃO DA SILVA

Master's Program in Biological Sciences

Approved:

_______________________
Igor C. Almeida, D.Sc. Chair

_______________________
Rosa Maldonado, Ph.D.

_______________________
Katja Michael, Ph.D.

_______________________
Stephen Crites, Ph.D.
Dean of the Graduate School
Dedication

To my beloved mother, Maria Luzia da Silva, who currently lives in Brazil.
Trypanosoma cruzi TRYPOMASTIGOTE
GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED MUCINS
AND A SYNTHETIC α-Gal-CONTAINING NEOGLYCOPROTEIN AS
POTENTIAL BIOMARKERS AND VACCINES FOR CHAGAS DISEASE

by

IGOR LEANDRO ESTÊVÃO DA SILVA

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Science

THE UNIVERSITY OF TEXAS AT EL PASO

August 2019
Acknowledgments

This work has been both a scientifically, highly productive, and intellectually stimulating sharing. The hard work has been paid off knowing that these moments of joy, learning, and unique lab environment will be unforgettable because they were lived with friends rather than colleagues. Here then, is a double dose of appreciation to those who deserve much more:

Dr. Igor Almeida, for all of the support, trust and help he has given me since day one. He has been very important to my development as a human being and as a researcher. His unrestricted mentorship allowed me to achieve great things as a master’s student.

My wife Jessica Estêvão, because she is incredibly amazing. She is my life’s cornerstone, my best friend, my beloved one. Her support is unconditional, pure; and I wouldn’t be who I am without her beside me.

My mother Maria Luzia da Silva (she gets two mentions), for being an inspiration, model, and hero during my entire life. She is priceless in the truest sense of the word.

My two fathers João Maria de Souza and Carlos Alberto Soares, my step mother Geralda Lemos Pereira which I have great admiration for, grandmother Margarida and my god mother Elizete Lopes Silva and furthermore relatives. I usually have to face most of my problems alone but sometimes they are there to pull me out of the lower points of my life. These relatives are beyond rare; they are always there for me.

My committee members Dr. Igor Almeida, Dr. Katja Michael, and Dr. Rosa Maldonado, for taking me on.

The present and former Almeida’s lab members: Uriel Rodriguez-Ortega, Brenda Zepeda, Alexa Alawneh, Bernice Caad, Jerry Duran, Nasim Hosseini, Maria Tays Mendes, Janet Olivas, Trini Ochoa, Nasim Salloum, Gloria Polanco-Anaya, and Veronica Escalante have been instrumental
for my time here at UTEP. Especial thanks to Uriel Rodriguez-Ortega, so called “CL-Elisa wall”, for his hard work as my lab partner but mostly for his unconditional friendship.

Dr. Das, thanks for making our days happier and enthusiastic!

The Maldonado’s lab members, UTEP professors, especially Renato Aguilera for the knowledge and support passed around.

The Department of Biological Science, The University of Texas at El Paso, and Graduate School for all generous scholarships and use of the amazing infrastructure such as, laboratories, library and Core Facilities.

Many thanks to the staff of the core facilities for the assistance and access to the equipment; for the personnel of the Bioscience building main office for assistance and their kindness.

Our great collaborators from Barcelona, Spain: Dr. Dr. Joaquim Gascon, Dr. Maria-Jesús Pinazo, Dr. Luis Izquierdo, and Dr. Julio Padilla; from Geneva, Switzerland: Dr. Isabela Ribeiro; from Venezuela: Dr. Oscar Noya, and Dr. Belkisyolé Alarcón de Noya; and from Texas, USA: Dr. John VandeBerg, and Susan Mahaney.

I thank the UTEP/BBRC Core Facilities: Biomolecule Analysis (BACF) and Genomic Analysis (GACF). The BBRC/UTEP Core Facilities are supported by a grant (2G12MD007592) from the National Institute on Minority Health and Health Disparities (NIMHD).

This work was supported by Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation (Graduate School, UTEP and National Institutes of Health (1U01AI129783-01A1, 1R21AI115451-01, and 2G12MD007592).
Abstract

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, is a neglected tropical disease that kills or permanently disable thousands of people annually. About 6-8 million people are estimated to be infected worldwide. Although many efforts have been made for the development of an effective immunotherapy, currently there is no vaccine to prevent or treat CD in humans. Despite their toxicity, the two current drugs for CD, benznidazole (BZN) and nifurtimox (NFX), have medium-to-high efficacy in the chronic stage of the disease and could save or improve the lives of thousands of patients. However, negative seroconversion in treated patients, as measured by the conventional serology, may take 10-20 years to occur. This is one the reasons that only 0.3% of chronic CD (CCD) patients are currently being treated. Identification of biomarkers (BMKs) to evaluate early response to chemotherapy for chronic CCD has become of paramount importance because of lack of validated early BMKs for CCD therapeutic responses. Therefore, clinical and laboratorial BMKs are urgently needed for the early assessment of treatment outcomes or disease progression, thus potentially new drugs, and/or different treatment regimens could be tested. The cell surface of infective *T. cruzi* trypomastigote form is covered by highly immunogenic glycosylphosphatidylinositol-anchored (GPI) mucin-like glycoproteins (tGPI-mucins). Almeida and colleagues have shown in a series of publications in the last several years that CCD patients have high levels of protective antibodies (Ch anti-α-Gal Abs) against the terminal nonreducing α-Gal glycotopes expressed on tGPI-mucins or TcMUCII mucins. Those authors have also shown that Ch anti-α-Gal Abs correlate with parasitological cure following chemotherapy with BZN, thus negative seroconversion for those antibodies could be considered a reliable criteria of cure in contrast to PCR assay. The Ch anti-α-Gal Abs recognize a major and immunodominant glycopeptide expressed on tGPI-mucins, the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα (Galα3LNα). In this project, I aimed to validate the synthetic glycopeptide Galα3LNα covalently conjugated to a carrier protein (bovine serum albumin, BSA), giving rise to a neoglycoprotein (NGP) named NGP24b (Galα3LNα-BSA), as a reliable surrogate BMK for the tGPI-mucins for early assessment of treatment outcomes (Chapter 1). Moreover, I evaluated Galα3LNα-HSA as potential therapeutic vaccine candidate (Chapter 2) in an experimental murine transgenic model of CD that mimics human antibody responses to α-Gal glycotopes.

**Keywords:** *Trypanosoma cruzi*; Chagas disease; diagnosis; chemotherapy; treatment follow-up; anti-α-Gal antibodies; biomarkers; trypomastigote mucins; synthetic neoglycoprotein; vaccine.
Table of contents

Acknowledgments ........................................................................................................ iv

Abstract ...................................................................................................................... vi

Table of contents ....................................................................................................... vii

List of tables ............................................................................................................... viii

List of figures ............................................................................................................. x

Chapter 1: tGPI-Mucins and an α-Gal-containing neoglycoprotein as candidates for Chagas disease biomarkers ................................................................. 1

Chapter 2: α-Gal-containing neoglycoprotein as candidates for Chagas disease vaccine ........ 48

Conclusions and Discussion ...................................................................................... 65

Appendix .................................................................................................................... 68

References .................................................................................................................. 69

Curriculum Vitae ....................................................................................................... 91
List of tables

Table 1 ........................................................................................................................................... 15
# List of figures

## Chapter 1:

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>36</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>41</td>
</tr>
</tbody>
</table>

## Chapter 2:

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>43</td>
</tr>
<tr>
<td>17</td>
<td>44</td>
</tr>
<tr>
<td>18</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>57</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>21</td>
<td>62</td>
</tr>
<tr>
<td>22</td>
<td>65</td>
</tr>
</tbody>
</table>
I. CHAPTER 1:

*Trypanosoma cruzi* trypomastigote glycosylphosphatidylinositol-anchored mucins (tGPI-mucins) and a synthetic \(\alpha\)-Gal-containing neoglycoprotein as candidates for Chagas disease biomarkers

1.1. Chagas disease

Chagas disease (CD) (American trypanosomiasis) is endemic in Latin America, where 6-8 million patients are currently chronically infected with *Trypanosoma cruzi*, the protozoan parasite causing this disease, and about 100 million are at risk of infection (Coura and Vinas 2010) (Figure 1). CD was first described in 1909 by the Brazilian physician, Carlos Chagas (Steverding 2014). Due to a marked increase in population migration from endemic countries, CD is becoming a considerable burden to Europe, Australia, Japan, and the U.S.A. (da Silva Valente, de Costa Valente et al. 1999, Perez-Molina, Norman et al. 2012). It is estimated that 300,000 individuals infected with *T. cruzi* currently live in the U.S.A., with 30,000-45,000 of them having developed cardiomyopathy and 315 having already disseminated the disease congenitally (Castro, de Mecca et al. 2006, Tanowitz, Machado et al. 2009, Custer, Agapova et al. 2012) (Figure 2). In Europe, Spain is the country with the largest number of immigrants, mostly from Bolivia, chronically infected with *T. cruzi* (Gascon, Bern et al. 2010).
1.2. *Trypanosoma cruzi* life cycle

*Trypanosoma (Schizotrypanum) cruzi* life cycle has been extensively discussed since 1909, when Carlos Chagas first described it alongside with the novel infections disease called Chagas. He also described its vector, host, epidemiology, and clinical manifestations of the disease (Chagas 1909, Brener 1973). The parasite circulates between insect vectors and mammalian hosts, and has been portioned into seven discrete typing units (DTUs), TcI-VI and TcBat, with reference to their known molecular genetics, eco-epidemiological features, and pathogenicity (Zingales 2018). Geographical distribution, pathogenesis, clinical features, and response to therapy has been related to this genetic diversity (Zingales, Miles et al. 2012, Zingales 2018).

*T. cruzi* has two life-cycle stages or forms in the mammalian hosts, including humans, and two forms in the reduviid vector (Perez-Molina and Molina 2018). Chagas disease is therefore referred to as a vector-borne disease. The life cycle of the parasite initiates when hematophagous triatomine insect vector (order Hemiptera, family Reduviidae, genera *Triatoma, Rhodnius*, and *Panstrongylus*), popularly known as *kissing bug*, ingest a blood meal from a mammal and metacyclic trypomastigotes, nonreplicative and infective forms, excreted with the insect’s feces, enter the body via the bite wounds or mucosal membranes such as the conjunctiva (CDC, Dias, Laranja et al. 1956, Burleigh and Andrews 1995) (Figure 3). Inside the host, the metacyclic trypomastigotes invade nucleated cells near the site of inoculation, where they rapidly escape the parasitophorous vacuole and differentiate into intracellular amastigotes in the cytoplasm. Intracellular amastigotes are replicative but noninfective, so they multiply by binary fission and, 3-4 days later, differentiate into infective trypomastigotes,
which within 2-3 days rupture the host cell membrane to reach the extracellular milieu and, eventually, the bloodstream. Trypomastigotes are not replicative but they can infect other cells nearby and start a new replication cycle inside of the host cells after differentiating into intracellular amastigotes. Ultimately, a triatomine bug can ingest a pleomorphic population of bloodstream trypomastigotes (and up to 10% amastigotes) during a bloodmeal from an infected mammal (Ley, Andrews et al. 1988, De Souza 2002), and parasites then pass into the insect’s midgut. There, trypomastigotes differentiate into noninfective epimastigote forms, which replicate by binary fission and differentiate into infective metacyclic trypomastigotes in hindgut, once the bloodmeal has been completely digested (Andrews, Hong et al. 1987, Perez-Molina and Molina 2018). At this point, the cycle is complete and can be restarted when the triatomine takes another blood meal (Figure 3).
Figure 1. Estimated global population infected by *Trypanosoma cruzi*. Figure taken from: https://www.cell.com/trends/parasitology/references/S1471-4922(15)00143-9 (Perez, Lymbery et al. 2015).

Figure 2. Estimated burden of Chagas disease in the United States. Figure taken from reference (Manne-Goehler, Umeh et al. 2016).
Figure 3. Trypanosoma cruzi natural life cycle. Figure copied from https://www.cdc.gov/dpdx/trypanosomiasisamerican/index.html (CDC).
In the natural environment, *T. cruzi* is transmitted through a variety of species of triatomine bugs, which act as vectors. There are 151 species of triatomine bugs and all are considered capable of harboring and transmitting *T. cruzi* (Vieira, Praca et al. 2018). However, other non-vectorial transmission mechanisms have been described, such as via blood products, organ transplant, and vertical transmission. In regions where infection is endemic, oral transmission has also been described through ingestion of food contaminated with feces from infected bugs (da Silva Valente, de Costa Valente et al. 1999, Prata 2001).

*T. cruzi* evades the protective immune response mounted by the host against the parasite by invading nucleated cells of the liver, colon, esophagus, heart, and peripheral nerve system as amastigotes. Following this invasion, *T. cruzi* amastigotes induce the chronic phase of the disease in which the parasite sheds its antigens, which elicit extensive chronic inflammations. These autoimmune-like inflammations cause hepatomegaly, mega colon, and cardiomyopathy that gradually destroy the infected tissues, resulting in a chronic disease that ends with heart failure in many of the patients (Rassi, Rassi et al. 2010).

Drug treatment of infected patients has a questionable efficacy during the chronic phase of the disease, and is frequently associated with severe side effects. Thus far, there are only two drugs (i.e., nifurtimox-NFX and benznidazole-BZN) available for CD treatment. Both drugs cause moderate to severe side effects (Castro, de Mecca et al. 2006, Perez-Molina, Norman et al. 2012). Although both are very efficacious in the acute phase, their efficacy is more limited during the chronic phase. In addition, both drugs demonstrate poor activity against many *T. cruzi* isolates, and have considerable side effects that can lead to the discontinuity of the therapy (Soeiro Mde and de Castro 2011). In the U.S.A., BNZ was recently approved for
the treatment of CD in children (ages 2-12) (Alpern, Lopez-Velez et al. 2017). The treatment for *T. cruzi* infection is strongly recommended in both acute and chronic stages of the infection despite of the fact that treatment may not be efficient in late stages of the infection (Bern, Montgomery et al. 2007, Rassi, Rassi et al. 2010, Viotti, Alarcon de Noya et al. 2014).

1.3. Biomarkers

There is cumulative evidence and specific needs for development of complementary biomarkers to evaluate early responses to treatment for CCD (Pinazo, Thomas et al. 2014). Consequently, there is a great necessity for the development of novel, more efficient biomarkers for CD that will make evaluation of early responses to treatment for CCD more efficient for millions of people worldwide.

Conventional serology (CS) techniques (i.e., enzyme-linked immunosorbent assay-ELISA, indirect immunofluorescence-IIF, and indirect hemagglutination-IHA), using parasite lysates (ELISA) or fixed parasites (IIF and IHA) have high sensitivity and good specificity and are largely used for CD diagnosis (Britto, Silveira et al. 2001, Cancado 2002, Rodriques Coura and de Castro 2002). However, these methods fail in performing an effective and fast clinical follow-up of CCD patients following chemotherapy with BZN or NFZ, especially in adult patients. The conversion from a positive to a negative serological state (or negative seroconversion) of CS tests may take years to decades to occur (Viotti, Vigliano et al. 2006, Fabbro, Streiger et al. 2007). In a recent meta-analysis study, Sguassero et al. observed that there was a much higher probability of seroconversion by the CS in children (1-19 years) than in adults (>19 years), after 11-13 years of follow-up (Sguassero, Roberts et al. 2018). Those
authors showed that most (90%) of the adult patients remain positive for the CS even 11 years following treatment. Similar trends had been previously reported by Fabbro et al. (Fabbro, Streiger et al. 2007), who observed that the probability of negative seroconversion for adult patients treated with NFX (n=27) was 43% after 27 years of follow-up. On the other hand, in patients treated with BZN (n=27), the probability of negative seroconversion was only 32% after 25 years of follow-up. For untreated patients (n=57), the probability of negative seroconversion was 0%, clearly demonstrating that although not every effective, the treatment with BZN or NFX could cure a significant percentage of patients. Nevertheless, these and other studies have unequivocally demonstrated that the assessment of posttreatment outcomes of existing or new drugs, or new regimens of current drugs in clinical trials is one of the greatest challenges in CD (Pinazo, Thomas et al. 2015). Therefore, biomarkers (BMKs) could potentially be useful to evaluate the efficacy of current drugs in a short period of time, and also to validate tools for use in clinical trials when testing new drugs or drug regimens. Biomarkers could then possibly help physicians with patient outcome and bring more precise insights about therapeutic responses (Pinazo, Thomas et al. 2015). Several advances in the research of BMK molecules have been made but only a few of them have been tested after specific treatment and using appropriately designed studies (Pinazo, Thomas et al. 2015). Attempts to generate reliable BMK(s) for early detection and effectiveness of CD chemotherapy have been carried out by several groups and some of them are noteworthy due to the fact that they showed earlier negative seroconversion following treatment or early disease progression (e.g., cardiac damage) after T. cruzi infection.

The efficacy of BNZ treatment of chronically infected children and adolescents in a placebo-controlled randomized trial was evaluated by chemiluminescent ELISA (AT CL-ELISA) using
purified trypomastigote-derived glycosylphosphatidylinositol-anchored mucin glycoproteins (tGPI-mucins), also known as F2, F2/3 (Almeida, Krautz et al. 1993, Almeida, Ferguson et al. 1994, Almeida, Covas et al. 1997), or AT antigen (de Andrade, Zicker et al. 1996). The AT CL-ELISA was used to measure the lytic anti-α-Gal antibody titers after treatment with BZN. Serum from all participants was positive at the beginning of the trial with both CS (ELISA, IIF, and IHA) and non-CVS (AT CL-ELISA). Although all CS tests remained positive after a three-year observation period, at the end of follow-up, 37 (58%) of the 64 BZN-treated participants and 3 (5%) of those who received placebo were negative for anti-\textit{T. cruzi} antibodies. In the six-year follow-up of that study, showed successful chemotherapy in 64.7% and 84.7%, respectively, by intention-to-treat and by per-protocol analysis as measured by seronegativity in the AT CL-ELISA using tGPI-mucins (Andrade, Martelli et al. 2004). The AT CL-ELISA proved to be the first molecular tool for the follow-up of chemotherapy of CCD patients. In the current study, we have evaluated the same immunoassay for the follow-up of adult CCD patients from Bolivia and Spain treated with BZN.

Another important BMK used for evaluation of the efficacy of BNZ treatment in children with CD was the recombinant \textit{T. cruzi} flagellar calcium-binding protein (FCaBP) (Engman, Krause et al. 1989), also known as F29 (Sosa Estani, Segura et al. 1998). Data showed that 35.2% and 62.1% of the 44 BNZ-treated children were seronegative for the F29 antigen six and 48 months post-treatment, respectively (Sosa Estani, Segura et al. 1998). Moreover, data obtained from other researchers confirmed that the ELISA using F29 as antigen was useful as an early indicator of negative seroconversion in treated adult CCD patients as compared to CS (Fabbro, Velazquez et al. 2013). Negative seroconversion for F29 occurred in 50% of treated patients
16 years after the treatment, whereas by the CS that was observed only after 26 years of follow-up.

Based on these studies by Andrade et al. (de Andrade, Zicker et al. 1996) and Sosa-Estani et al. (Sosa Estani, Segura et al. 1998), a group of international CD experts invited by the Pan-American Health Organization (PAHO)/World Health Organization (WHO) decided to recommend the BZN treatment for children and adolescents (up to 15 years of age) with CD (OPS/OMS 1998). More recently, the negative seroconversion for the same two BMKs (i.e., tGPI-mucins and F29) was considered by FDA as criteria of cure to approve BZN for the treatment of CD in children (ages 2-12 years) (FDA 2017).

In other studies, a correlation between the results from CS tests and a set of 16 protein groups that were identified and incorporated into a multiplex bead array format was observed (Cooley, Etheridge et al. 2008, Viotti, Vigliano et al. 2011). Moreover, a set comprising four recombinant proteins (KMP11-H70-PFR2-Tgp63) and its differential reactivity correlated with the clinical status of the patients, following BZN treatment (Fernandez-Villegas, Pinazo et al. 2011, Fernandez-Villegas, Thomas et al. 2014). However, no statistically significant drop in reactivity was observed against total soluble T. cruzi proteins.

qPCR for amplification of T. cruzi DNA is currently the leading test for assessing the response to treatment in a short period of time in patients with chronic infection (Pinazo, Thomas et al. 2014). Nevertheless, parasite levels in bloodstream are not stable during the chronic phase of infection and may not be detected by PCR due to sensitivity, especially during the extensive trypanocidal treatment (Gomes, Lorena et al. 2009, Murcia, Carrilero et al. 2010, Perez-Ayala,
Perez-Molina et al. 2011). A negative PCR result does not mean a parasitological cure or a response to a specific treatment. This technique is considered very useful, however, it is not available in regular health care centers (Pinazo, Thomas et al. 2015).

Short RNA ligands called aptamers such as Aptamer L44 (Nagarkatti, Bist et al. 2012, Nagarkatti, de Araujo et al. 2014), biochemical biomarkers such apolipoprotein (APO) and fibronectin fragments (Ndao 2012, Santamaria, Chatelain et al. 2014), cytokines and surface markers (van Kooten and Banchereau 2000, Laucella, Postan et al. 2004, Chamekh, Vercruysse et al. 2005, Albareda, Laucella et al. 2006), chemokines (Falcao, Correa-Oliveira et al. 2002, Tunon, Blanco-Colio et al. 2014), hypercoagulability biomarkers (Pinazo, Tassies et al. 2011), and several other molecules have been proposed as biomarkers of treatment outcomes in T. cruzi infection (Table 1). Despite of the fact that some of these molecules are promising and can be used to characterize treatment response, most of them have not yet been explored as a surrogate marker of parasitological cure or have limitations hampering sensitivity. Moreover, lack of further assessment of the response to chemotherapy in well-controlled, prospective clinical trials has hampered the application of any of these potential BMKs in the clinical settings and, therefore, a significant breakthrough in the follow-up of treated CD patients (Pinazo, Thomas et al. 2015).

Cardiac markers, such as troponin I, marker of early stages of the disease, and natriuretic peptides have been used in several works to determine disease progression at the myocardial level (Puyo, Scaglione et al. 2002, Ribeiro, Reis et al. 2003, Heringer-Walther, Moreira et al. 2005, Puyo, Scaglione et al. 2005). Several other biochemical molecules that test tissue damage, such as glutamic oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline
phosphatase, acid maltase, alpha-hydroxybutyric dehydrogenase (or LDH1), adipokines and angiotensin-converting enzyme, have also been assessed to evaluate any possible early heart problems (Alarcon-Corredor, Carrasco-Guerra et al. 2002, Combs, Nagajyothi et al. 2005, Wang, Moreira Mda et al. 2010). It is important to emphasize that there are no reports of studies using these BMKs to assess therapeutic efficacy (Pinazo, Thomas et al. 2015).

Glycosylphosphatidylinositols (GPIs) are a class of natural glycosylphospholipids that anchor proteins and glycoproteins (via their C-termini) such as mucins, as well as lipophosphoglycans (via the reducing end of the chain) to the membrane of eukaryotic cells, including T. cruzi. GPI anchors are widely present in parasitic protozoa, which are the most diverse and amongst the most ancient group of organisms in the eukaryotic kingdom (Sogin, Gunderson et al. 1989). The surface of T. cruzi trypomastigotes is covered by GPI-anchored glycoproteins such trans-sialidases (TSs), mucin-associated surface proteins (MASPs), and tGPI-mucins (mainly, from the TcMUCII subfamily, and glycoinositolphospholipids (GIPLs) (Acosta-Serrano, Almeida et al. 2001, Acosta-Serrano, Hutchinson et al. 2007) (Figure 4, left panel).

tGPI-Mucins contain the linear immunodominant glycootope Galα1,3Galβ1,4GlcNAcα (Galα3LNα) (Figure 4, middle and right panel), which induce high levels of protective T. cruzi-specific anti-α-Gal antibodies in ChD patients (Almeida, Ferguson et al. 1994). This is the only tGPI-mucin O-glycan that has been fully characterized to date and it is strongly recognized by Ch anti-α-Gal Abs, but only weakly by anti-α-Gal Abs from healthy individuals (normal human serum (NHS) anti-α-Gal Abs) (Almeida, Ferguson et al. 1994, Schocker, Portillo et al. 2018).
Among mammals, the natural anti-α-Gal antibody is produced only in humans, apes, and Old World monkeys, whereas other mammals synthesize the α-Gal epitope and thus cannot produce this antibody (Galili, Clark et al. 1987, Galili, Mandrell et al. 1988, Galili 2017). Therefore, the therapeutic effect of α-Gal-/sialic acid (SA)-containing liposomes may be found in anti-α-Gal Ab-producing primates and not in other mammals, unless they are engineered to lack α-Gal epitopes as in α1,3-galactosyltransferase (α1,3GalT)-KO mice and α1,3GalT-KO pigs. However, the α-Gal epitope is absent in birds and in other no mammalian vertebrates because the gene encoding the α1,3GalT, the enzyme that synthesizes α-Gal epitopes, appeared only in mammals (prior to divergence of marsupials from placental mammals) and is absent in no mammalian vertebrates. Chickens and other birds lack the α-Gal epitope and produce the natural anti-α-Gal Ab (Galili, Mandrell et al. 1988).

The α-Gal residues expressed in the tGPI-mucins are recognized by anti-α-Gal Abs that are produced during the acute and chronic stages of CD (Almeida, Milani et al. 1991, Almeida, Krautz et al. 1993, Almeida, Ferguson et al. 1994). CD anti-α-Gal Abs (Ch anti-α-Gal) has greater affinity for parasite surface antigens than do natural anti-α-Gal antibodies (Almeida, Ferguson et al. 1994). Our laboratory purified GPI-mucins-like glycoproteins from trypomastigote forms of the T. cruzi parasite (tGPI-mucins) with terminal α-galactosyl glycotopes, which are highly immunogenic to humans (Almeida, Ferguson et al. 1994). Because the exact structures of the immunogenic carbohydrate epitopes in the tGPI-mucins remain unknown, a small library of α-galactosyl residue saccharides have been previously synthesized and conjugated to a carrier protein in order to determine which epitope could...
possibly work as a BMK for CD. Based on previous experiments we selected the α-Gal-based synthetic neoglycoprotein NGP24b as a biomarker candidate for CD diagnosis and early assessment of therapeutic outcomes of CCD because of its efficiency in differentiating CD from NHS reactivity in a chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA).

In this project, we propose to use this specific BMK (α-Gal epitope) for CD diagnosis and treatment follow-up based on a CL-ELISA, using tGPI-mucins (from tissue culture-derived trypomastigotes from the Y strain) (Almeida, Covas et al. 1997) and the synthetic NGP24b (Schocker, Portillo et al. 2016, Schocker, Portillo et al. 2018). The development of new CD biomarkers is very important since T. cruzi lysates that are regularly used in serological assays have a high level of non-specificity due to non-homogeneous T. cruzi lysate batches that are inconsistent. NGPs could eliminate the reproducibility problem of serological assays using T. cruzi lysates because the former, in contrast to the latter, have precise synthetic and specific structures. Furthermore, the CL-ELISA technique using NGP24b could provide a more rapid and sensitive test and could avoid very demanding long-term patient follow-up as observed in the conventional serology (CS) tests.
<table>
<thead>
<tr>
<th>Parasite biomarkers</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasite molecules</strong></td>
<td></td>
</tr>
<tr>
<td>rTc24</td>
<td>(Krautz, Galvao et al. 1995)</td>
</tr>
<tr>
<td>Complement regulatory protein</td>
<td>(Meira, Galvao et al. 2004)</td>
</tr>
<tr>
<td>F-1V fraction and EXO</td>
<td>(Moretti, Cervetta et al. 1998)</td>
</tr>
<tr>
<td>F29 (FCABP)</td>
<td>(Sosa Estani, Segura et al. 1998, Fabbro, Velazquez et al. 2013)</td>
</tr>
<tr>
<td>Ag13</td>
<td>(Sanchez Negrette, Sanchez Valdez et al. 2008)</td>
</tr>
<tr>
<td>P2β</td>
<td>(Fabbro, Olivera et al. 2011)</td>
</tr>
<tr>
<td>Recombinant proteins set (multiplex)</td>
<td>(Viotti, Vigliano et al. 2006, Cooley, Etheridge et al. 2008)</td>
</tr>
<tr>
<td>KMP11, H70, PFR2, Tgp63</td>
<td>(Fernandez-Villegas, Pinazo et al. 2011, Fernandez-Villegas, Thomas et al. 2014)</td>
</tr>
<tr>
<td><strong>Parasite DNA/RNA amplification techniques</strong></td>
<td></td>
</tr>
<tr>
<td>Polymerase chain reaction techniques</td>
<td>(Britto, Cardoso et al. 1999, Britto, Silveira et al. 2001, Sanchez Negrette, Sanchez Valdez et al. 2008, Fabbro, Olivera et al. 2011)</td>
</tr>
<tr>
<td>Aptamers L44</td>
<td>(Nagarkatti, Bist et al. 2012, Nagarkatti, de Araujo et al. 2014)</td>
</tr>
<tr>
<td><strong>Host response/damage biomarkers</strong></td>
<td></td>
</tr>
<tr>
<td>Immunological markers</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>CD62L, CD127</td>
<td>(Bustamante, Craft et al. 2014)</td>
</tr>
<tr>
<td>CD27, CD28</td>
<td>(Appay, van Lier et al. 2008)</td>
</tr>
<tr>
<td>TEM CD55RA+ CCR7</td>
<td>(Albareda, Laucella et al. 2006)</td>
</tr>
<tr>
<td>CD40L</td>
<td>(Chamekh, Verrecruysse et al. 2005, Habib, Noval Rivas et al. 2007)</td>
</tr>
<tr>
<td>Interleukin (IL)-10</td>
<td>(Sousa, Gomes et al. 2014)</td>
</tr>
<tr>
<td>Ab amastigotes, trypomastigotes, epimastigotes</td>
<td>(Alessio, Cortes et al. 2014)</td>
</tr>
<tr>
<td><strong>Biochemical biomarkers</strong></td>
<td></td>
</tr>
<tr>
<td>Selenium</td>
<td>(Rivera, de Souza et al. 2002)</td>
</tr>
<tr>
<td>Apolipoprotein A1 and fragments (F)</td>
<td>(Santamaria, Chatelain et al. 2014)</td>
</tr>
<tr>
<td>Endogenous thrombin potential, F1+2</td>
<td>(Pinazo, Tassies et al. 2011, Pinazo, Posada Ede et al. 2016)</td>
</tr>
</tbody>
</table>

Table 1. Summary of major biomarkers. Modified from (Pinazo, Thomas et al. 2015).
Figure 4. Schematic representation of the *T. cruzi* trypomastigote plasma membrane. **Left panels**: Infective *T. cruzi* trypomastigote form has a very complex surface coat, consisting of glycoconjugates (glycoproteins and glycolipids), such as GIPLs and three major families of GPI-anchored proteins (GPI-APs) (i.e., tGPI-mucin, trans-sialidase-TS, and mucin-associated surface protein-MASP). **Middle panel**: the tGPI-mucin comprises a polypeptide core heavily substituted by O-glycans linked to threonine residues on the protein. **Right panel**: the tGPI-mucin molecule encompasses terminal, nonreducing α-galactopyranose (α-Galp) residues, which are the major targets for the highly abundant protective anti-α-Gal Abs, elicited during both the acute and chronic phases of CD. The hypothetical linkages (*) of the branched O-glycans are based on partial liquid chromatography-tandem mass spectrometry (LC-MS/MS) data analysis, following β-elimination and permethylation of tGPI-mucin-derived O-glycans (Almeida et al., unpublished data). Figure copied and text modified from Ortega-Rodriguez et al., 2019 (Ortega-Rodriguez, Portillo et al. 2019).
II. Research Hypothesis

During *T. cruzi* infection, parasite-specific anti-α-Gal antibodies (Ch α-Gal Abs) are produced (Milani and Travassos 1988, Almeida, Milani et al. 1991, Gazzinelli, Pereira et al. 1991). These antibodies have different specificities than natural anti-α-Gal antibodies (NHS α-Gal Abs) (Almeida, Ferguson et al. 1994, Galili, Wang et al. 1999, Galili 2017). Thus, we hypothesize that tGPI-mucins and NGP24b, which contain terminal α-Gal residues, are efficient BMKs for diagnosis and follow-up of chemotherapy of adult CCD patients.

III. Goals and Objectives:

In this project, we propose to investigate how tGPI-mucins and NGP24b behave as BMKs for diagnosis (Objective 1) and early assessment of treatment outcomes (Objective 2) in adult CCD patients.

Objective 1 will provide information about the diagnostic potential of the two antigens.

Objective 2 will provide information about the prognostic potential of the two antigens.

IV. Specific aims:

Specific Aim 1: To perform serological immunoassays using tGPI-Mucins and NGP24b as biomarkers for diagnosis of CCD patients.
Specific Aim 2: To perform serological immunoassays to evaluate tGPI-mucins and NGP24b as biomarkers for early assessment of the chemotherapeutic outcomes with benznidazole.
V. Timeline and Project View

Protocol: New tools for evaluation of Chagas disease treatment

BIOMARCHA Study (ISGlobal – Barcelona)
Patient Groups:
1. Participants with no Chagas disease will be evaluated as a Control Group
2. Participants diagnosed with Chagas disease and treated with BZN will be followed-up as a Case Group

Total number of participants: 63 (all from Barcelona)

**Figure 5. Scheme of the protocol.** Sixty-three CD patients were randomly assessed to benznidazole treatment or placebo. Serum was collected right before beginning of treatment (time 0), and 2, 6, 12, and 24 months after treatment. CL-ELISA for antibody detection was performed for all patients and all time points.

VI. Material and Methods

6.1. Ethics Statement

This study was implemented in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki after the approval by the ethical committees of the participating institutions (ISGlobal/Hospital Clinic-Barcelona, and UTEP). The clinical part of the study was carried out at
Hospital Clinic-Barcelona, and the immunoassays with tGPI-mucins and NGP24b were performed at UTEP. All serum samples were provided by HCB to UTEP with no patient’s identifiers.

6.2. Inclusion and exclusion criteria and drug treatment:

The study population were patients from the ISGlobal/Hospital Clinic, Barcelona, Spain.

Inclusion Criteria:

- Patients from endemic areas (Latin America, mostly Bolivia).
- Older than 18 years old and younger than 50.
- With serological confirmation of Chagas disease infection with two different techniques.
- Indeterminate or initial cardiac form (Kuschnir I).
- Not previously treated for Chagas disease.

Exclusion Criteria:

- Co-morbidity: previous cardiac disease from other etiology (ischemic, alcoholic or hypertensive), active inflammatory or immunology diseases for another agent. Hepatic disfunction
- Pregnancy or lactation

6.3. Sample collection:

Sera were collected on day zero of treatment; and months 2, 4, 6, 12, and 24 after BZN treatment began by Dr. Joaquim Gascon’s group at ISGlobal/Hospital Clinic-Barcelona, Barcelona, Spain. Samples were frozen and shipped in dry ice to UTEP for immunoassays.
6.4. Purification of GPI-mucins from *T. cruzi* trypomastigotes

6.4.1. Mammalian tissue-culture-derived *T. cruzi* trypomastigotes (TCTs)

Briefly, LLC-MK2 cells were cultured in DMEM containing 10% hi-FBS and antibiotics. Cells were infected with TCTs and incubated at 37°C with 5% CO2 humid atmosphere for 3 days. Three days post-infection (dpi), supernatant was removed and cells were washed three times with PBS. Fresh DMEM containing 10% hi-FBS and antibiotics was added to the flask. Six dpi, supernatant was collected and placed into 50-ml conical tubes for centrifugation and obtainment of pellet parasite (P1). P1 was incubated at 37°C, for 2 hr, to allow TCTs to swim up towards the surface. Supernatant was removed and PI was washed with 10-15 ml PBS, and centrifuged for 10 min, at 3,220 x g, 4°C. Washing and centrifuging steps were repeated until all DMEM color was not visible in the pellet. Remaining PBS in the supernatant was removed and pellet was frozen at -80°C for storage. P1 was lyophilized before tGPI-Mucin purification.

6.4.2. Purification of tGPI-Mucins

Purification of tGPI-Mucins was performed by Uriel Ortega-Rodrigues from Dr. Almeida’s lab according to the following protocol (Ortega-Rodriguez, Portillo et al. 2019). The following procedure is performed with a minimum of 1 x 10^10 TCTs, which are obtained from ten T175 flasks of TCT-infected LLC-MK2 cells at 6–7 days posts-infection (dpi).

**Protocol steps:**

1. Add 2 mL MeOH directly to lyophilized parasite pellet (P1), vortex briefly, and transfer the pellet suspension to a MeOH-rinsed borosilicate glass tube with PTFE-lined cap.
2. Add another 2 mL MeOH, followed by 2 mL CHCl3 and 1.6 mL H2O (M:C:W, 2:1:0.8, v/v/v); vortex tube vigorously for 2 min.

3. Centrifuge for 10 min at 3000%g, 4°C.

4. Carefully remove supernatant, which will be enriched with lipids (i.e., neutral lipids and phospholipids) and tGIPs (~90% of the total), and transfer it to a fresh glass tube with PTFE-lined cap.

5. Add 2 mL MeOH and 4 mL CHCl3 to the partially delipidated pellet, and vortex for 2 min. Centrifuge for 10 min at 3000%g, at 4°C, and transfer supernatant to tubes containing the organic/lipid extracts.

6. Repeat step 5 twice.

7. Add 4 mL MeOH, and 2 mL CHCl3 and 1.6 mL water (M:C:W, 2:1:0.8, v/v/v) to the C:M (2:1, v/v)-delipidated pellet and vortex for 2 min. Centrifuge for 10 min at 3000%g, at 4°C, and transfer supernatant to the tube containing the organic/lipid extracts obtained from delipidation steps.

8. Repeat step 7 once. Carefully dry the C:M-/C:M:W-delipidated pellet and organic/lipid extracts under a low, constant stream of nitrogen gas.

9. Once dried, keep both pellet and organic/lipid extracts at 20°C (short-term) or 80°C (long-term).

11. Add 4 mL 9% 1-ButOH directly to the dry delipidated parasite pellet, and incubate at 4°C, overnight (O/N) while rocking to ensure maximum recovery of GPI-anchored proteins (GPI-APs).
12. Centrifuge for 10 min, at 3000\(^{\circ}\)g, 4\(^{\circ}\)C, to pellet the delipi-298 dated parasites.

13. Remove and transfer supernatant, enriched in GPI-Aps (GPI-AP fraction), to a fresh methanol-rinsed glass tube with PTFE-lined cap.

14. Repeat steps 11–13 twice. However, the second and third 9% 1-ButOH extraction steps should be done for only 1 h each.

15. Centrifuge tube for 10 min, at 3000\(^{\circ}\)g, 4\(^{\circ}\)C, to pellet parasites, and transfer supernatant to the tube containing the GPI-AP fraction obtained in step 13.

16. Lyophilize the combined GPI-AP-enriched fractions, obtained in steps 13 and 14.

17. To partition GPI-APs from any remaining GIPLs not extracted by C:M and C:M:W, add 1 mL 1-ButOH and 1 mL deionized water to dry, combined GPI-AP fraction, and vortex for 1 min. Centrifuge for 10 min, 3000\(^{\circ}\)g, 4\(^{\circ}\)C.

18. Remove upper phase (91% 1-ButOH fraction), which is enriched with tGIPLS (~10% of the total), and transfer to a fresh MeOH-rinsed glass tube with PTFE-lined cap. Transfer lower phase (9% 1-ButOH fraction) rich in GPI-APs, to a fresh MeOH-rinsed glass tube with PTFE-lined cap.

19. Repeat steps 17 and 18.

20. Lyophilize the 9% 1-ButOH fraction (enriched with GPI-APs), and store the 91% 1-ButOH fraction (enriched with tGIPLs) at 80\(^{\circ}\)C.

21. Prepare the OS column by packing up to 6 cm resin to the 10 mL glass column.
22. Wash the column with 10 column volumes of Buffer A, using a Master flex Platinum L/S-13 silicone tubing (90 cm), and set flow rate to 12 mL/h in the peristaltic pump.

23. Carefully create a gradient of 30 mL of Buffer A in the left chamber and 30 mL of Buffer B in the right chamber.

24. Run a 5–60% 1-PropOH gradient to clean the OS column, using a 12 mL/h flow rate. Finally, re-equilibrate the column using 10 column volumes of Buffer A.

25. In parallel, set the fraction collector to collect sixty 1 ml fractions.

26. Resuspend dried 9% 1-ButOH fraction (rich in GPI-APs) in 2 mL of Buffer A.

27. Gently vortex, centrifuge (10 min, 3000×g, 4°C) or filter (0.45μm low-protein-binding PTFE syringe filter) to remove any insoluble debris, and apply the supernatant or filtrate onto the column using a flow rate of 4 mL/h. Be careful not to dry the resin.

28. Run a 5–60% 1-PropOH gradient (prepared as described in step 24) to elute the OS column, using a 12 mL/h flow rate. Collect sixty 1 ml fractions. Store them at 4°C.

29. Determine the 1-PropOH concentration in every third fraction by using a refractometer. Generate a standard curve by measuring the refractive index of 5, 10, 20, 30, 40, 50, 60, 70, and 80% 1-PropOH, each containing 0.1 M NH₄Ac, pH 7.2. Use 50–60μL minimum of each fraction for the measurement. Carefully recover the fraction following measurement, dry the refractometer lens with Kimwipes (or equivalent tissue paper), wash the lens with 100–200μL 70% ethanol, and wipe dry.
30. Test reactivity of each fraction (1μL) to Ch anti-α-Gal Abs (or to ChSP, if purified anti-α-Gal Abs are not available) via CL-ELISA.

31. Pool fractions showing the strongest reactivities to Ch anti-α-Gal Abs (or ChSP) that elute between 20 and 30% 1-PropOH. tGPI-Mucins with an alkylacylglycerol (AAG) GPI-anchor typically elute within this range; however, lyso-tGPI-mucins that contain a single C16:0-alkyl group elute earlier, between 8 and 15%.

32. Lyophilize tGPI-mucin-enriched fraction, redissolve it in 20% 1-PropOH, and store it at 20ºC (short-term) and 80ºC (long-term).
6.5. Synthesis of Gal\(\alpha(1,3)Gal\beta(1,4)\text{GlcNac}\alpha\)-BSA (NGP24b)

Synthesis of NGP24b was performed by Alba Montoya at Dr. Katja Michael’s laboratory at the Department of Chemistry and Biochemistry, UTEP. The target mercaptopropyl trisaccharide Gal\(\alpha(1,3)\)Gal\(\beta(1,4)\text{GlcNac}\alpha\) (Gal\(\alpha\)LN\(\alpha\), structure (Str) 69) (Figure 6) was synthesized by an elevated temperature glycosylation using the know allyl GlcNAc acceptor (Str 59) and a large excess of the acetylated tricholoroacetimidate alpha-Gal donor (Str 60) to give the Gal\(\beta(1,4)\text{GlcNac}\alpha\) disaccharide (Str 61) in a high yield of 70%. Saponification gave the deacetylated disaccharide (Str 62), then \(p\)-methoxybenzylolation at position 3 of the galactose residue via its tin acetal gave (Str 63), followed by benzylation of the 5 remaining hydroxyls to afford (Str 64). Oxidative cleavage of the \(p\)-methoxybenzyl (PMB) group with DDQ furnished the Gal\(\beta(1,4)\text{GlcNac}\alpha\) acceptor (Str 65). This new disaccharide acceptor (Str 65) was glycosylated with the known di-\text{-}\text{tert} butylsilylidene equipped \(\alpha\)Gal trichloroacetimidate donor (Str 8), using TMS-\text{OTf} catalysis to give trisaccharide (Str 66) (achieving very good separation from the SM, having for the 1\text{st} time a NMR characterization of the compound fully protected). Then, the di-\text{-}\text{tert} butylsilylidene group was cleaved with a large excess of 70\% HF-pyr to give (Str 67), followed by radical addition using AcSH and AIBN under UV light yielding (Str 68). Finally, a saponification was done it to get the desired epitope (Str 69) ready for conjugation to a carrier protein (Scheme 11).
Figure 6. Synthesis of mercaptopropyl-derivative of trisaccharide Galα(1,3)Galβ(1,4)GlcNac. Conditions: a: BF₃-Et₂O, DCM, 35-40°C, 3 h (70%); b: NaOMe, MeOH, r.t., 3 h (97%); c: Bu₂SnO, MeOH, reflux, 8 h; PMB-Cl, Bu₄NBr, benzene, reflux, 12 h (78%, 2 steps); d: BzCl, pyr (61%); e: DDQ, DCM/H₂O, r.t., overnight (77%); f: TMSOTf, DCM, 0 °C, 2.5 h, molecular sieves 4 Å (46%); g: HF-Pyr, THF, 0°C then r.t., 1.5 h (70%); h: AcSH, AIBN, THF, UV light (350 nm), 12 h (72%) and i: NaOMe, MeOH, r.t., 3 h (quant.). Figure elaborated by Alba Montoya and Dr. Katja Michael (Dept. of Chemistry, UTEP).
6.6. Chemiluminescent ELISA

Briefly, 96-well polystyrene microplates (Maxisorp, Nunc, Thermo Scientific) were coated overnight at 4°C with 25 ng/well of NGP24b or 1:32 μL/well of tGPI-Mucins in 200 mM carbonate-bicarbonate buffer, pH 9.6 (CBB). Plates were blocked with 200 μL 1% BSA-PBS (BSA, Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C before washing three times with PBS-0.05% Tween 20 (Sigma-Aldrich) (PBS-T) using a microplate washer (Elx50 washer, BioTek). Human sera at 1:800 dilution (in PBS-T + 1% BSA) were analyzed in triplicate. Plates were incubated for 1 h at 37°C. Plates were then washed three times with PBS- 0.05% Tween 20. Goat anti-human IgG antibody (Thermo Scientific; 50 μL, 1:10,000 dilution) in PBS-T + 1% BSA was added and incubated for 1 h at 37°C. Plates were washed to eliminate non bond antibodies and Horseradish peroxidase (HRP)-streptavidin (Invitrogen; 50 μL, 1:5000 dilution) in PBS-T + 1% BSA was added and plates were incubated for 1 h at 37°C. Plates were washed again and the reaction was developed with Super-Signal Chemiluminescent Substrate (Thermo Fisher Scientific) (SSLE: SSP : CBB-B - 1:1::8, v/v/v). Relative luminescence units were measured using a microplate reader with luminescence detector (Luminoskan Ascent - Labsystems). Positive (ChHSP) and negative (NHSP) controls tested the protocol functionality and assay reactivity of each microplate. Plate cutoff in each plate was calculated by using six technical replicate Relative Luminescence Unit (RLU) values of a pool of 10 NHS. The average RLU value of these six technical replicates was added to the standard deviation multiplied by a standard deviation multiplier factor (Cutoff = average NHSP + SD x f), as described by Frey et al (Frey, Di Canzio et al. 1998).
6.7. α-Galactosidase treatment

This protocol was performed in conformity of Al-Salem’s work (Al-Salem, Ferreira et al. 2014). In summary, to test the specificity of the IgG antibodies against with tGPI-Mucins or NGP24b, we immobilized plates with antigens pre-treated with green coffee bean α-galactosidase (G8507, Sigma-Alrlich). MaxiSorp Nunc polystyrene microplate wells were coated with 25ng/well of NGP24b in carbonate/bicarbonate (CBB) solution and incubated overnight at 4°C. The plate was blocked with 200 μl 1% BSA-PBS for 1hr 37°C, and washed three times with 200 μL PBS-T. In parallel, two hundred microliters of α-galactosidase (in ammonium sulfate suspension, 50 units/mg protein) were centrifuged at 10,000 xg for 10 min at 4°C to remove the excess ammonium sulfate. The supernatant was discarded and the pellet containing the enzyme was gently redissolved in ice-cold 100 mM potassium phosphate buffer (pH 6.5). Fifty microliters of the enzyme solution (0.01 units/μL) were added to each well, and the plate was incubated for 24 hr at 37°C. The microplate was washed three times with 200 μL PBS-T and the chemiluminescent ELISA was performed as described above.
VII. Results

7.1. Purification of tGPI-Mucins

tGPI-mucins extracted by organic solvents and purified by hydrophobic interaction chromatography using octyl-Sepharose (Figure 7a-b). Major peaks eluting between 20 and 30% 1-ProOH, likely corresponding to tGPI-mucins, were reactive with Ch anti-α-Gal Abs. This is in accordance with previous results from our laboratory (Almeida, Ferguson et al. 1994). Minor peaks reactive with Ch anti-α-Gal Abs, and likely corresponding to lyso-tGPI-mucins, were observed at 10-15% 1-ProOH. The purified tGPI-mucins were then analyzed by CL-ELISA with serum pools and individual sera of CCD patients and NHS individuals (Figure 7c-d). Strong reactivities with both pools and individual sera of CCD patients, and no or little with NHSP and individual NHS samples, were observed. Taken together, these results indicate that the purified tGPI-mucins had the chromatographic and immunoreactivity profiles as previously described (Almeida, Krautz et al. 1993, Almeida, Ferguson et al. 1994, Almeida, Covas et al. 1997).
Figure 7. Purification and immunoreactivity analysis of tGPI-mucins. (a) General scheme for purification and functional analysis of tGPI-mucins. Parasites are first delipidated, followed by extraction of GPI-APs with 9% 1-BuOH. GPI-APs are enriched by 1-BuOH/H₂O partition, and tGPI-mucins are purified by hydrophobic-interaction chromatography (HIC) with Octyl-Sepharose. The tGPI-mucin-containing fractions are detected by CL-ELISA using Ch anti-α-Gal Abs. Positive fractions are combined, lyophilized, resuspended in 1 mL 20% 1-PrOH and quantified by myo-inositol analysis, as described (Almeida, Ferguson et al. 1994). The purified tGPI-mucins are titrated with a pool of sera from patients with chronic ChD (ChSP) and a pool of normal human sera (NHSP), before further assays with individual sera from ChD patients. (b) Chromatography elution profile of tGPI-mucins on OS-HIC. α-Gal-Enriched fractions (lyso-tGPI-mucins, tGPI-mucins, and tGIPLs) are located by CL-ELISA with Ch anti-α-Gal Abs. RLU, relative luminescence units. (c) Titration of the tGPI-mucins was performed with a pool of sera from patients with chronic ChD (ChSP) (n=10) and a pool of normal human sera (NHSP) (n=10), at different dilutions. Ch and NHS anti-α-Gal antibodies were purified from ChSP and NHSP, as described (Almeida, Ferguson et al. 1994). (d) Reactivity of purified tGPI-mucins with individual sera from patients with chronic ChD (ChS), ChSP, and NHSP. The tGPI-mucin CL-ELISA titer was calculated by dividing the test sample average RLU value by the cutoff, calculated as the mean value of 10 NHSP + 10 SD. Horizontal black line: titer equal to 1.0. Green dashed line: titer <1.0 and equal or greater than 0.9 (Almeida, Covas et al. 1997). Data interpretation: positive result: titer ≥ 1.0; inconclusive result: titer <1.0, >0.9; negative result: titer <0.9. ChS and ChSP samples (c, d) were from IMT/FM/UCV, and NHSP sample (d) was from ISGlobal-Barcelona. Sera were collected from patients strictly following the International Ethical Guidelines for Biomedical Research Involving Human Subjects and protocols approved by the Institutional Review Boards of IMT/FM/UCV and ISGlobal-Barcelona. Figure and text copied/adapted from (Ortega-Rodriguez, Portillo et al. 2019).
7.2. Synthesis of Galα(1,3)Galβ(1,4)GlcNAcα-BSA (NGP24b)

NGP24b synthesized as described in section 6.5 above was analyzed by matrix-assisted laser-desorption mass spectrometry (MALDI-TOF-MS) as described (Schocker, Portillo et al. 2016, Schocker, Portillo et al. 2018) (Figure 8). We observed by MALDI-TOF-MS that 24 units of the glycan were covalently attached to each BSA molecule.

Figure 8. Conjugation of the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα to BSA. (A) Conjugation a: TCEP, phosphine buffer pH 7.2 and maleimide-activated BSA. (B) MALDI-TOF mass spectrum of Galα(1,3)Galβ(1,4)GlcNAcα-BSA (NGP24b). m/z, mass to charge ratio.
7.3. Chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for antibody detection in patients’ sera before and after BZN treatment

We then analyzed IgG response to the purified tGPI-Mucins (Almeida, Ferguson et al. 1994) and the synthetic neoglycoprotein NGP24b (Schocker, Portillo et al. 2016, Schocker, Portillo et al. 2018) by CL-ELISA in CCD patients diagnosed from Barcelona. CD human serum pool (ChHSP) and normal human serum pool (NHSP) were serially diluted (1:200, 1:400, and 1:800) in PBS-0.05% Tween 20 + 1% BSA. NGP24b was serially diluted at 100, 50, 25, 12.5, 6.25, and 3.125 ng/well in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6 (CBB)), and tGPI-Mucins was serially diluted at 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 μl/well in coating buffer for optimization of the antigens and antibody dilutions (Figures 9 and 10).
Figure 9. Serial dilution of the serum at different tGPI-Mucin concentrations by CL-ELISA. Antigen was very reactive against ChHSP even at 1:32/well dilution but not with NHSP.

Figure 10. Serial dilution of the serum at different NGP24b concentrations by CL-ELISA. NGP24b was highly reactive to ChHSP but not NHSP. The 25 ng Ag/well and 1:800 serum/antibody dilution were used to further screen the individual patients’ sera.
7.4. tGPI-Mucins and NGP24b as biomarkers for the follow-up of the CD chemotherapy in humans and nonhuman primates

In addition to binding antigen, antibodies participate in a broad range of others biological activities. When considering the role of antibody in defending against disease, one must remember that antibodies generally do not kill or remove pathogens solely by binding to them. In order to be effective against pathogens, antibodies must not only recognize antigen but also invoke responses – effector functions- that result in removal of the antigen and death of the pathogen (Kindt Thomas J 2007). There are four major human IgG subclasses: IgG1, IgG2, IgG3, and IgG4. Most IgG subclasses can activate a collection of serum glycoproteins called the complement system. Complement includes a group of proteins that can perforate cell membranes (Kindt Thomas J 2007). The quantification of total serum immunoglobulins was performed in all 63 patients to analyze possible changes in total IgG antibody production according to disease stage, regardless of the antibody specificity.

We screened the sera of 63 patients from Barcelona, Spain, by CL-ELISA with tGPI-mucins or NGP24b as antigens, before and after benznidazole administration. Significant decrease in reactivity against NGP24b and against tGPI-Mucins occurred 24 months post-treatment in 55% and 58% respectively. The overall decrease in titers showed that four patients previously positive, before treatment, became serum-negative after 24 months of BNZ treatment (Figure 11).
Figure 11. CL-ELISA titers of all patients before and after BNZ treatment in patients from Spain. Serum samples were tested by CL-ELISA with purified tGPI-Mucins and NGP24b in triplicate before treatment (time 0), and 24 months after treatment with Benzimidazole (BNZ). Each circle or square represents the average of three simultaneous determinations. [99.5% CI for all data collected] [p value = 0.0003 for NGP24b and p= 0.0069 for tGPI-Mucins].
Antibody levels against fractions obtained from purified tGPI-Mucins at different dilutions and synthetic α-Gal-containing neoglycoprotein were studied in 63 treated patients (24 months after treatment with benznidazole). Positive serology remained present in the majority of the patients after treatment but those patients had significant decrease in their titers (Figure 12). tGPI-Mucins showed interesting results even when using a larger dilution (1:64). These results corroborate the tGPI-mucin immunogenicity and specificity to the α-Gal epitope. The NGP24b titers are very consistent and show similar profile than the tGPI-Mucins ones. The overall decrease in titers after treatment (ranging from 26.7% when using tGPI-Mucins at 1:32 dilution and serum at 1:800 in patient#27, up to 72.5% when using NGP24b at 25 ng/well dilution and serum at 1:800 dilution in patient#4) was detected by both antigens, which demonstrate that both antigens are powerful biomarkers for follow-up of CD treatment.
We observed a significant reduction in the titers of trypanolytic anti-α-Gal antibodies, as measured by CL-ELISA, among BZN-treated patients (Figure 12). We then performed statistical analysis and linear regression of the data of the patients that were submitted to BNZ treatment (Figure 13). The linear regression of NGP24b showed the same pattern and trend of the tGPI-mucins. The BMK candidates predicted similar seroconversion time as showed in patient # 033. Our proposed
biomarkers demonstrated efficiency even at early stages of the disease treatment. It was possible to assess patient outcome as early as 2 and 4 months after treatment.

Figure 13. Representative curves of benznidazole-treated patients in Spain using NGP24b and tGPI-Mucins as biomarkers for CCD. Serum samples were tested by CL-ELISA with purified tGPI-mucins and NGP24b in triplicate before treatment (time 0), 02, 06, 12, and 24 months after treatment with BNZ. Each circle represents the average of three simultaneous determinations. Codified patients are represented by numbers (#033, 040, 059, and 065). The green number and arrow represent the estimated seroconversion time. Logarithmic, power, or exponential equations and r values for each titration curve are shown. The horizontal green dotted lines represent the cut-off titer (1.00). [99.5% CI for all data collected] [p value< 0.0001 for all patients].
Subsequently, we analyzed the reactivity of sera from three individual CCD patients to tGPI-mucins and NGP24b, before and after α-galactosidase treatment. The specificity of individual Chagas sera directed mainly to the terminal α-Gal epitopes present in tGPI-Mucins and NGP24b was showed in Figure 14. The enzymatic treatment caused a broad reduction in reactivity for both antigens proving the specificity of the antibodies to the α-Gal epitopes.

![Figure 14](image)

**Figure 14. Reactivity of individual patients’ sera on KM24b/NGP24b and tGPI-Mucins before and after α-galactosidase treatment.** Serum samples were tested by CL-ELISA with purified tGPI-mucins and NGP24b in triplicate before BNZ treatment (time 0), and 24 months after drug treatment. Codified patients are represented by numbers (# 004, 021, and 033). Samples were submitted to α-galactosidase treatment and CL-ELISA was performed right after.

When looking at the all patients’ combined results, we noticed 49.6% decrease in the CL-ELISA reactivity where tGPI-mucins were used as antigens and an expressive 81.2% reduction when using NGP24b as antigen (Figure 15). This result shows that the majority of the antibodies are mainly specific to the terminal α-Gal residues, since both tGPI-mucins and NGP24b contain this terminal nonreducing glycotope in their structures.
Figure 15. Reactivity of all combined patients’ sera with tGPI-mucins and NGP24b in CL-ELISA, before and after α-galactosidase treatment of the immobilized antigen.
The BMKs tGPI-Mucins and NGP24b were tested with sera from nonhuman primates (baboons). Animals were vaccinated with α-Gal-based vaccine and then they were infected with *T. cruzi* twice. We ran this experiment using three antigens side by side and observed that all antigens could track the infections (Figure 16 A-C). Sera from animals vaccinated with NGP24b were also capable of recognizing the specific antigen (Figure 16B). As expected, the branched antigen NGP11b (Galα(1,6)[Galα(1,2)]Galβ-BSA) (Figure 16C), which also contains terminal, nonreducing α-Gal epitopes, confirmed the same trend shown by NGP24b.

tGPI-Mucins and NGP24b were tested again as a BMK for the follow up of CD treatment, now using a naturally *T. cruzi*-cynomolgus monkey (macaques, *Macaca fasciculari* or *M. cynomolgus*) of southeastern Asia, Borneo, and the Philippines (Figure 17). In summary, those animals were divided in three different groups: First group received optimal BNZ treatment, second group receive intermittent BNZ treatment, and the third group received E1224 treatment. Sera were collected at several time points from the monkeys and each group had at least one placebo animal. Some animals were immunosuppressed by drug induced immunosuppression. For immunosuppression protocols, see reference (Diehl, Ferrara et al. 2017). Some animals were euthanized and quantitative PCR was analyzed in the many different kind of tissues: left ventricle, right ventricle, adipose tissue, brain, colon, esophagus, and skeletal muscle. NGP24b was able to track the BNZ treatment in monkey#20461 (Figure 17A) as good as tGPI-Mucins did. In group two that also receive BNZ treatment (intermittent one), it was also possible to see the nice trend in curve made by NGP24b after drug treatment. It was observed that the group that received E1224 was not presenting any expressive response to drug treatment.
Figure 16. Reactivity of tGPI-Mucins, NGP24b Galα(1,3)Galβ(1,4)GlcNAcα, and NGP11b in baboon sera by CL-ELISA. (A) tGPI-Mucins were used as antigen at 1:64 ul/well (=1.56x10^6 parasite eq.). (B) NGP24b was used at 25 ng/well. (C) NGP11b was used at 50 ng/well.
Figure 17. tGPI-Mucins and NGP24b in comparison with two other antigens, and qPCR from blood and tissues after optimal BNZ treatment (A), intermittent BNZ treatment (B), and E1224 drug treatment (C). IS, Immunosuppression; E, euthanasia.
This result corroborates the previous observation where it was shown that E1224 is a drug that has a suppressive effect on parasite clearance, but failed to sustain efficacy until 12 months of follow-up in a clinical trial with 231 patients (Torrico, Gascon et al. 2018). Reactivity against the recombinant *T. cruzi* flagellar calcium-binding protein (F29) (Sosa Estani, Segura et al. 1998) was analyzed by ELISA in parallel with the other BMK candidates (tGPI-mucins and NGP24b) for use as monitoring method to test the efficacy of BZN in those monkeys in the chronic phase of CD. It’s noteworthy to mention that F29 protein antigen, which is proven on the literature to be an useful BMK for follow-up of Chagas treatment (Fabbro, Velazquez et al. 2013), had similar trend but much less reactivity in comparison with tGPI-mucins and NGP24b. qPCR results also corroborates with tGPI-mucins and NGP24b reactivity.
VIII. Conclusion and discussion

The aim of this study was to provide insights about the α-Gal-based glycoprotein and tGPI-mucins as candidate BMKs in chronic *T. cruzi* infection. Studies have demonstrated the binding of anti-α-Gal antibodies to live parasites and the subsequent complement-mediated or -independent lysis of *T. cruzi* (Almeida, Milani et al. 1991, Pereira-Chioccola, Acosta-Serrano et al. 2000). There is evidence that the glycoconjugates from host cell-derived trypomastigotes that are recognized by Ch anti-α-Gal are mucin-like glycosylphosphatidylinositol (GPI)-anchored molecules bearing several oligosaccharide chains, not yet fully characterized (Almeida, Ferguson et al. 1994). Experiments remain to be performed and therapies with inclusion of new drug regimens could be better evaluated using NGP24b and tGPI-mucins to access diagnosis and treatment outcomes. In addition, these antigens are very promising for the elimination of false-negative serum in blood banks and, therefore, avoid infection of patients during blood transfusion. New forms of treatment would benefit millions of infected people worldwide and certainly these BMKs will give us better insights on patients’ treatment.

Both antigens used in this project showed to be very powerful tools in diagnosing CD. NGP24b, which is a synthetic compound could therefore substitute parasite lysates as antigens in CS assays broadly used in CD diagnosis in clinical labs, blood banks, and research. Parasite lysates are not reliable due to non-homogeneous *T. cruzi* lysates and lack of specificity and its replacement would benefit health care centers, researchers, and the population in general. Our data support the hypothesis that the synthetic α-Gal glycoprotein NGP24b could be used as BMK for diagnostic and prognostic of CD, thus being an alternative for the challenges of purifying tGPI-mucins.
We need to better understand how BMKs work, what they do under a variety of experimental conditions, and how they may be manipulated for more reliable diagnostic and/or prognostic results. Further understanding and development of pivotal biomarkers in normal immune protection, immunodeficiency, and pathological state depends upon continual expansion of our knowledge of these molecules.

IX. Future Experiments

- We plan to evaluate and compare the patients’ titer levels and its correlation with the qPCR data.
- Combination of NGP24b and tGPI-Mucins could improve reactivity of the NGP and should be tested in different ratios.
- Quantification of tGPI-Mucins has been analyzed in collaboration with the Complex Carbohydrate Research Center, Athens, GA to better standardize our protocols.
- Search for other new CCD biomarkers such as tGPI-mucins from other strains and other parasite forms should be evaluated since patients from different regions could be exposed to different parasite genotypes.
- Evaluation of the NGP in different animal models.
- Development and evaluation of new NGP according to the structure of the parasite surface that are still unknown and hasn’t been characterized yet.
CHAPTER 2:

α-Gal-containing neoglycoprotein as candidates for Chagas disease vaccine

Abstract

We aim to evaluate the Galα3LN glycotope coupled to human serum albumin, as a potential therapeutic vaccine candidate for CD in the murine model of infection. Based on our previous studies in acute phase of CD, we concluded that α1,3-galactosyltransferase-knockout (α1,3GalT-KO) mice immunized with a synthetic α-Gal-containing neoglycoprotein (αGal-NGP) gained protection against the parasite *T. cruzi*. This vaccine was able to reverse the tissue damage caused by the parasite, mainly heart abnormalities. Due to its very impressive protection against the parasite in acute phase, we intend to test the vaccine against previous *T. cruzi* infection and also we want to see the long term effect of the immune protection in the context of a therapeutic vaccine.

I. Introduction - Vaccine

Many attempts to generate a prophylactic or preventive vaccine for CD have been conducted in the last three decades, including use of the attenuated parasite, purified protein, recombinant protein and DNA and, more recently, replication-deficient bacteria and recombinant viral vectors to reduce acute parasitism and heart inflammation and chronic myocarditis (Gourbiere, Dorn et al. 2012). Moreover, no vaccines for the prevention and/or treatment of CD are currently available (Coura and Borges-Pereira 2010, Steverding 2014). Consequently, there is a great necessity for
the development of novel, more efficient therapies for CD that will make treatment available for millions of people worldwide.

The surface of parasitic protozoa has a complex glycocalix coat, which is majorly composed of glycosylphosphatidylinositol (GPI) (Figure 4). GPI-anchored polysaccharides and glycoproteins appears from the phospholipid layer and the major surface glycoproteins of *T. cruzi* are the mucin-like proteins. Their sequences are rich in Ser and Thr residues, which are acceptor sites for the addition of O-linked oligosaccharides. The abundant amount of carbohydrates confers a strong hydrophilic character and probably an extended conformation (Schenkman, Ferguson et al. 1993, Previato, Jones et al. 1994, Almeida, Camargo et al. 2000, Buscaglia, Campo et al. 2006). Structural and immunological evidence of the O-linked carbohydrate structures show the presence of galactofuranose is restricted to strains of *T. cruzi* (Jones, Todeschini et al. 2004).

The production of Galα1,3Gal epitopes in man and Old-World primates has been evolutionarily diminished via the suppression of the gene encoding the alpha 1,3 galactosyltransferase. Therefore, free, non-reducing terminal α1,3Gal epitope (Galα1-3Galβ1-4GlcNAc-R) is highly immunogenic to humans and we mount a strong immune response to it because we do not produce glycoconjugates that contain galactofuranose due to inactivation of the α1,3-galactosyltransferase (α1,3-GT or α1,3-GalT) gene 20–28 million years ago in our ancestral primates (Galili, Clark et al. 1987).
Anti-α-Gal Abs are the major protective lytic antibodies in both acute and chronic Chagas disease (Almeida, Milani et al. 1991). These α-Gal glycotopes induce high levels of *T. cruzi*-specific anti-α-Gal antibodies in ChD patients (Ch anti-α-Gal Abs (Milani and Travassos 1988).

We recently performed a study using an α-Gal-based glycovaccine for experimental acute Chagas disease and it demonstrated that animals vaccinated with α-Gal-based glycovaccine were fully protected against lethal *T. cruzi* challenge by inducing a strong anti-α-Gal antibody mediated humoral response (Portillo, Zepeda et al. 2019).

This project seeks an investigation of how well Galα3LN-HSA behaves as a therapeutic Chagas vaccine since linear α-Gal glycotopes are absent in humans (Macher and Galili 2008).

**Objective one:** By using a reliable animal model such as α1,3-GalT-KO mice, will help us to understand the limitations hampering the discovery of new vaccines, and provide us information about the candidate vaccine efficiency.

**Objective two:** will give us insights about the adding and protective effect that a combination therapy, vaccine plus BNZ treatment, can provide in order to improve or maintain the life quality of the tested animals.
II.  Research Hypothesis

Our hypothesis is that Galα3LN-HSA vaccination can modify the immunological unbalance that concurs to Chagas’ heart disease to improve prognosis. We expect to bring insights on the biological processes contributing to beneficial effects of the Galα3LN-HSA on clinical signs of chronic experimental CD. The use of a vaccine with LMPLA adjuvant (Matyas, Muderhwa et al. 2003) may help to decrease the high amount of drugs needed for parasite control. These drugs (BNZ and NFX) are very toxic, so the use of less drugs would be beneficial for patients with CD.

III.  Goals and Objectives:

In this project we propose to study a therapeutic glycoprotein-based vaccine (Galα3LN-HSA) using α1,3-Galactosyltransferase knockout (α1,3GalT-KO) mice, which closely resemble humans in their humoral response to αGal epitopes.

IV.  Specific aims

Specific aim 1: To carry out therapeutic immunization of Galα3LN-HSA vaccine alone or combined with adjuvant and test its efficacies.

Specific aim 2: We intend to combine a drug delivery (benznidazole) with the NGP vaccine and evaluate the benefits of drug vaccine combination.
V. Timeline and project view

**Protocol: Therapeutic immunization of GaLaT-KO with GaLa3LN-HSA**

**Vaccination groups (n=5 per group):**
1. GaLa3LN-3-HSA + L-MPLA
2. GaLa3LN-3-HSA
3. L-MPLA
4. PBS

**Figure 18. Scheme of the protocol.** Twenty mice were divided in four different groups. Serum was collected at days post infection (DPI): 0, 90, 97, 104, 111, 118, 140, 160, and 180. Chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for antibody detection was performed for all animals and all time points. Organs were harvested at endpoint, 180 DPI. Immunizations occurred at 90, 97, 104, and 111 DPI.

VI. Material and Methods

6.1. Experimental infection and vaccination

Mice were obtained from the animal facilities of The University of Texas of El Paso (UTEP, USA) and were housed under specific pathogen-free conditions in a 12-h light dark cycle with access to food and water *ad libitum*. Five to seven week old female C57BL/6 (H-2b) mice were intraperitoneally (i.p.) infected with 100 blood trypomastigotes of the Colombian *T. cruzi* DTU I
strain/genotype that has been prepared by passage through C57BL/6 every 30 days. Four immunizations with Galα3LN-HSA were administered ninety days post infection on days 90, 97, 104, and 111 to determine the therapeutic nature of Galα3LN-HSA.

Mortality were recorded weekly. Sex and age-matched non infected (NI) controls were analyzed in parallel. According to experimental designs, groups of mice were euthanized under anesthesia and followed by UTEP Laboratory Animal Resources Center protocols.

**6.2. Drug treatment**

Benznidazole drug is produced by Sigma-Aldrich. Benznidazole will be formulated in distilled water containing 0.5% methylcellulose and 0.5% Tween 80. During a treatment course, each mouse will receive 0.2 ml of drug suspension per dose by oral gavage.

**6.3. Antibody detection using CL-ELISA**

Antibodies (total IgM, total IgG and its subtypes (IgG1, IgG2A, IgG2B, IgG3), IgE, and IgA) against the Galα3LN-HSA will be analyzed using a chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) as described in chapter one with some modifications. Titration was performed to optimize the best antigen/serum concentration. The analysis of isotype switching will determine if there are any vaccine-induced anti-α-Gal-antibodies representing the therapeutic effectors. Briefly, the protocol is as following: Immobilize antigen (Ag) according to the desired concentration. Add Ag on the microplate wells to a final volume of 50 μL, diluted in 200 mM CBB by using a multichannel pipette (20–200 μL) and reservoir boat. Incubate for 16 h or overnight at 4C. Remove the residual Ag by inverting the microplate and tapping it onto absorbent towels. Do
not wash. Block free sites with 200μL per well of 1% BSA or 5% skin milk in PBS. Seal the microplate with plastic wrap and incubate for 1 h at 37°C. Wash 3 times with 200 μL per well of PBS-T using an automatic plate washer. Add 50 μL of serum at 1:100 dilution, in PBS-T. Repeat steps 6 and 7. Dilute and prepare fresh secondary antibody (i.e. donkey anti-mouse IgG (H + L) biotinylated antibody) at 1:2,000 dilution in PBS-T +1% BSA. Add 50 μL of diluted secondary antibody per well. Repeat steps 6 and 7. Dilute high-sensitivity neutravidin-horseradish peroxidase (NA-HRP) to a ratio of 1:5000 in PBS-T +1% BSA. Add 50μL of diluted NA-HRP per well and cover plate with aluminum foil. The reagent is light sensitive, so keep it protected from direct light. Turn off the lights in the lab if it’s possible to do so. Incubate for 1 h at 37°C. Repeat step 7. Combine Super Signal ELISA Pico Stable Peroxide Solution, Super Signal ELISA Pico Luminol Enhancer, and CBB/0.1% BSA in a 1:1:8 ratio (v/v/v). Add 50 μL per well.

Read luminescence immediately, using a microplate luminometer. Readings are expressed as relative luminescence units (RLU). Notes: Controls: CD mouse sera pool (ChMSP) and Normal mouse sera pool (NMSP) controls are used to test the protocol functionality and assay reactivity of each microplate. We used three wells of the plate for positive controls (ChHSP) at 1:100 sera dilution and nine wells for the negative control (NHSP) at 1:100 sera dilution and three wells for blank (50 µl of PBS-TB for blank).

Plate cutoff in each plate is calculated by using nine technical replicate Relative Luminescence Unit (RLU) values of the NMSP (from 10 different patients). The average RLU value of these nine technical replicates was added to the standard deviation multiplied by a standard deviation
multiplier factor (Cutoff = average NHSP + SD x f), as described by Frey et al (Frey, Di Canzio et al. 1998).

6.4. Parasitemia

Five μL of total blood, obtained by venipuncture of the mouse tail, was placed between a microscope slide and a cover slide, and TCTs (Colombiana strain) were counted on a hemocytometer. Data were plotted as the number of TCTs per microliter of blood.

6.5. Real-time quantitative RT-PCR for analysis of parasite burden

For real-time quantitative RT-PCR (RT-qPCR), mice organs were harvested for semiquantitative parasite load quantification (Ramirez, Cura et al. 2015), washed to remove blood clots, weighed and frozen in RNAlater (#AM7021, Life Technologies, USA).

Tissue was homogenized in sterile, ice-cold PBS using sterile gentle MACS M tubes on the gentle MACS dissociator (Miltenyi Biotec, Auburn, CA). Samples were spiked with 5μL of a 40 pg/μL of linearized pUC57 plasmid, as an internal amplification control (IAC) (Duffy, Bisio et al. 2009). Extraction of genomic DNA was done from 20-30 mg of tissue by using the High Pure PCR template preparation kit (Roche) according to the manufacturer’s instructions.

Following DNA extraction, samples were diluted to 20 ng/μL and a total of 100 ng of DNA was used for a final volume of 20μL. Amplification of T. cruzi satellite DNA was done by using the specific primers (Piron, Fisa et al. 2007, Duffy, Cura et al. 2013) Cruzi 1, and Cruzi 2, both at 750
nM, and the TaqMan probe Cruzi 3 at 50 nM. Both the forward (5′–ACCGTCATGGAACAGCGTA–3′) and the reverse (5′–CTCCCGCAAACCCCTATAAAT–3′) primers for the IAC were used at 100 nM, and the TaqMan probe was used at 50 nM. Standard curves were done with a spiked pool of naive tissue lysate (heart, and colon) with 1 × 10^5 parasites/mL and serially diluting 1/10 in naive mouse DNA. Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA) was set to run at 50 °C for 2 min, 94 °C for 10 min, followed by 40 cycles at 95 °C and 58 °C for 1 min; fluorescence was collected after each cycle. All samples were run in duplicate.

6.6. Immunohistochemistry

The mice were euthanized under anesthesia and their hearts were removed, embedded in tissue-freezing medium (Tissue-Tek, Miles Laboratories, USA) and stored in liquid nitrogen. The phenotypes of the inflammatory cells (CD4+, CD8+, F4/80) colonizing the heart tissue and the T. cruzi parasitism will be characterized and analyzed as previously described (Silverio, Pereira et al. 2012).

6.7. Cytokine profile

Cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, MIG, RANTES, M-CSF, G-CSF, GM-CSF, LIF, LIX, VEGF, Eotaxin, TNF-α, and IFN-γ) were detected in serum using a commercial Millipore cytokine kit according to the manufacturer’s instructions. Samples were
assayed with suitable controls provided by manufacturer for the construction of standard curves. The fluorescence produced by the beads were measured on a Milliplex system (Millipore Inc., USA) and analyzed using the software contained in equipment.

VII. RESULTS

7.1. Parasitemia

Monitoring of parasitemia would indicate us the best inoculation. Since we desired to induce CCD in the animals rather than having some deaths, we could not challenge the animals with high levels of parasites. Mice were challenged with different parasite inoculations ($10^2$; $10^3$ and $10^4$ trypomastigotes of Colombiana strain) (Figure 19).

![Graphs showing parasitemia](image)

**Figure 19. Parasitemia.** Groups of mice were inoculated with $10^2$, $10^3$, and $10^4$ *T. cruzi* trypomastigotes (Colombiana strain).
7.2. Cytokines

Expression of anti-inflammatory and pro-inflammatory cytokines may play a relevant role in determining the clinical presentation of chronic patients with CD and suggests the occurrence of specific immune responses, probably associated to different T. cruzi DTUs (Poveda, Fresno et al. 2014). Biomarkers in CD to monitor disease progression and response to therapy are desperately needed (Requena-Mendez, Lopez et al. 2013, Pinho, Waghabi et al. 2016). In this project we tested the sera from mice before and after immunizations with Galα3LN-HSA alone, and combined with LMPLA adjuvant.

Regarding circulating cytokines, IL-1α displays a wide variety of biological activities on many different cell types, including T cells, B cells, and monocytes. Receptors for IL-1 are also found on the other leukocytes, including eosinophils and dendritic cells, as well as on nonimmune system cells such as fibroblasts, vascular endothelial cells, and some cells of the nervous system. The in vivo effects of IL-1 include induction of fever, the acute phase response, and stimulation of neutrophil production.

IFN-γ sources are CD4+ and CD8+; and NK cells and it affects activation, growth, and differentiation of T cells, B cells, and macrophages as well as NK cells; up-regulates MHC expression on antigen-presenting cells; signature cytokine of TH1 differentiation; weak antiviral and anti-proliferative activities (Kindt Thomas J 2007).
IL-2, which stimulates growth and differentiation of T cells, B cells, and NK cells, increased after vaccinations in L-MPLA group and decreased in the Galα3LN-HSA + L-MPLA group. No significant change was observed in levels of IL-1α, IFN-γ, IL-1β, IL-3, IL-4, IL-9, IL-10, and IL-12(p40) in the Galα3LN-HSA group or Galα3LN-HSA + MPLA group (Figure 20).
Figure 20. Analysis of serum cytokines. Cytokine measurements were performed by sandwich ELISA. Vaccinated mouse serum cytokines in comparison with nonvaccinated mouse ones. Results are representative of two biological replicates.
7.3. Antibody Isotyping

In this study, where the serology for anti-*T. cruzi* IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, IgE and IgA in animals with CDD were followed, we tried to correlate the levels of antibody titers, and cytokine levels, to cardiac disease severity.
Figure 21. Immunoglobulin test by CL-ELISA before and after therapeutic vaccination.
In addition to binding antigen, antibodies participate in a broad range of other biological activities. When considering the role of antibody in defending against disease, one must remember that antibodies generally do not kill or remove pathogens solely by binding to them. In order to be effective against pathogens, antibodies must not only recognize antigen but also invoke responses effector functions that result in removal of the antigen and death of the pathogen (Kindt Thomas J 2007).

The quantification of total serum immunoglobulins was performed in all 20 animals to analyze possible changes in total antibody production according to disease stage, regardless of the antibody specificity. The total levels of total IgA, and IgE were similar among all groups, without correlation with total antibody production and development of cardiac disease stage. IgE antibodies mediate the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives, and anaphylactic shock (Kindt Thomas J 2007). In this experiment, the Galα3LN-HSA vaccine did not produce IgE, which is really good because it’s not inducing allergic reactions. IgA constitutes only 10% to 15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears, and mucus of the bronchial, genitourinary, and digestive tracts (Kindt Thomas J 2007). We did not detect IgA production in the Galα3LN-HSA neither in the Galα3LN-HSA +LMPLA group.

However, the level of total IgG, IgG1, IgG2b, and IgM was higher in the Galα3LN-HSA + LMPLA adjuvant compared with Galα3LN-HSA alone. IgG, the most abundant class in serum, constitutes about 80% of the total serum immunoglobulin. There are four human IgG subclasses: IgG1, IgG2, IgG3, and IgG4. Most IgG subclasses can activate a collection of serum glycoproteins called the
complement system. Complement includes a group of proteins that can perforate cell membranes (Kindt Thomas J 2007). IgM and total IgG antibodies present at 90 DPI are due to the fact that these mice were infected with T. cruzi at day zero, beginning of the experiment, therefore those antibodies were produced against the parasite. These protective lytic T. cruzi-specific anti-α-Gal antibodies produced by the vaccinated groups (Galα3LN-HSA + LMPLA and Galα3LN-HSA alone) could be mounting an immune defense against the T. cruzi infection, therefore avoiding cardiac problems in the mice and decreasing parasite load in the tissues. To clarify this hypotheses, we decided to perform qPCR in the tissues such as heart and colon tissue were the parasite has a preference to be located at and because those are vital organs.

7.4. qPCR performance by Equivalence Partitioning Analysis based on calibration curves using the Comparative Threshold Method (Ct-method)

This experiment embraced the evaluation of DNA samples from parasite stocks representative of the T. cruzi DTU I, Colombiana strain. We expected parasitic loads in CCD samples from PBS, and LMPLA group (control groups) to be higher than in Galα3LN-HSA vaccinated mice. We analyzed parasite burden in the Colon of all twenty animals, including all groups, and we found out that four out of five animals in the Galα3LN-HSA +LMPLA group had parasite loads inferior than all other groups but one animal in the Galα3LN-HSA + LMPLA group reached concentrations of up to 3.3 Log 10 par. eq./100 ng (Figure 22A). Parasitic loads in the heart of the mice showed that vaccinated group failed to develop protection against T. cruzi infection (Colombiana strain). Blue and white rounded shaped circles represent the two groups that received the Galα3LN NGP
therapeutic vaccine and data bellow (Fig. 20 B.) showed higher concentration of parasite load in these two groups.

![Graph showing parasite load in colon and heart](image)

**Figure 22.** Quantitative real-time PCR (qPCR) analysis of parasitic load in colon and heart.

### VIII. Conclusion and Discussion

The aim of this study is to provide insights about the α-Gal-based glycoprotein as a candidate therapeutic vaccine in chronic *T. cruzi* infection animal model for Chagas heart disease. Studies have demonstrated the binding of anti-Gal antibodies to live parasites and the subsequent complement-mediated lysis of *T. cruzi* (Almeida, Milani et al. 1991, Almeida, Ferguson et al. 1994). There are evidences that the glycoconjugates from cell derived trypomastigotes that are recognized by CD anti-Gal are mucin-like glycosyl-phosphatidylinositol (GPI)-anchored molecules bearing several oligosaccharide chains (Almeida, Ferguson et al. 1994). In this project we assumed that the anti-Gal antibodies would give protection to the animals and that the
activation of B cells via major histocompatibility complex (MHC) class II to CD4+ T cells could be mediated through human serum albumin protein (in form of peptides) in order to trigger isotype switching (IgM to IgG). The immunoglobulin data made us optimistic about this new and first Chagas vaccine. IgE antibodies may be related to allergic or autoimmune reactions, therefore a safe vaccine should not enhance its production. It’s important to emphasize that we did not found IgE in the serum of vaccinated group. Besides the fact that IgM and IgG levels were high in the vaccinated groups, we noticed that these antibodies alone were not enough to decrease parasitic loads in colon and heart of mice as measured by qPCR. A C-reactive protein test was performed and data showed no discrepancy among groups that received vaccine and groups that did not (data no shown).

Regulation of IFN-gamma and chemokine expression, are responsible for the control of inflammation and immunopathology observed in the cardiac tissue of animals infected with T. cruzi (Talvani, Ribeiro et al. 2000). Cytokines and chemokines are important tools against T. cruzi infection (Poveda, Fresno et al. 2014). In this project, no significant chance was observed in levels of cytokines that could favor the Galα3LN vaccinated group.

Experimental therapeutic studies with inclusion of drugs combined with the vaccine and adjuvant are very promising and should be pursued. New forms of treatment would benefit millions of infected people worldwide and certainly this vaccine would prolong and improve their lives.
IX. Future Experiments

- We plan to evaluate the vaccine combined with benznidazole drug. Is there a dose dependent manner when drug is combined with vaccine treatment?
- Is there any immune differences when using different parasite strains? New approaches can be done using different strains to generate chronic disease.
Appendix

List of publications and manuscripts

Book chapter:


2. Two more manuscripts in preparation
References:


Gomes, Y. M., V. M. Lorena and A. O. Luquetti (2009). "Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies?" *Mem Inst Oswaldo Cruz* **104 Suppl 1**: 115-121.


Curriculum Vitae

Igor Estêvão was born in Minas Gerais-Brazil in 1981. He acquired his Bachelor in Biomedicine in Summer 2008 in his home country. He also worked in clinical lab for several years. His goal to pursue a career in the field of Science has implications on his past background and future aimed achievements. The majority of his work and volunteer experience has involved clinical and research laboratory work. As a volunteer (2016-2017) in Dr. Almeida’s lab he dedicated his best efforts toward his education and goals and as a result, he was offered a position as core coordinator in the UTEP Biomolecule Analyses Core Facility (BACF). He soon attained full-time employment with the Border Biomedical Research Center. This facility facilitates and expand the pathobiology research at UTEP by strengthening the research infrastructure of the institution. He was then accepted to the Master of Science program at UTEP in Spring 2018 under the mentorship of Dr. Igor C. Almeida. He is involved and several clinical trials and serological experiments that are taking place in different parts of the world such as Biomarcha project from Spain, Bendita project from Bolivia/Brazil, Fexi 001 from Spain, Mexico project, Venezuela project, Cynomolgus project in collaboration with Texas Biomed and UTRGV University, Baboon project from El Paso/San Antonio-Tx, and Pregnant Women Chagas diagnosis project from El Paso-Texas. In parallel, he worked extensively in a project involving the use of a novel synthesized glycoprotein as therapeutic vaccine for Chagas disease in murine models. He is co-author in one book chapter and has two more manuscripts in preparation. Igor Estêvão got accepted into the Biosciences PhD – Fall 2019 program at The University of Texas at El Paso (UTEP) and got the prestigious four year “The University of Texas at El Paso Doctoral Excellence Fellowship” to keep moving forward with his researches. He hopes that his goal in acquiring a Science Master degree, posteriorly a doctor of philosophy degree, and finishing his research projects can help not only himself but everyone else that may be benefited by his science.

Permanent address: El Paso, Texas, U.S.A