ENDOTHELIAL, IMMUNOLOGICAL AND STRUCTURAL ASSESSMENT OF PLACENTAL SPECIMENS FROM *T. cruzi* VERTICAL TRANSMISSION CASES IN SANTA CRUZ DE LA SIERRA – BOLIVIA

by

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Dissertation Abstract

The role of the placenta in the vertical transmission of *T. cruzi, as well as the risk factors that contribute to vertical transmission, are* not well defined.. Few studies have evaluated the use of the placenta for diagnosis of Chagas disease in newborns. Accurate identification of infected newborns is a high priority in control programs since the cure rates are high. Using immunohistochemistry, we evaluated endothelial and structural impairment of placental cells, as well as features of the immune response that may be contributors in congenital Chagas disease.

Immunohistochemistry markers of disturbed placental barrier were standardized and matched to obstetrical and neonatal data. No differences were found in placental measurements of neonatal and obstetrical characteristics in cases of vertical transmission. Conventional testing, achieved diagnosis after 6 months of age. In contrast, TESA-blot and quantitative polymerase-chain reaction (q-PCR) (on cord blood or umbilical tissue) identified the majority of cases at birth. Successful immunohistochemistry techniques were identified for further evaluation.

We also assessed placental barrier impairment linked to endothelial cells and dysfunctional endothelial tight junction' proteins using markers of angiogenesis. Expression of endothelial tight junction proteins was increased at fetal blood vessels and decreased at syncytiotrophoblast. Markers of angiogenesis showed decreased expression at the placental barrier. Dysregulated angiogenesis and impaired cell-to-cell contact at the

syncytiotrophoblast layer are related to placental barrier impairment and may be associated with congenital infection.

The expression of structural proteins was increased in fetal blood vessels and the syncytiotrophoblast, while the expression of tight junction proteins was decreased in the cytotrophoblast. Expression of mediators of innate immune response was increased in the syncytiotrophoblast and fetal blood vessels. Expression of markers of macrophage activation was decreased in the syncytiotrophoblast. Increased expression of structural proteins at the syncytiotrophoblast is related to impaired placental barrier and may be associated with *T. cruzi* vertical transmission.

In conclusion, molecular tests for diagnosis of congenital Chagas Disease should be implemented in poor resource settings. Signs of impaired placental barrier and immune response are documented in this study. Future research identifying placental biomarkers at maternal serum would be very useful for perinatal diagnosis.

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Dedication

To God

To Santa Maria

To Lisbeth, Andres y Beatriz,

To my parents, Jose y Carmen

To Dr. Robert H. Gilman

To my brothers, Jose y Marcos

To my dear Nicolaza

Table of Contents

List of Tables and supplemental tables

TABLES

SUPPLEMENTAL TABLES

List of Figures

List of abbreviations

Abbreviation	Full Name
BSA	Bovine serum albumin
CAV-1	Caveolin-1
CD	Cluster Designation
CLAU1	Claudin 1
CLAU5	Claudin 5
CMV	Cytomegalovirus
Cyt-7	Cytokeratin 7
DAB	Diamin Benzidine
DALY	Disability-adjusted life years
EA-ELISA	Enzyme-linked immunosorbent assay based on epimastigote-derived antigens
Fg	Fibrinogen-like protein
FPA	Fibrinopeptide A
GIPLs	Glycoinositol phospholipids
GPI	Glycosylphosphatidylinositol
HAI	Hemagglutination inhibition assay
hCG-Beta	Human chorionic gonadotrophin beta
HIAR	heat-induced antigen retrieval
HRP	Horse radish peroxidase
IFAT	Indirect immunofluorescence tests
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
IQR	Inter quartile range
M+B-	Mother positive -Baby negative
M+B+	Mother positive-Baby positive
M-B-	Mother negative-Baby negative
MyD	Myeloid Differentiation Primary Response
NCDCP	National Chagas Disease Control Program
NO	Nitric Oxide
PAHO	Pan American Organization
PBS	Phosphate-buffered saline
PLAP	Placental Alkaline Phosphatase
PlGF	Placental Growth Factor
PRR	Pattern Recognition Receptor
q-PCR	Real time Polymerase Chain Reaction
RPMI medium	Roswell Park Memorial Institute medium
SMA-Alpha	Smooth Muscle actin Alpha
TESA-blot	Trypanosoma excreted-secreted antigens blot
Th	T-Cell helper
TLR	Toll-like Receptor

TMA	Tissue Micro Array
VE-Cadherin	Vascular Endothelial -Cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor
WHO	World Health Organization
ZO1	Zonula Occludens

Chapter One - Introduction

The first two cases of congenital Chagas Disease were described by Carlos Chagas in 1901 in a set of twins. Parasites were identified during the autopsies of these twins who presented with neurological complications at birth. Chagas disease can produce abortions, premature births as well as fetal target organ injury including damage of the liver, spleen, heart and central nervous system. The Pan American Health Organization (PAHO) indicates that about 14,000 cases occur annually in Central and South America. Congenital infection occurs at any time during pregnancy and with any of the pregnancies, for that reason every serologically positive mother is a potential mother transmitting the disease to her newborn. (WHO, 2006)

Early diagnosis of *Trypanosoma cruzi* vertical infection has many advantages. Follow-up may be easier to coordinate if mothers know that their infant has Chagas disease when they leave the hospital. The drugs to treat Chagas disease are better tolerated in infants and young children, and the therapy is more likely to be effective when administered close to the time of infection. (WHO 2002, Carlier 2005, Oliveira *et al.* 2010; Carlier *et al.* 2011, Yoshida *et al.* 2011, Tornheim *et al.* 2013)

Few studies have evaluated the placentas of neonates diagnosed with Chagas disease. Bittencourt in 1963 studied the placentas of nine cases of congenital Chagas disease and found that the macroscopic appearance was very similar to that observed in cases of syphilis. The villi and villous tree were edematous, Hofbauer cells were identified and foci of lymphocytic infiltration and local necrosis were also documented. Sometimes there is loss of the entire chorionic epithelium with fibrinoid granulomatous reaction with

round, epithelioid and giant cells. Langhans cells were not present. (Bittencourt 1963) Moya in 1979 analyzed the placentas of 18 newborns diagnosed with congenital Chagas disease and found amastigotes surrounded by a chronic inflammatory mononuclear infiltrate localized in the decidua and the amniochorionic plate. No abnormalities were observed at gross examination compared to normal placental specimens. Neither parasites nor lesions were observed at the level of the chorionic villi. Of all the 18 cases, only 8 (44 %) were identified as *T. cruzi* infection in the newborn by detectable parasites in blood. Only in 5 of the 18 cases parasites were found also in cord tissue. (Moya 1979) Tafuri in 1984 reported the very first assessment of *T. cruzi* infected human placenta by electron microscopy, describing fibroblastic and collagen proliferation of the walls of small arteries and arterioles, infiltration of Hofbauer cells with amastigotes and parasites in degenerated muscle cells of the vascular walls. (Tafuri 1984) Fretes in 1995 assessed 11 placentas, 6 of them from mother serologically positive for *T. cruzi* infection and 5 from controls. No information was provided regarding the mother-to-child transmission on the 6 seropositive specimens. However, in 2 of the 6 placentas from serologically positive mothers, there was diminution and occlusion of the lumen of the chorionic blood vessels with hyaline aspect of their walls. (Fretes 1995)

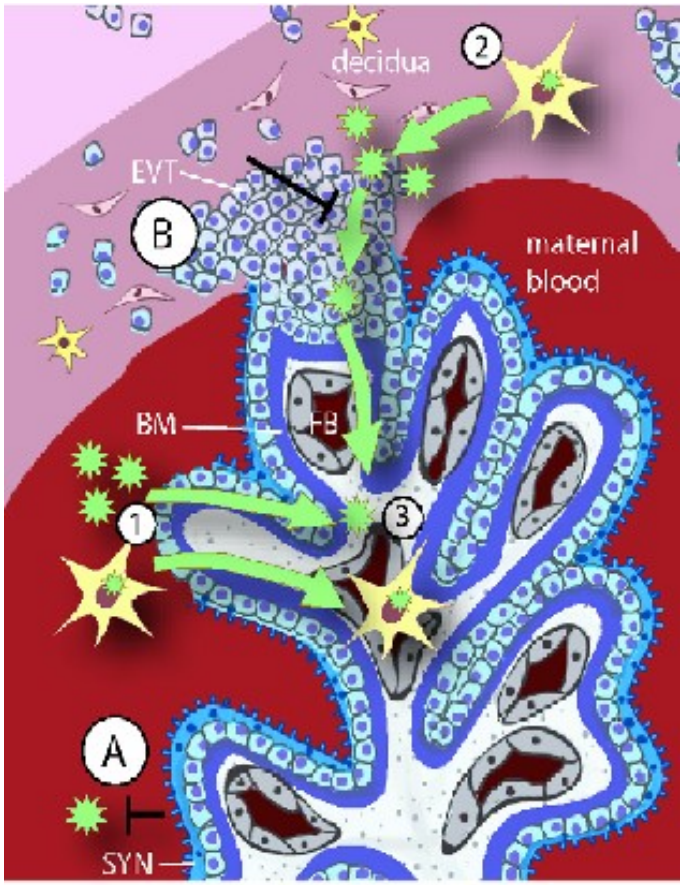
The usefulness of placental and umbilical cord tissues for detection of *T. cruzi* vertical transmission cases is uncertain. This is because the placenta plays a containment role for parasites between the mother and the fetus, and thus the presence of parasites in the placenta does not necessarily indicate congenital infection, instead it may be considered a risk factor. (Reyes *et al.* 1990, Torrico *et al.* 2004, Luquetti *et al.* 2005, Ponce *et al.* 2005, Virreira *et al.* 2006, Roddy *et al.* 2008; Sosa-Estani *et al.* 2008; Carlier *et al.* 2011) Additional risks factors for transmission

of Chagas disease to the offspring include: (1) the level of parasitaemia in the pregnant woman, with the risk of *T. cruzi* congenital infection being higher in those neonates born from pregnant women with high parasitaemia than those born from mothers with low levels of detectable parasitaemia, and (2) the disruption on the placental barrier due to the presence of structural alterations of the maternal-fetal transition. (Carlier et al. 2012)

There are also mechanisms protecting the fetus against the infection during pregnancy. Most of these mechanisms are linked to the intact formation and preservation of placental villi microstructure constituted by two layers of trophoblasts, the syncytiotrophoblast and the cytotrophoblast. The syncytiotrophoblast, the outer layer, prevents intercellular penetration and mixing between maternal blood and fetal tissue, and the cytrotrophoblast (Langerhans cells), the inner layer, provides protection by covering the extra villous tissues including the chorionic plate. The risk to the fetus is considered high is disruptions of this barrier occurs after the 12th week of gestation, time at which maternal blood supply to the fetus starts. (Moya *et al.* 1979; Abrahams and Mor 2005; Carlier 2005, Romero *et al.* 2007, Carlier *et al.* 2011; Mor and Cardenas, 2010) To consider a microorganism a threat to the fetus, the cell layer of syncytiotrophoblast should be disrupted or the pathogen should gain access to the decidual compartment or to the placental villi. The risk of transmission is higher if placental barrier rupture is found compared to minimal or no risk when there is no placental involvement (Figure 1). (Bittencourt, 1963; Carlier, 2005; Redline 2006; Schijman, 2006; Carlier *et al.* 2011; Mor and Cardenas, 2011)

The interaction between the virulence factor specific to *T. cruzi* and the factors described above also influences the risk of congenital infection. In infected pregnant women, the trypomastigotes found in the intervillous space, may penetrate the

trophoblast and nest and multiply as amastigotes in Hofbauer cells. This nest is lysed later releasing parasites into the interstitial space causing necrosis and inflammatory reaction in chorionic villi. (Schijman *et al.* 2006, Carlier *et al.* 2011)



"A schematic depiction of the human maternal–fetal interface is used to illustrate the barriers and possible pathways for pathogen transmission. The two sites of direct contact between maternal and fetal cells are the blood–syncytiotrophoblast (SYN) (a) and the uterus–extra villous trophoblast (EVT) (b) interfaces. Both of these trophoblast subpopulations have defense mechanisms against infection, and underneath the trophoblast barrier, the basement membrane (BM) presents an additional physical barrier. Failure of the placental barrier can occur in the presence of high pathogen titers or multiple infections. Damage of the syncytiotrophoblast enables pathogens (green stars) that are free in maternal blood or inside of maternal leukocytes (yellow cells with green stars) to cross into fetal tissues (1). However, animal and organ culture models agree that most placental infections originate in the uterine decidua (2), which is minimally accessible from the maternal blood. Pathogens can reach the decidua only by dissemination in maternal cells, most likely leukocytes. If the defense mechanisms of the EVT are overcome, the infection may spread to the fetal blood (FB) (3), act as a nidus for maternal reinfection, and/or cause trophoblast death resulting in placental insufficiency or spontaneous abortion. Finally, some pathogens may reach the fetus by traveling within maternal leukocytes on their natural way to the fetus."

(Modified from Robbins and Bakardjiev, 2012)

Figure 1. Placental colonization by pathogens.

The immune system plays a very particular role during pregnancy by allowing tolerance to the fetus and also protecting the fetus against pathogens. (Munn *et al.* 1998; Thelon and Heine, 2003; Moor and Cardenas, 2010) Cytotrophoblast cells express Toll-like receptor (TLR) 2, which upon activation initiates a Myeloid Differentiation Primary Response (MyD) 88-dependent intracellular response. The downstream effects of TLR-2-MyD88 activation target the trypomastigotes by: (1) releasing of nitric oxide (NO) and pro-inflammatory

cytokines, including interleukin (IL) 12; and (2) inducting a T-Cell helper (Th) 1 response mediated by glycosylphosphatidylinositol (GPI) and anchored mucin -like glycoproteins. Following injury of the trypomastigotes, Tc52, a protein from the *T. cruzi* becomes released. Tc52 protein is composed by two sites, the G (glutathione binding) site, responsible for TLR-2 activation, and the H (hydrophobic domain) site associated with survival and virulence of the parasite (Buschiazzo *et al.* 2002, Frasch 2000, Jacobs *et al.* 2009). A MyD88-independent pathway is also operative in the response of the cytrotrophoblast cells against *T. cruzi* trypomastigotes via activation of TLR-4 by glycoinositol phospholipids (GIPLs). Upon TLR-4 activation, TRIF (domain containing adapter-inducing interferon - β) triggers the production of type 1 interferon (IFN) (α and β) and activation of IRG [Immunity Related Guanosine Triphosphatases (GTP)] proteins (immune related GTPases), involved in immune responses against intracellular pathogens (Oliveira *et al.* 2010; Palm and Medzhitov 2009 , Taylor *et al.* 2004) The switch to the Th1 cytokine profile (IFN-γ , TNF-α , IL-12) transforms natural killer cells (NKs) into lymphokine-activated killer cells (LAKs), which exert cytolytic actions by stimulating the production of immunoglobulins (capable of activating the complement cascade), decreasing OX2 (CD200), and leading to Fibrinogen-like protein (Fg) 2 production. Downstream events include the production of IL-8, which attracts and activates neutrophils to attack the fetal-placental structures and induces the formation of clots occluding the maternal-fetal circulation resulting in ischemia, necrosis and death of fetal tissues. These histopathological findings are documented in placentas of women infected with *T. cruzi* who have presented with abortions, premature babies, or stillborn. (Bittencourt , 1963 ; Raghupathy 1997 ; Thellin and Heinen , 2003 ; Torrico *et al.* 2004 ; Kayama and Takeda , 2009)

Biomarkers of angiogenesis have been previously tested for malaria placental infection (Ruizendaal *et al.* 2015) and pre-eclampsia (Hupertz, 2015). Expression of angiogenic factors is decreased in association with endothelial dysfunction in the presence of *T. cruzi* (Ramirez *et al.* 2012) and *Toxoplasma gondii.* (Hunter *et al.* 2001). Vascular Endothelial Growth Factor (VEGF) and Placental Growth Factor (PlGF) showed a pivotal role in neoangiogenesis associated with inflammatory conditions. (Clark *et al.* 1998) However, no previous studies have described the relationship between angiogenic factors, adherens and tight junction proteins in maintaining the integrity of the endothelial barrier in placenta of neonates diagnosed with congenital Chagas disease. Vascular Endothelial -Cadherin (VE-Cadherin), a marker of disturbed endothelial barrier, is closely linked to Occludin in vitro (Dye *et al.* 2001) that decreases in association with vascular endothelial dysfunction produce by malaria (Gillrie *et al.* 2007), and embryonic death in fetuses expressing defects of angiogenesis. (Gavard *et al.* 2008) Other markers of disturbed epithelial barrier such as Caveolin-1 (CAV-1) and Occludin are increased in placental specimens linked to hypoxia (Jiang *et al.* 2014) and bacterial infection with ischemia, respectively. (Marchiando *et al.* 2010)

This dissertation focuses on the usefulness of Tissue Micro Arrays (TMAs) and individual assessment of images obtained after immunohistochemical (IHC) procedures applied to randomly selected biopsies from a nested case-control study in order to identify impairment of the placental barrier in congenital Chagas disease. (See the section "Use of immunohistochemistry, individual analysis of images and tissue micro arrays to assess placental specimens from *T. cruzi* vertical transmission cases"). In addition, markers of angiogenesis and cell-to-cell contacts in endothelial cells of the placental barrier were measured comparing placental specimens from congenital Chagas disease to

controls in a nested case-control study performed in Santa Cruz de la Sierra – Bolivia (See the section "Endothelial Features of Human Placentas from Full-term Pregnancies Resulting in Congenital *T. cruzi* transmission In Bolivia"). Lastly, immunological and structural components of the placental barrier were assessed in order to identify differences potentially linked to the mechanisms of congenital *T. cruzi* infection. (See the section "Immunological and Structural Features of Human Full Term Placentas that are Associated with *T. cruzi* vertical transmission In Bolivia").

Chapter Two - Background

Chagas Disease is a vector-borne disease caused by the protozoan parasite *Trypanosoma cruzi. T. cruzi* is carried in the gut of blood-sucking triatomine bugs. Transmission occurs when infected bug feces are inoculated through the bite site or intact mucous membranes; *T. cruzi* can also be transmitted through transfusion, transplant and congenitally. Most recently, oral transmission was also described.

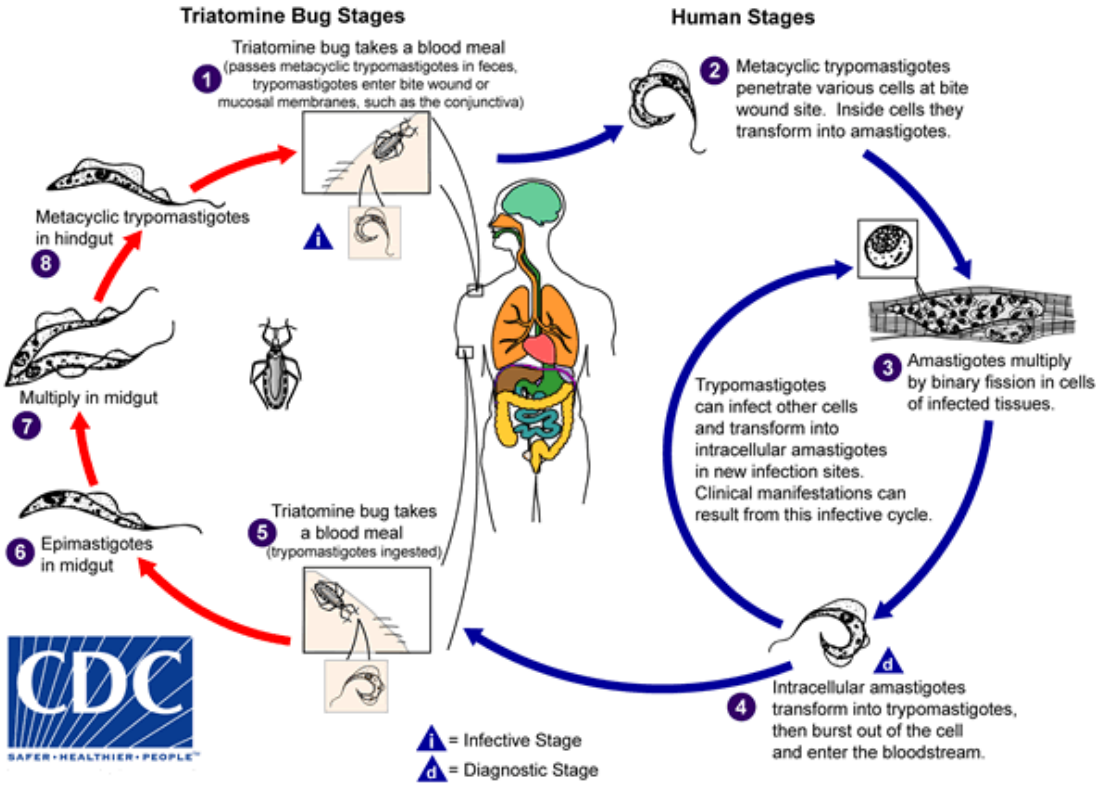


Figure 2. Life Cycle of *Trypanosoma cruzi*

http://www.cdc.gov/parasites/chagas/biology.html

II.1. Etiology and life cycle

Host infection occurs when infected triatomine insect vectors *(Triatoma infestans, T. rhodnius, T. panstrongylus)* locally called vinchuca in Bolivia, Argentina, Chile and

Paraguay; barbeiro in Brazil; chirimacha in Peru and tipo in Colombia; takes a blood meal and release their feces containing trypomastigotes near the bite sites. Differentiation into intracellular amastigotes occurs within the invaded cells near the wound. After multiplying by binary fission, intracellular amastigotes differentiate into trypomastigotes and then enter the blood stream. Once into the circulation, trypomastigotes infect tissues in different sites and transform into intracellular amastigotes at the new sites of infection. Bloodstream trypomastigotes are unable to replicate and only amastigotes can replicate when entering a new cell. Vectors become infected when bite and feed blood with circulating parasites from an infected host. Ingested trypomastigotes transform into epimastigotes at the insect midgut, differentiating and multiplying at the same site, and becoming infective metacyclic trypomastigotes at the hindgut of the vector. (Figure 2)

II.2. Modes of transmission

II.2.1. Vector

Triatoma infestans is the main vector for Chagas disease transmission in South America. (Klug 1834) This vector is thought to have originated in Bolivia and transported later to the neighbor countries where it has established reproductive populations. PAHO reported *T. infestans* was eliminated from Uruguay in 1997, from Chile in 1999, and from Brazil in 2006. (Coura & Dias 2009) However, Brazil still reports residual foci in the states of Rio Grande do Sul and Bahia. (Figure 3)

Figure 3. Geographical distribution of *T. infestans* in Central and South America

http://www.emedmd.com/content/chagas-disease

Bolivia reports cases in a variety of regions and altitudes, ranging from the high altitude Andean valleys to the Gran Chaco area (Figure 4) (Noireau 2009) Failure of elimination efforts at the Gran Chaco region indicate the need for sustained and continued monitoring and coordination of the Control Programs and the government. (Gürtler 2009)

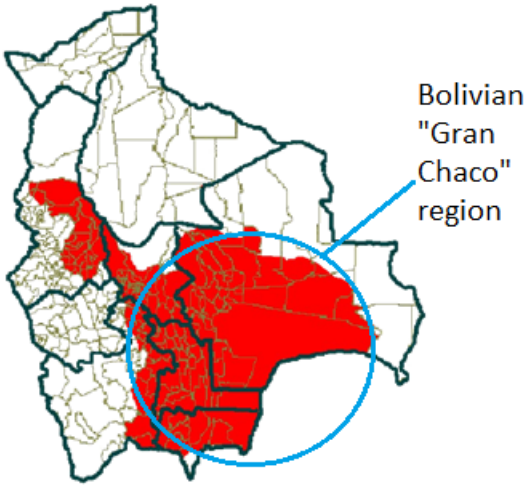


Figure 4. Bolivian National Control Program –Bolivian regions with reports of *T. Infestans* presence . (2010,NCP personal communication, not published)

II.2.2. Blood transmission

In 1945 transmission via blood transfusion was reported for Chagas disease. (Dias *et al.* 1945) Due to increased emigration from South America of patients with Chagas disease, the blood transfusion mechanism of transmission is still considered an important route in non-endemic countries.(Coura 1966, Wendel & Dias 1992, Schmunis 2007, Coura & Viñas 2010) Although the incidence of new infections via blood transfusions has decreased as a result of improved control at blood banks in endemic regions, these control measures may be insufficient for control as many non-endemic countries such as Spain, the USA, and many Asian countries are receiving thousands of migrants from endemic areas who carry Chagas disease. (Coura & Viñas 2010, Coura *et al.* 2014)

II.2.3. Oral transmission

The oral route of transmission is still controversial. The first outbreak of potential oral transmission in humans was documented in Brazil in 1968 and involved 18 people

and six deaths. (Silva *et al.* 1968, Guimaraes *et al.* 1968) Other Brazilian outbreaks included fewer fatal cases and infected patients. (Shikanai-Yassuda *et al.* 1991) The last report with great international repercussion involved 103 acute Chagas disease cases in Caracas, Venezuela. (Alarcon *et al.* 2010) This report described for the first time contaminated guava juice as the source of infection in an urban oral Chagas disease outbreak originated from an inner-city household, where rodents and peridomestic triatomins are thought to maintain the transmission.

II.3. Chagas Disease – Clinical manifestations, diagnosis and treatment.

Chagas disease leads to more morbidity and mortality in the Americas than any other parasitic disease. In 2002 estimates for the American region, Chagas disease accounted for 6 times as many disability-adjusted life years (DALYs) lost as malaria.(PAHO 2006, WHO 2002, WHO 2002) An estimated 8 million people are currently infected; 20-30% of these will develop symptomatic, potentially life-threatening Chagas disease. (WHO 2002, Bern *et al.* 2009)

Although an autochthonous enzootic cycle and competent vectors exist across the southern half of the country, the vast majority of human *T. cruzi* infections in the USA affect immigrants infected in their home countries.(Bern *et al.* 2008, Bern *et al.* 2007) While Chagas disease is increasingly recognized as an important public health issue in North America, data about its frequency in Canada, Mexico, and the USA are limited.(Buekens *et al.* 2008, Yabsley *et al.* 2002) In 2006 researchers estimated that 40,000 infected women of child-bearing age lived in the USA, resulting in 189 congenital infections occurring per year, and making the case for universal screening for congenital *T. cruzi* in the USA.(CDC 2006, Navin *et al.* 1985, Yabsley *et al.* 2002)

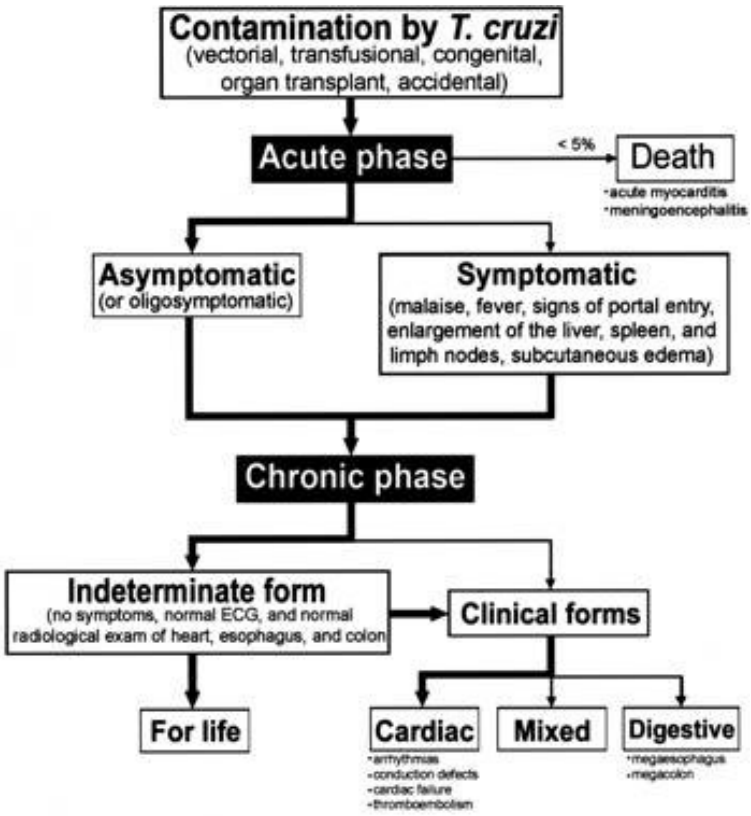


Figure 5. Natural history of Chagas disease. "There are two phases of the human disease: the acute, which occurs immediately after infection, lasts from a few weeks to months, and is usually asymptomatic; and the chronic, which occurs in approximately 30 percent of cases. The thickness of arrows indicates the relative probability of a depicted pathway." (Rassi *et al.* (2007).

After a mainly undetectable acute phase of the disease, infected individuals progress to the chronic phase characterized by positive serology and low or undetectable levels of parasitaemia.(Hidron *et al.* 2011, Samuels *et al.* 2013) Individuals not developing signs and symptoms related to targeted organs (mainly heart, esophagus and intestine) are thought to live in an indeterminate phase of the disease. However, nearly 20-30% of infected individuals will develop symptomatology of Chagas disease, more often cardiomyopathy. Early manifestations of the disease are conduction defects, especially right bundle branch block and/or left anterior fascicular block, which later may progress to higher grade heart block and complex ventricular arrhythmias. (Maguire, 1987) In patients with more advanced Chagas

cardiomyopathy, congestive heart failure, ventricular aneurysm, and complete heart block are signs of worst prognosis, including high rates of mortality and sudden death. (Figure 5) (Rassi, 2010)

II.3.1. Congenital Chagas Disease

Congenital *T. cruzi* infection causes a spectrum of clinical manifestations.(Freilij *et al.* 1995, Maguire 2004) Disease severity may be related in part to the period of pregnancy during which transmission occurs.(Andrade *et al.* 1982, Azogue *et al.* 1991, Shippey *et al.* 2005) Although data are sparse, transmission early in pregnancy appears to increase the risk of spontaneous abortion (Bittencourt *et al.* 1972), while infection after 22 weeks of gestation is more likely to lead to a late stillbirth or infected live-born infant.(Bittencourt *et al.* 1974, Menezes *et al.* 1992) Infected live-born infants fall into 3 clinical categories: (1) severe disease at birth with high risk of neonatal death (Bittencourt *et al.* 1981), (2) apparently well at birth with progression to serious complications in the first weeks to months, and (3) asymptomatic throughout infancy, but carrying 20-30% risk of chronic cardiac or gastrointestinal Chagas disease decades later.(Luquetti *et al.* 2005) Most infected newborns are asymptomatic or have subtle findings, and fall into categories 2 or 3. Very few infected infants would be detected by even the most rigorous newborn examination. (Basombrio *et al.* 1999, Blanco *et al.* 2000, Carlier *et al.* 2003) For infants with no or mild symptoms at birth, the sequelae of *T. cruzi* infection likely could be entirely prevented, if curative antitrypanosomal treatment is administered early in life. (Andrade *et al.* 2004, Andrade *et al.* 1996, Luquetti *et al.* 2005, Russomando *et al.* 1998, Schijman *et al.* 2003)

II.3.2. Field Epidemiology- Congenital Chagas Disease

The prevalence of *T. cruzi* infection in pregnant women in different countries of South America ranged from 2% to 51% in some urban centers and from 23% to 81% in some rural areas. (WHO 2002) The likelihood of vertical transmission from women seroreactive to *T. cruzi* has been extremely variable among countries and geographical areas, ranging from 0.7% to 4% in Argentina, 10.5% in Paraguay, and 2% - 21% depending on birth weight in Bolivia.(Arcavi *et al.* 1993, Basombrio *et al.* 1999, Bittencourt *et al.* 1985, Blanco *et al.* 2000, PNCCC 2007, Sanchez *et al.* 2005, Schenone *et al.* 2001, Sosa-Estani *et al.* 2005, Torrico *et al.* 2004, Virreira *et al.* 2007, Bern *et al.* 2009, Rendell *et al.* 2015) Factors reported to increase the risk of transmission include younger maternal age, HIV and, in an animal model, parasite strain.(Bittencourt *et al.* 1992, Freilij *et al.* 1995, Moretti *et al.* 2005, Sartori *et al.* 2007)

The Pan American Health Organization estimates that 26% of new infections now occur through mother-to-child transmission; as other routes diminish, the proportion attributable to congenital infection will grow.(PAHO 2006, WHO 2002) *T. cruzi* vertical transmission can occur from women themselves infected congenitally, perpetuating the disease in the absence of the vector. (Schijman *et al.* 2006, Freilij *et al.* 1995) The screening performed by the National Control Program in Bolivia in all endemic departments employs prenatal serological testing, followed by microscopic examination of concentrated cord blood from infants of seropositive mothers. (Azogue *et al.* 1995, Carrasco *et al.* 2003) For infants not diagnosed at birth, conventional IgG serology is recommended after 6 months of age.(PNCCC 2007) Similar programs exist in Argentina and Brazil. (Blanco *et al.* 2000, Neto *et al.* 2004)

Because of logistical challenges, <20% of at-risk infants complete all steps of the algorithm.[PAHO 2006, PNCCC 2007]

II.4. Congenital Chagas disease: Relevance and diagnostic tools

Early diagnosis and thus treatment of congenital *T. cruzi* infection is linked to better tolerance and effectiveness of the treatment and to prevention of the late consequences of untreated Chagas disease. If identified early enough after birth, the infected newborns can be treated and cured, decreasing rates of chronic sequelae and transmission to their offspring. Early diagnosis and treatment is therefore a high priority in control programs as it has a multiplying effect.[Neto *et al.* 2004, Okumura *et al.* 2004, Russomando *et al.* 1998, Schijman *et al.* 2003]

T. cruzi vertical transmission cannot be prevented, and the effects of anti-*T. cruzi* treatment of women in reproductive age, aimed at preventing or reducing the likelihood of vertical transmission remain unknown.[Sosa-Estani *et al.* 1999] Early detection of the congenitally infected infant ensures over 90% of therapeutic success using Nifurtimox (Lampit®, Bayer).[Bern *et al.* 2007, Schijman *et al.* 2003, WHO 2002] Until recently, only acute and congenital Chagas disease were thought to be treatable. However, in the 1990s, two placebo-controlled trials of Benznidazole treatment in children with indeterminate phase Chagas disease demonstrated approximately 60% cure of infection as measured by conversion to negative serology 3-4 years after the end of treatment. [Sosa-Estani *et al.* 1999] Follow-up of one of these treatment cohorts suggested that the earlier the children were treated, the higher the rate of cure. [Andrade *et al.* 2004]

II.4.1.- Conventional diagnostic methods for vertical *T. cruzi* transmission

A.- Direct parasitological methods. These methods based on microscopic visualization of parasites have the advantage of very high specificity when performed by an experienced laboratory, but lack sensitivity. Testing a single specimen by the Strout method will miss 50% or more of infected infants. (Bern *et al.* 2009, Rendell *et al.* 2015)

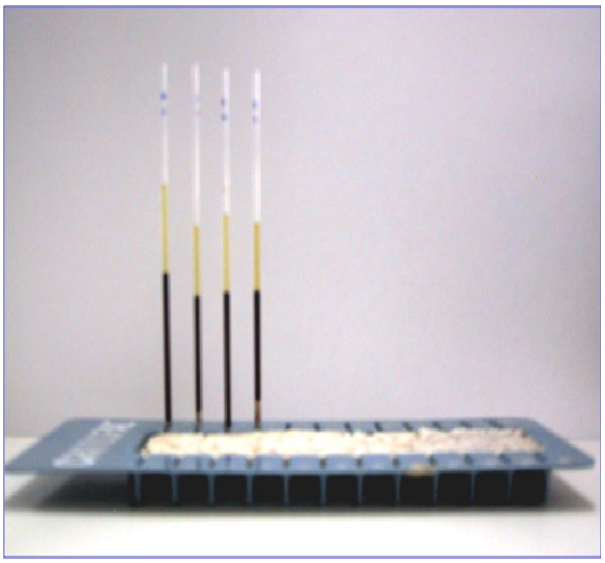


Figure 6. "Micromethod" test

Photography courtesy of Dr. Faustino Torrico

Concentration methods yield better sensitivity than direct examination of fresh blood, are inexpensive and produce rapid results. The microhematocrit method, (Figure 6) which has replaced the classic Strout method as the most widely used technique, consists of collection of cord or neonatal blood in 4-6 microhematocrit tubes followed by centrifugation and direct examination of the buffy coat. (Azogue *et al.* 1985, La Fuente *et al.* 1984, Freilij *et al.* 1983) However, in a study that examined cord blood from 78 neonates with *T. cruzi*-positive placentas, the sensitivity of the microhematocrit method was 61%. (Azogue *et al.* 1995)

Of the 35 infants whose placentas were positive but cord blood was negative, 27 completed 4 more microhematocrit examinations at 4, 7, 15 and 30 days of age. All had circulating parasites found on at least one examination, but all 4 examinations were necessary to diagnose all of the infants (cumulative detection on day 0: 61%, day 4: 67%, day 7: 83%, day 15: 94%). Examination of frequent repeated specimens is considered not be practical for large scale screening.

B. <u>Histologic examination of the placenta.</u> Histology has high sensitivity for identification of infected infants, but its specificity has not been definitively determined. The literature suggests that not all pregnancies with positive placental histopathology result in infected infants (Torrico *et al.* 2004), but relevant data are sparse. One of the few studies that directly examined this issue was performed in 1988-1989 in Santa Cruz - Bolivia demonstrating very high sensitivity and specificity.(Azogue *et al.* 1991) In this study, placentas from 820 pregnancies were examined by histopathology, and *T. cruzi* was demonstrated in 78. None of the infants from the 742 pregnancies with negative placental pathology had positive cord blood. Infants from pregnancies with positive placentas but negative cord blood were also followed by the microhematocrit method in serial heel stick specimens. Among those that completed follow-up, all were found to be infected. Nevertheless, these data need to be confirmed in further studies. Moreover, pathologic examination of the placenta may not be a practical method for screening because of the resources it would require.

C. <u>Classical indirect parasitological techniques:</u> Xenodiagnosis, hemoculture and suckling mouse inoculation can have high sensitivity and specificity in expert hands.(Freilij

et al. 1983) However, each of these techniques has stringent requirements and requires substantial resources and expertise (laboratory-reared triatomine colonies for xenodiagnosis, labor-intensive techniques and a contamination-free environment for hemoculture, and animal facilities for mouse inoculation), making them impractical for large-scale screening. Moreover, results from these assays are not available for 30-60 days.

II.4.2.- Conventional serologic diagnosis for older children and adults

A. <u>IgG serologic testing</u> is the recommended diagnostic modality for Chagas disease in older children and adults. (WHO 2002) The standard algorithm is to screen blood with one conventional assay and to confirm positives with a second test based on a different principle. The conventional assays are enzyme-linked immunosorbent assay based on epimastigote-derived antigens (EA-ELISA), hemagglutination inhibition assay (HAI), and indirect immunofluorescence tests (IFAT). (Camargo *et al.* 1969) Two positive tests are required to confirm the diagnosis. This algorithm has disadvantages both for prenatal Chagas disease screening in mothers and post-natal screening in newborns. For expectant mothers, who often present to the hospital for the first time already in labor, serologic test results will be not be available until days to weeks after mother and child have returned home, making follow-up difficult. For infants before the age of 6-9 months, positive results by standard IgG-based serologic tests may be due to passive transfer of maternal antibodies; the positive predictive value of these tests is therefore low. Anti-*T. cruzi* IgM assays have proved neither sensitive nor specific, possibly due in part to suppression of fetal IgM production by circulating maternal IgG antibodies. (Reyes *et al.* 1990) In addition,

some infants with congenital *T. cruzi* infection are negative in both IgG and IgM based assays. (Reyes *et al.* 1990)

II.5.. IHC Markers of Placental Barrier disfunction

II.5.1. Immunological Markers

A.-Toll like receptor 2 (TLR-2)

Increased expression of TLR-2 in placental cells during the second quarter of pregnancy represents a protective mechanism at the immunologically sensitive period in fetal life. (Rindsjo *et al.* 2007) However, expression of TLR-2 is more prevalent in the third quarter of pregnancy in endothelial cells, macrophages, fibroblasts and syncytiotrophoblast. (Koga *et al.* 2009)

Term placenta has a TLR expression pattern characterized by positive immunoreactivity for TLR-2 in the cytoplasm of syncytiotrophoblast. (Koga *et al.* 2009, Holmlund *et al.* 2002) Although the type of delivery may affect the expression of TLR-2 in the placenta, infection, fetal survival and gestational age are more important factors regulating TLR-2 expression in trophoblast. (Rindsjo¨*et al.* 2007)

All eukaryotes have glycosylphosphatidylinositol (GPI)-anchored proteins, but some parasites including *T. cruzi*, *Plasmodium falciparum*, *Toxoplasma gondii* and *Leishmania major*, express from 10- to 100-times more GPI-anchored proteins and these GPI anchors are structurally different from the GPI anchors produced on host cells. (Kayama *et al.* 2010) TLR-2 and TLR-4 recognize patterns associated with membrane of pathogens like *T.*

gondii, Trypanosoma cruzi, and *P. falciparum*. Such TLR expression is strongly enhanced during infection of the placenta. (Błaszkowska *et al.* 2014) Expression of TLR-2 was found to be higher in viral and bacterial infection compared to toxoplasma infection also showing strong reactivity of TLR-2 and TLR-4 in the trophoblasts in term placentas. (Hayati *et al.* 2010, Holmlund *et al.* 2002) The level of TLR expression in the placenta is controlled by each pathogen and/or endogenous molecule produced during inflammation, as a feedback mechanism to enhance or inhibit immune responses. One of the functions of TLRs is related to their ability to recognize not only microbial ligands, but also the "danger signals" released by damaged cells. (Koga *et al.* 2009)

Among the TLR family members, TLR-2 was the first receptor shown to be involved in *T. cruzi* induced innate immune response. GPI anchored mucin glycoproteins, distributed in the membrane of the cell surface of trypomastigotes of *T. cruzi* that induce the synthesis of proinflammatory cytokines and NO in innate immune cells. (Kayama *et al.* 2010) TLR-2 activation leads to a better immune response to *T. cruzi*, avoiding harmful response by exacerbated and proinflammatory cytokines, which induce lethal damage in the host. It is likely that TLR-2 has an immuno-regulatory role, as knockout mice for TLR-2 produces increased levels of proinflammatory cytokines. (Pellegrini *et al.* 2011)

B. Toll-like receptor 4 (TLR-4)

In the first quarter of pregnancy, TLR-4 is expressed in villous and extra villous trophoblast but is absent in cells from the syncytiotrophoblast. (Schatz *et al.* 2012) At term, the placenta is highly immunoreactive for TLR-4 in the cytoplasm of syncytiotrophoblast. (Schatz *et al.* 2012, Koga *et al.* 2009) Similarly, TLR-4 is highly expressed in cultured

syncytiotrophoblast and endothelial cells identified by IHC and confirmed by PCR and Western blot. (Ma *et al.* 2007)

Recruitment of innate immune cells is enhanced by stimulation of TLR-4. Since trophoblast cells express those receptors, they may modulate the maternal immune system in infective conditions. (Bernardi *et al.* 2012, Abrahams *et al.* 2005) Trophoblast cells constitutively produce chemokines that are important for crosstalk with local immune cells. In the presence of bacterial and viral components, is thought that trophoblast cells generate an inflammatory cytokine/chemokine response via the TLR-4-NF-kB pathway. (Bernardi *et al.* 2012)

Hypoxic trophoblast cells showed increased TLR-4 expression and endothelial permeability. TLR-4 and Caveolin 1 (CAV-1) may act together on the hypoxic trophoblast induced permeability of the endothelial cell monolayer. (Jiang *et al.* 2014)

TLR-4 is importantly involved in recognizing the GPI anchor of *Trypanosoma cruzi* and it was observed that the expression of TLR-4 results in resistance to *T. cruzi* infection. (Kayama *et al.* 2012)

C. CD3, CD4 and CD8

While CD3, CD4 and CD8 are known markers of cells from lymphocytic lineage, CD4 and CD8 are also identified in Hofbauer cells within the cytotrophoblast. (Lairmore *et al.* 1993) CD3 is identified in the chorionic villi, and CD3/CD4 ratio was found higher in cases of villitis of unknown etiology (VUE) and chorioamnionitis. (Kim *et al.* 2008) . The induced immune response against *T. cruzi* may provide protection as well as result in

injury. CD4 and CD8 T cells, as well as B cells are involved in systemic immunity. (Pellegrini *et al.* 2011) However, further studies are needed to identify to confirm this finding and the potential utility in early diagnosis of vertical *T. cruzi* transmission.

D. CD163

CD163 marker is expressed on macrophages and on Hofbauer cells within the placenta that are thought to belong to the monocyte-macrophage lineage. (Zaccheo *et al.* 2009) Hofbauer cell population remains constant throughout gestation, occupying about 40% of stromal cells of the villi and is in close anatomical proximity to the fetal trophoblast and villous capillaries. (Kim *et al.* 2008) The contact of macrophages with *T. cruzi* trypomastigotes induces the formation of protuberances in the macrophage cellular membrane. (Romano et al. 2012) Additionally, Hofbauer cells may internalize the parasites via phagocytosis, (Shippey et al. 2005) although this observation is controversial.

E. CD14

Monocyte subsets can be distinguished based on the expression of CD14, an important component of the lipopolysaccharide (LPS) recognition system. (Faas *et al.* 2014) CD14, jointly with TLR-4 signaling, activates inflammatory cascades. Increased CD14 immunoreactivity in activated Hofbauer cells on villitis of unknown etiology (VUE) has been reported. (Kim *et al.* 2008) Again, the role in the pathogenesis of *T. cruzi* and transmission to the fetus is not known.

F. CD56

The Neural Cell Adhesion Molecule (NCAM) also called CD56 is expressed in Natural Killer cells and was described in decidual cells from miscarriage specimens. (Papamitsou *et al.* 2015) Altemani described in 2000 that Chagasic villitis with an inflammatory infiltrate was composed mainly of CD68+ macrophages, T lymphocytes, and a few natural killer cells. (Altemani *et al.* 2000)

II.5.2. Placental Vascular Endothelial Cell Markers

A. Angiogenesis Factors

Immunohistochemical techniques have localized VEGF protein expression in villous trophoblast during normal pregnancy at term. (Shore *et al.* 1997) During pregnancy, the placenta expresses angiogenic growth factors from the VEGF family. In the first trimester of pregnancy, Placental Growth Factor (PlGF) is expressed primarily in the extra villous Trophoblast (EVT) cells within the maternal decidua, but later be abundant expression is observed in the villous trophoblast. VEGF and PlGF have a vital role in the development of placental vasculature. (Andraweera *et al.* 2012)

VEGF is a mitogen and survival factor for endothelial cells. Decreased VEGF expression results in vascular regression in several physiological and pathological conditions. (Szpera-Gozdziewicz *et al.* 2014) The PlGF is predominantly expressed in the placenta, heart and lungs. Increased PIGF leads to disturbed VEGF-dependent angiogenesis in several pathological conditions. (Andraweera *et al.* 2012) In situ hybridization studies demonstrate that PlGF gene expression is activated in different placental cell populations. (Clark *et al.* 1998)

Injury of a blood vessel leading to disruption of the endothelial cell lining results in a rapid adherence of platelets to the exposed sub-endothelium. This event is mediated by the von Willebrand factor (vWF). The role of vWF as a diagnostic biomarker has been examined in two small studies of sepsis, with less than 20 subjects each. Both studies reported that plasma vWF levels were elevated in critically ill patients with sepsis as compared with healthy adults, patients with Systemic inflammatory response syndrome (SIRS), and non-septic hospitalized patients. (Page *et al.* 2013) vWF was also shown to be a marker strongly expressed in the endothelium from maternal large vessels. (Dye *et al.* 2011) It was also found immunohistochemical reactivity for vWF in the fetal villous endothelium and in the syncytiotrophoblast of normal-term placentas. (Parra-Cordero *et al.* 2011) The utility of the vWF in diagnosis of *T. cruzi* infection has not been studied.

B. Endothelial tight junction proteins

Claudin-1 was shown to be an important tight junction protein maintaining continuity in barriers and was previously found in large vessels of the full-term placenta. (Leach *et al.* 2002) Occludin may not have a structural role as tight junction protein alone and is being considered as part of a tight junction proteins complex with Claudin 1.(Leach *et al.* , 2000) Occludin expression was decreased in choriammnionitis which is thought to cause tight junction instability but does not affect Adherens junctions. (Tosseta *et al.* , 2014)

VE-Cadherin is the major endothelial molecule in the group of adherens junctions. (Dye *et al.* 2001) and was described to be expressed in endothelial cell-cell contacts. When increased angiogenesis, VE-Cadherin transduces the angiogenic effect of VEGF producing increased permeability in the endothelial barrier. (Leach *et al.* 2002) CAV-1 is the

main structural component of endothelial caveolae and regulates endothelial transcytosis. In vitro associated activity of CAV-1with TLR-4 leads to increased permeability in the endothelial barrier of hypoxic trophoblast cultured cells. (Jiang *et al.* 2014)

II.5.3. Structural Markers

A. Cytokeratin 7

Cytokeratin-7 expression is characterized in trophoblast cells during parasitic infections such as toxoplasmosis (Xu *et al.*, 2012) as well as bacterial infection such as listeriosis. (Zeldovich *et al.* 2011) Bacteria infect fetal trophoblasts cells and spread to the nearby syncytiotrophoblast cells covering the placental villi. (Le Monier *et al.* 2006) In viral infections such as cytomegalovirus (CMV), cytokeratin-7 is also identified in the chorionic villi of the placenta (Maidji 2010) with increased expression particularly in the cytotrophoblast more than in syncytiotrophoblast cells. (Bayer *et al.* 2015, Luo *et al.* 2009, Klimann *et al.* 1986, Mouillet *et al.* 2010)

B. Human Chorionic Gonadotrophin – Beta (hCG-Beta)

Human chorionic gonadotropin (hCG) is a glycoprotein with biologically similar activity to luteinizing hormone (LH). (Katabuchi *et al.* 2008) The syncytiotrophoblast also synthesizes hormones like progesterone and human chorionic gonadotropin (hCG), which are essential for maintaining healthy viable pregnancy. (Jones *et al.* 2006) Chorionic villous macrophages may locally degrade placental hCG molecules, minimizing hCG transmission to the fetus. (Katabuchi *et al.* 2008) Increased hCG-β secretion is described following in vitro *T. cruzi* infection of BeWo cells. (Liempi et al. 2014) The role of these changes is not known.

C. Placental Alkaline Phosphatase (PLAP)

Placental alkaline phosphatase (PLAP) is enriched in syncytiotrophoblast and microvilli membrane. (Kumpel *et al.* 2011) However, further increased PLAP is documented in fetal blood vessels in tunica intima and media as a result of CMV infection. (Akyürek *et al.* 2000) Increased PLAP in cultured human placental trophoblast inoculated with *T. cruzi* decreases the invasion of the parasite. (Priotto *et al.* 2009)

D. Structural tight junction proteins

ZO-1 is a peripheral tight junctional molecule present in all clefts and integral in placental tissues. (Lievano *et al.* 2010) Claudin 5 expression is decreased in pre-eclampsia. (Morita et al. 1999) The role of changes in expression of these tight junction molecules in the setting of parasitic infections, specifically those leading to vertical *T. cruzi* transmission, needs study.

E. Fibrinopeptide A

As a precursor of fibrin, Fibrinopeptide A increases in malarial infection of the placenta as well as pre-eclampsia. Fibrinopeptide A is associated with fibrin deposits at the inter-villous space (Ahmed *et al.* 2014) and the trophoblast. (Blazkowska *et al.* 2014) To this day no studies on the role of fibrinopeptide A in vertical *T. cruzi* transmission have been published.

Chapter Three - Objectives and Methodological Overview

III.1. Objectives

The main objective of this thesis is to contribute to the understanding of the role of the placenta in the *T. cruzi* vertical transmission that would be suitable for the evaluation of future diagnostic and implementation of prevention strategies. The specific aims for this thesis are, (1) to evaluate the usefulness of immunohistochemistry to assess the expression of markers in placental tissue; (2) to assess angiogenesis and endothelial impairment of the placental barrier in *T. cruzi* vertical transmission; and (3) to evaluate the structural impairment of the placental barrier and the immune response as a result of *T. cruzi* vertical transmission.

III.2. Methodological Overview

The role of the placenta in *T. cruzi* vertical transmission remains unclear. Few placental proteins have been evaluated *in-vitro* by using highly pathogenic *T. cruzi* strains against cultured trophoblast cells. In this dissertation we nested a case control study in an ongoing cohort study to contribute to the knowledge that could lead to better understanding of the placental role in *T. cruzi* vertical transmission.

We evaluated the usefulness of immunohistochemistry to assess the expression of endothelial, structural and immune markers in placental specimens from *T. cruzi* vertical transmission cases. To that end, we nested a case control study including placental specimens from 3 study groups (mother + /baby +; mother +/ baby - ; and mother -/ baby -) to: (1) describe the epidemiologic differences in the transmission between cases and controls; (2) assess the weaknesses in the diagnosis of *T. cruzi* vertical infection, and (3)

standardize, measure, and identify a set of markers of the placental barrier using immunohistochemistry techniques. The specimens included in the nested case-control study were randomly selected from all the available placental samples obtained as part of the larger cohort study, which ran from October 2007 to October 2014.

The placental barrier is known to possess endothelial activity. This dissertation is aimed to assess the expression of angiogenic factors at the placental cells as well as the expression of endothelial function markers at the placental barrier. For this we performed immunohistochemistry analysis using a set of endothelial markers focusing angiogenic factors and endothelial tight junction proteins. Individual analysis of the images obtained from each marker were assessed by location at the placental villi (syncytiotrophoblast, cytotrophoblast and fetal blood vessels) to determine if endothelial expression of angiogenesis and tight junction protein markers is altered in cases of vertical *T. cruzi* transmission.

Finally, to better understand the immune response due to congenital *T. cruzi* infection and to assess structural integrity of the placental barrier when vertical *T. cruzi* transmission occurs, we assessed the immunohistochemical expression of (1) a set of markers related to immune pattern recognition and immune cells including macrophages and Natural Killer cells; and (2) a set of markers known to be part of the placental barrier structure as well as tight junction proteins associated with placental barrier integrity.

Chapter Four – Use Of Immunohistochemistry, Individual Analysis Of Images And Tissue Micro Arrays To Assess Placental Specimens From *T. cruzi* vertical Transmission Cases.

IV.1. Abstract

Background

Mechanisms involved in *T. cruzi* vertical transmission are not well identified. Few studies *in-vivo* and more recent *in-vitro* studies in placental tissues have described molecules that could be related to *T. cruzi* vertical transmission. An interruption in the placental barrier should be identified in order to better describe the mechanisms of transmission. Due to chronic placental inflammation, *T. cruzi* vertical transmission may be the result of a defective placental barrier, altered on its (1) structural integrity associated with impaired activity of structural tight junction proteins and weaker activity of structural proteins associated with syncytiotrophoblast continuity; (2) endothelial cell activity associated with endothelial barrier dysfunction due to decreased expression of angiogenesis factors which is related to leakier endothelial tight junctions; and (3) innate immune response, mediated by Pattern Recognition Receptors (PRRs) such as TLR-2 and TLR-4 at the placental cells which could be found to be increased producing increased inflammation associated with increased activity of macrophages, monocytes and Natural Killer cells at the placental villi.

Methods

Placental specimens from *T. cruzi* vertical transmission cases and controls were obtained from an ongoing Congenital Chagas Disease Study in order to perform Immune Histo Chemistry (IHC) and image analysis. The use of Tissue Micro Arrays (TMAs) for standardization of proven techniques of IHC was tested to obtain successful procedures to identify differences between the study groups. Individual analysis of images was also tested in order to describe such differences. Advantages and disadvantages of TMA use and the resulting methods were also discussed.

Results

No statistical differences were found among the main macroscopic characteristics of the placenta. Median gestational age was lower in cases of *T. cruzi* vertical transmission cases although they still are full term pregnancies. About 88% of cases included in this study would be correctly identified at birth if q-PCR were available and implemented in those settings. High intra- and inter-reader agreement was found when individual reading of images was performed. Scoring of specific locations in the tissue was also performed providing more information in the assessed tissues.

Conclusions

Use of TMAs when performing time-consuming techniques such as IHC was found to reduce time and costs when assessing high number of samples. Individual readings could provide focal information in heterogeneous tissues as placenta. Activity of structural, immunological and endothelial molecules in placental tissues from *T. cruzi* vertical transmission cases was shown at this study.

IV.2. Introduction

The vast majority of congenital *T. cruzi* infections is clinically asymptomatic. (Torrico et al. 2004, Bern et al. 2009, Kaplinski *et al.* 2015, Rendell et al. 2015) Symptomatic babies usually present nonspecific signs of illness such as low birth weight, hepato-splenomegaly, anemia and jaundice. (Punukollu et al. 2007; Schijman *et al.* 2006) Around 7 months of age, IgG antibodies from the mother are no longer detectable and if congenital infection occurred the newborn's antibodies are evident by conventional serology for IgG. Similar to adults infected by vectors, about 30% of congenitally infected patients develop complications in the chronic phase of the disease. (Carlier et al. 2011; Punukollu et al. 2007; Rassi et al. 2010; Schijman et al. 2006) Identifying and treating *T. cruzi* infection as early as possible in life produces better adherence and tolerance to treatment, as well as less severe adverse effects during treatment. (Carlier et al. 2011, Sosa-Estani *et al.* 2008)

To make the diagnosis of vertical *T. cruzi* transmission, an umbilical cord blood sample must be taken at delivery or the infant will need a peripheral blood draw after delivery. The micromethod is a buffy coat smear where parasites are directly observed by microscopy and is currently used to test infants for congenital Chagas disease in endemic countries. A previous study performed in Santa Cruz, Bolivia identified only four of ten infected babies on their first month of life, but none of them were identified in cord blood specimens. (Bern et al. 2009) In order to perform this method of concentration, at least three capillary tubes are needed and observation must be for no less than 5 minutes. (La Fuente et al. 1985, Umezawa et al. 1996 Ponce et al. 2005, Virreira et al. 2006, Roddy et al. 2008, Sosa- Estani et al. 2008, Ramirez et al. 2009)

The mechanisms by which some mothers transmit *T. cruzi* infection to their newborns and the risk factors for this infection are not well identified. Factors such as

very high parasitaemia in the mother after the second trimester of pregnancy and high parasitaemia at delivery have been described in the literature. (Bittencourt 1963, Carlier 2005, Redline 2006, Schijman 2006, Carlier et al. 2011, Mor and Cardenas 2010, Bern *et al.* 2009, Rendell *et al.* 2015, Kaplinski *et al.* 2015)

While there have been in-vitro studies of trophoblast molecules that could be associated with *T. cruzi* vertical transmission, few *in vivo* studies have conducted a comparison of placental tissues from humans. (Sartori et al. 2002, Priotto et al. 2009, Mezzano et al. 2005, and Sartori et al. 2003)

Few studies have evaluated the possibility of using placental tissue and umbilicus in diagnosis of congenital Chagas disease. (Bittencourt 1963, Moya 1979, Tafuri 1984, Fretes 1995) The placenta itself plays a containment role for parasites between the mother and the fetus, so that the presence of parasites in the placenta is not indicative of congenital infection, but is considered a risk factor. (Reyes et al. 1990; Torrico et al. 2004, Luquetti et al. 2005, Virreira et al. 2006, Sosa-Estani et al. 2008; Bern et al. 2009, Carlier et al. 2011) To consider a microorganism a threat to the fetus, the cell layer of syncytiotrophoblast must be interrupted or the pathogen must gain access to the maternal side of the placenta or to the placental villi. The risk of transmission is higher if rupture of the placental barrier is identified. (Bittencourt et al. 1963; Carlier et al. 2005; Redline et al. 2006; Schijman et al. 2006; Carlier et al. 2011; Mor and Cardenas, 2010).

During pregnancy, the placenta expresses angiogenic growth factors from the VEGF family. *In-situ* hybridization studies showed that different placental cell populations produce VEGF and PlGF and that high levels of VEGF and PlGF are found in maternal circulation and may be affecting the function of endothelial cells leading to increased vascular permeability and edema. (Clark *et al.* 1998) No previous placental studies explored if angiogenesis factors are altered in congenital parasitic infections. (Carlier *et al.* 2012) Acute *Toxoplasma* infection may produce hypoxic conditions inhibiting angiogenesis (Hunter *et al.*

2001) and *T. cruzi* was also found to have antiangiogenic activity in animal studies related to tumor growth, relating this finding to the capacity of *T. cruzi* calreticulin to inhibit capillary morphogenesis, proliferation and migration of endothelial cells. (Lopez *et al.* 2010)

Endothelial tight junction proteins such as occludin and caveolin (Dye *et al.* 2001, Gavard et al. 2008) and adherens junctions such as VE-Cadherin were previously described by their capacity to maintain the barrier permeability in close relationship to angiogenesis in malaria infection (Gillrie *et al.* 2007) and hypoxia. (Jiang et al. 2014)

The immune system plays a very peculiar role during pregnancy. the mother needs to maintain tolerance to the fetus and protect the fetus against pathogens. (Munn *et al.* 1998; Thellin and Heinen 2003; Mor and Cardenas 2010) Cytotrophoblast cells express TLR-2 in response to *T. cruzi* infection and TLR-2 activation leads to apoptosis of cytotrophoblasts during the first quarter of the pregnancy with the potential for barrier disruption that facilitates fetal infection. (Abrahams and Mor 2005; Savino et al. 2007; Kayama and Takeda 2009; Palm and Medzhitov, 2009) The cytotrophoblast is also able to activate TLR-4 mediated by glycoinositol phospholipids (GIPLs). (Abrahams and Mor 2005) Due to the high parasitaemia found previously in chronically infected pregnant women with Chagas disease in Bolivia (Bern *et al.* 2009, Rendell *et al.* 2015. Kaplinski *et al.* 2015), we hypothesize that transplacental transmission of *T. cruzi* infection occurs late in pregnancy (Carlier *et al.* 2012) as a product of constant exposure of circulating *T. cruzi* against the placental barrier.

Due to chronic placental inflammation, *T. cruzi* vertical transmission may be the result of a defective placental barrier du to (1) alteration of structural integrity associated with impaired activity of structural tight junction proteins and weaker activity of structural proteins associated with syncytiotrophoblast continuity; (2) endothelial cell

activity associated with endothelial barrier dysfunction due to decreased expression of angiogenesis factors which is related to leakier endothelial tight junctions; and (3) innate immune response, mediated by Pattern Recognition Receptors (PRRs) such as TLR-2 and TLR-4 at the placental cells which could induce inflammation associated with increased activity of macrophages, monocytes and Natural Killer cells at the placental villi.

Performing immunohistochemistry (IHC) in specimens that were randomly allocated in Tissue Micro Array (TMA) blocks, this study aims to assess the alterations associated with a leaky placental barrier in specimens from *T. cruzi* vertical transmission cases. We performed techniques aimed to standardize, measure and identify the expression of a set of markers that were previously described to be associated with structural integrity, endothelial activity and immunological response of the cells constituting the placental barrier against congenital infections.

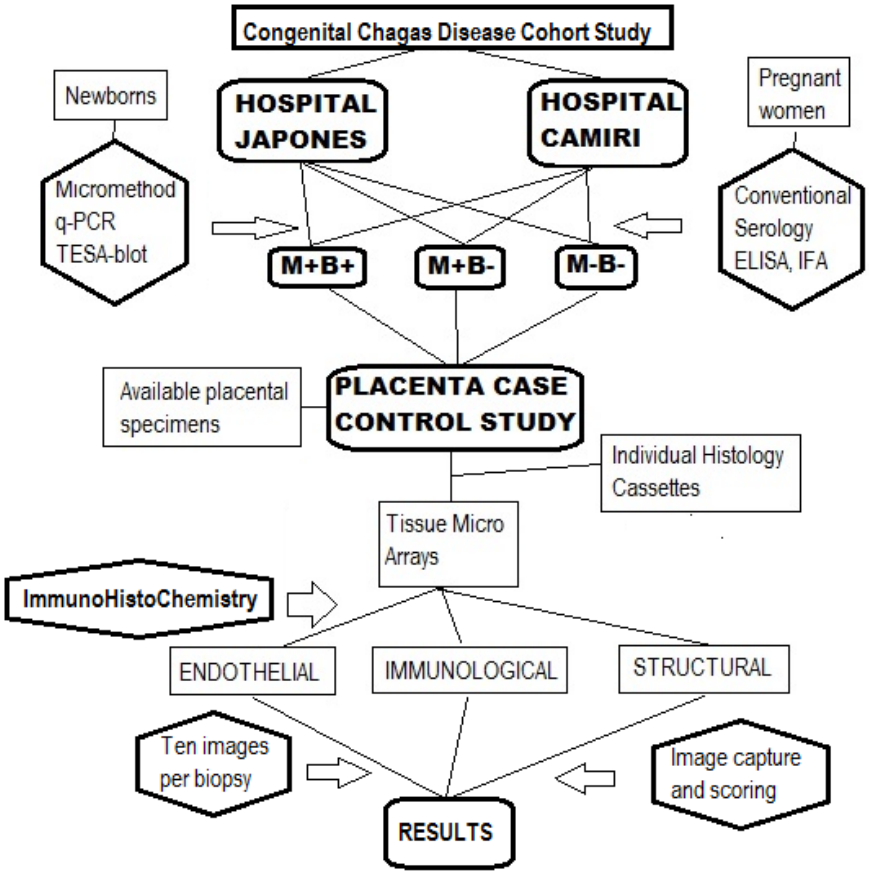
IV.3. Methods

We nested a retrospective case-control study with placental specimens from a congenital Chagas disease cohort study performed at Hospital Universitario Japones and Hospital Camiri in the department of Santa Cruz, Bolivia from 2007 to 2013. Our nested study was designed including available placental specimens from 3 different groups: (1) Chagas disease' seronegative mothers with uninfected newborns (M-B-), (2) Chagas disease' seropositive mothers with uninfected newborn (M+B-) and (3) Chagas disease' seropositive mothers with infected newborn (M+B+). (Figure 7)

Briefly, a source epidemiologic study was previously performed where Chagas disease screening was done in all pregnant mothers presenting for delivery at the study

sites and requested placental tissue from normal deliveries and C-sections. Screening at the time of enrollment was done using rapid tests and then confirmed by two serologic tests. In order to identify *T. cruzi* vertical transmission cases, all serologically positive mothers were invited to participate in a nested cohort study after delivery where diagnostic follow-up was performed in all returning children, performing tests at one, three, six and nine months of age. Micromethod was performed at all visits, serology for diagnosis at 9 months of age. Trypanosoma excreted-secreted antigens blot (TESA-blot) was carried out on available blood specimens. (Bern *et al.* 2009, Rendell *et al.* 2015, Kaplinsky *et al.* 2015)

Figure 7. Flowchart describing the source cohort study and placental specimen collection and processing



T. cruzi infection in pregnant women was confirmed performing 2 conventional serologic tests: Chagatest Recombinante ELISA (Wiener Laboratories, Rosario, Argentina), and Indirect Hemo-agglutination assay (IHA). Congenital infection of the newborn was identified by one positive direct parasitic test (micromethod) (La Fuente *et al.* 1985) before 6 months of age or later in life if ELISA or IHA were found positive until 9 months of age disregarding the serological diagnosis of the mother. Real time Polymerase Chain Reaction (q-PCR) technique in cord blood and umbilicus tissue, TESA-blot (Umezawa *et al.* 2001) and IFA were other methods performed for diagnosis at the cohort study and were previously published by Bern *et al*. , Rendell *et al*. and Kaplinski *et al*. (Bern *et al.* 2009, Rendell *et al.* 2015, Kaplinski *et al.* 2015) Infected babies identified during the study were reported to the National Control Program in order to be treated according to Bolivian National Guidelines for Chagas Disease Control.

IV.3.1. Experimental diagnostic methods:

A. <u>Molecular methods (qPCR)</u>: Standard phenol-chloroform DNA extractions were performed. (Bern *et al.* 2009) The primer set Cruzi 1 (5'–ASTCGGCTGATCGTTTTCGA–3') and Cruzi 2 (5'–AATTCCTCCAAGCAGCGGATA–3') was used to amplify a 166 base-pair DNA fragment. The probe Cruzi 3 (5'–CACACACTGGACACCAA–3') was labeled with 5′FAM (6—carboxyfluorescein) and 3′MGB (minor groove binder). TaqMan Human RNase-P detection reagent (Applied Biosystems) was included as an internal control; results were considered valid only if the internal control was efficiently amplified. A non-template negative control was included in each run. PCR standard curves were generated

by inoculating a blood clot specimen with 1 x 10^6 *T. cruzi* Y strain trypomastigotes, followed by extraction and serial dilutions. The detection limit was determined to be 1 parasite/ml. A positive result was defined by a cycle threshold (Ct) value below the Ct value of the detection limit standard, which fell consistently between 37 and 38 cycles. The individual specimen parasite loads were calculated based on the standard curve included in batch run. (Bern *et al.* 2009, Rendell *et al.* 2015)

B. IgM <u>Trypomastigote excreted-secreted antigens (TESA)-blot</u>: Western blots were performed and consecutive bands at 130–200 kDa on IgM TESA-blot demonstrate antibodies to Shed Acute Phase Antigens (SAPA), indicating acute or congenital infection. (Rendell et al. 2015, Umezawa et al. 1996) Nonspecific bands are usually found below 95kDa. TESA antigens are obtained following the method of Umezawa. (Umezawa *et al.* 1996) Briefly, the supernatants of infected LLC-MK2 cells are harvested four days after infection with 5x10^6 trypomastigotes of *T. cruzi* Y strain. After four days' growth, the medium is changed. After three washes with serum-free RPMI, the cells are incubated for 24 hours in serum-free media. The medium is centrifuged (2800 X g 10 minutes at four degrees centigrade), filtered through a cellulose-acetate membrane (0.2 μm) and then stored at -70^{o}C. Protein levels are determined by the Bradford method. The Western blot is performed as described by Umezawa (Umezawa *et al.* 1996): the supernatant (150 μl) from infected cultures is diluted (vol/vol) in SDS – sample buffer (6 mM Tris HCL, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and boiled for 5 minutes at 100 ^{o}C. Samples are loaded on a 7% polyacrylamide minigel. Then they are transferred and blocked with skim milk. Blots are incubated with sera diluted 1/200 in

Tris buffered saline with 1% skimmed milk and bands are detected using antihuman anti IgM conjugated to peroxidase followed by hydrogen peroxide and OPD. (Umezawa *et al.* 1996)

IV.3.2. Specimen collection and storage:

All the specimens included in this study were collected from all deliveries, vaginal or cesarean section (C-section), of pregnant women presenting to Hospital Universitario Japones and Hospital Municipal Camiri in Santa Cruz de la Sierra, Bolivia, between October 2007-October 2014 These pregnant women were part of an ongoing study related to congenital Chagas disease and were invited to participate in a cohort study for diagnosis follow-up of their babies until they were 9 months old.

Demographic, perinatal and neonatal characteristics were obtained from the information gathered in the source cohort study. Briefly, trained nurses at both sites interviewed pregnant mothers for characteristics of the pregnancy and recorded this information and delivery data in study forms. All possible placental specimens were collected regardless of their infection status. Information related to measurements and macroscopic characteristics was recorded in study forms for further analysis. (Bern *et al.* 2009, Kaplisnki *et al.* 2015, and Rendell *et al.* 2015)

Cord blood samples were collected right after the umbilical cord was clamped and cut following the delivery. Samples were obtained by un-clamping the umbilical cord at the placental end until 10 mL were obtained and before the placental detachment occurred.

Umbilical tissues specimens were collected from the remaining umbilical cord left at the baby's end and after the assessment performed by the neonatologist. A one-centimeter length of umbilical cord tissue was collected and processesed for analysis.

All specimens collected were maintained in a biospecimen repository at the Universidad Catolica Boliviana San Pablo in Bolivia and were later transported to the Research laboratory at Universidad Peruana Cayetano Heredia in Lima-Peru in order to perform all the immunohistochemistry experiments.

IV.3.3. Placental specimen processing:

Placental tissue was collected right after delivery or C-section and rinsed with Phosphate-buffered saline (PBS) at pH=7.2 (neutral). One centimeter diameter full thickness biopsies were obtained from a random location of the organ, through fully rinsed with sterile neutral PBS and then fixed in 10% formalin buffered in neutral PBS for 24 hours to be later saved in neutral PBS until embedded in paraffin blocks for standard histological procedures. (Burton *et al.* 2015, Kim *et al.* 2015, Benirschke 1998) Once infection status of the participants were determine in the source cohort study, available placental specimen cassettes of 24 cases of transmission (M+B+) were identified and 25 specimen cassettes from M-B- participants and 28 specimen cassettes from M+B- participants were selected from all available placental specimens from both groups using random numbers. Five-millimeter diameter biopsies from each specimen cassette were randomly allocated in a total of 7 Tissue Micro Array (TMA) blocks containing 11 biopsies each.

Random allocation of each biopsy in the TMA block was performed using random numbers and each block included proportional number of biopsies from each group.

(Figure 8) Duplicated TMA blocks were created including the same specimens, but with different random allocation in the block. Histological sections of 4 µm were cut and placed in poly-l-lysine pre-coated slides for staining.

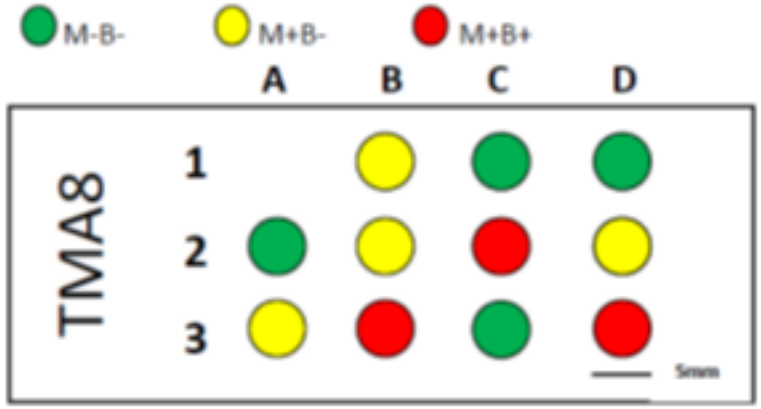


Figure 8. Slide schematic for Tissue Micro Arrays (TMAs).:TMAs were generated for this study obtaining each specimen from a paraffin embedded tissue block using a 5mm biopsy needle. Readings were performed using coordinates. Slot A1 is empty to ensure direction and orientation of the slide.

Maps of each TMA describing the position of each specimen at the slide and containing the origin code from each placental specimen were created and saved in order to ensure masked processing of the samples. (Hamilton *et al.* 2014, Skacel *et al.* 2002) Histological slides were obtained from the TMAs and were rehydrated using standard histological procedures. (Burton *et al.* 2015, Kim *et al.* 2015, Benirschke 1998)

IV.3.4.IHC standardization:

In order to obtain satisfactory staining results from the IHC procedures (Marsch *et al.* 2015, Warford *et al.* 2014, Leong *et al.* 2010), several methods were tested and compared against to the results obtained for publication on the Human Protein Atlas (http://www.proteinatlas.org). (Navani 2016)

Table 1. Primary antibodies used in the immunohistochemistry at the study.

Marker	Antibody	Source
Angiogenesis Markers		
VEGF-C	Anti-VEGFC Antibody IHC-plus™ (LS-B11404)	LSBio
PlGF	Anti-PLGF antibody (ab9542)	Abcam
Endothelial Markers		
CD31	Anti-CD31 antibody (ab76533)	Abcam
vWF	Anti-Von Willebrand Factor antibody (AB7356)	Millipore
Endothelial Tight junction proteins Markers		
VE-Cadherin	Anti-VE Cadherin antibody (ab33168)	Abcam
Occludin	Anti-Occludin antibody (ab64482)	Abcam
CLAU-1	Anti-Claudin 1 antibody (ab15098)	Abcam
Cav-1	Anti-Caveolin-1 antibody (ab2910)	Abcam
Placental Barrier Structural Markers		
SMA-Alpha	Anti-alpha smooth muscle Actin antibody (ab124964)	Abcam
PLAP	Anti-Placental alkaline phosphatase (PLAP) antibody (ab118856)	Abcam
FPA	Anti-Fibrinopeptide A antibody (ab92572)	Abcam
hCG-Beta	Anti-hCG beta antibody (ab53087)	Abcam
Cyt-7	Anti-Cytokeratin 7 antibody (ab68459)	Abcam
Placental Barrier Structural Tight junction proteins markers		
Zonula Occludens 1	Anti-ZO1 tight junction protein antibody (ab187012)	Abcam
CLAU-5	Anti-Claudin 5 antibody (ab15106)	Abcam
Immune response markers		
TLR-2	Human TLR-2 Affinity Purified (af2616)	R&D Systems
TLR-4	Human TLR-4 Affinity Purified (af1478)	R&D Systems
Immune cells markers		
CD163	Anti-CD163 antibody (ab87099)	Abcam
CD14	Anti-CD14 antibody [EPR3653]	Novus Biologicals
CD8	Anti-CD8 antibody (ab4055)	Abcam
CD4	Anti-CD4 antibody [EPR6855] (ab133616)	Novus Biologicals
CD3	Anti-CD3 antibody (ab16044)	Abcam
CD 56	Anti-NCAM antibody [EP2567Y] (ab75813)	Novus Biologicals

Once staining results from the standardization were similar to those published in the atlas, TMA slides were stained following the successful procedure. Briefly, heat-induced antigen retrieval (HIAR) methods including boiling and microwave heat incubation were performed. Several antigen retrieval incubation times and solutions were also tested, including acid and alkaline solutions and detergents (TWEEN® 20 BioXtra). Enzymatic antigen retrieval including trypsin, pepsin and proteinase K were also performed. (Yamashita *et al.* 2014, Leong *et al.* 2010) Endogenous peroxidase activity was blocked in all the procedures testing several incubation times. A standard 60-minute incubation at room temperature was performed for protein blocking solutions including sera from different species, 2% non-fat milk, and 2% bovine serum albumin (BSA). Three primary antibody concentrations (including the manufacturer's recommendation) were tested in order to identify the most appropriate level for each antibody (See list of primary antibodies in Table 1). Prediluted (Ready-to-use) secondary antibodies (Biotin-labeled Goat IgG – (KPL-71-00-37) and Biotin-labeled Rabbit IgG (KPL-71-00-37) antibodies were used and selected to avoid cross-reaction to primary antibody source species. Horseradish peroxidase (HRP)-streptavidin (Abcam-ab7403) was used in order to amplify the reaction and Diamin Benzidine (DAB) Substrate Kit (Abcam, ab64238) was used to perform the staining reaction.

IV.3.5. Individual Image analysis

Ten images of each biopsy allocated on the TMA-slides were obtained using Carl Zeiss Microscope and a 5MP camera attached to the microscope. All images were captured using ZEN® software (ZEISS Microscope software) and no enhancement or alteration of the images was performed. A code identifying the position of the biopsy at

the TMA slide was assigned accordingly to the TMA map. Two independent readers blinded for the infection status/study group allocation assessed ten field images from aleatory non-contiguous regions of each biopsy. When assessing IHC, a score for immune-staining was used following the methods provided by Katz et al. (Katz *et al.* 2009) Briefly, the staining intensity was rated according to the following scale: no visible staining = 0, faint staining = 1+, moderate staining = 2+ and strong staining = 3+. This method has been used and verified by others. (Luo *et al*., 2002; Patel *et al*., 2008) Images were rated according to the intensity of staining using the same score scale at 3 tissue locations: the syncytiotrophoblast, the cytotrophoblast, and fetal blood vessels. Fields containing placental villi were included in analysis and those containing decidual tissue were excluded.

Aleatory readings of the duplicate slides were performed by the two examiners to assess consistency of the scoring performed to each image.

IV.3.6. Data Analysis:

Variables with normal distributions were analyzed based on medians and standard deviations, and the variables that were non-normally distributed were analyzed based on medians and quartiles. All of the placental parameters evaluated were transformed to a logarithmic (Ln) scale before statistical analysis. One-way ANOVA tests with Bonferroni correction were used to determine the differences between groups. Kruskal-Wallis test was an alternative test to ANOVA if data was not normally distributed. Student's t-tests or Mann-Whitney U-tests were used when appropriate.

In order to evaluate the agreement of histological readings between readers, we performed an un-weighted kappa analysis as a measurement of interobserver reproducibility. Summed interobserver reproducibility of the study, and kappa values to each diagnostic category were calculated in the case and control material. Similarly, we performed a kappa analysis in order to evaluate the percent agreement of three readings of the same slide for each reader.

IV.3.7. Ethics

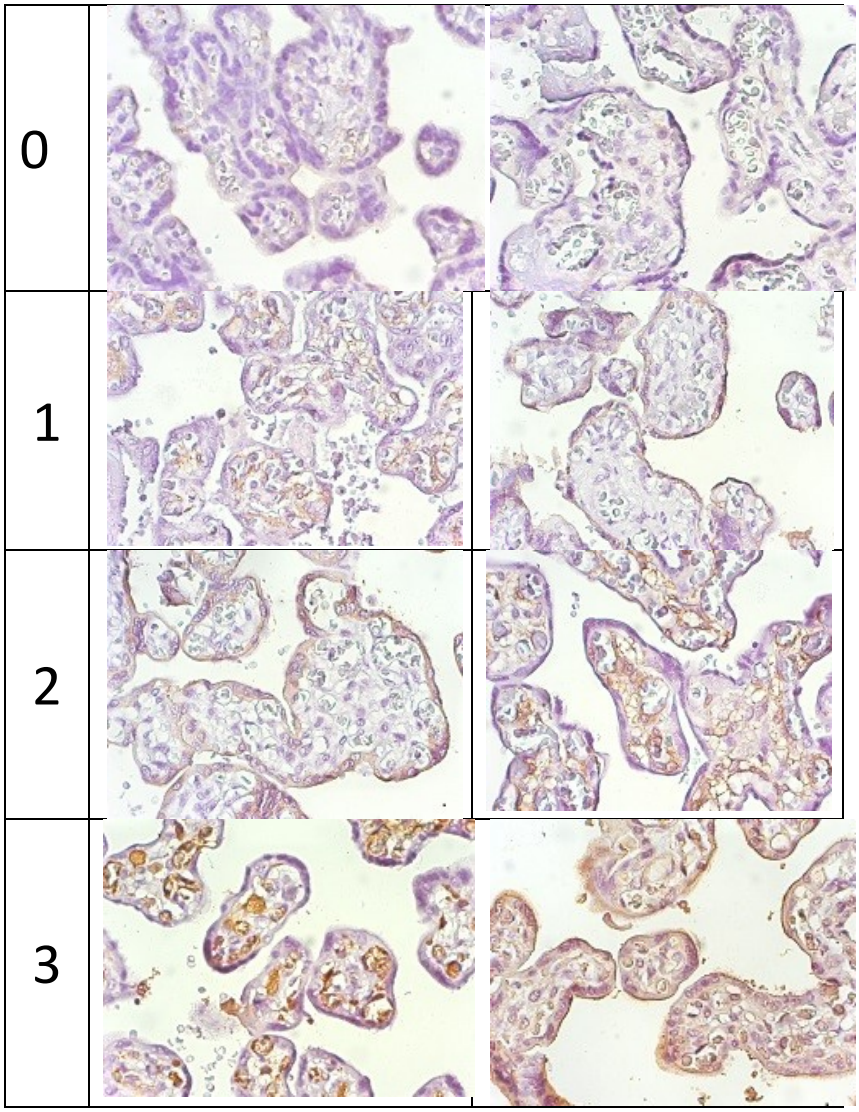
All specimens and data were collected according to the approved Cohort Study protocol and informed consents were obtained to participate in the study. The IRBs from Johns Hopkins School of Public Health, CDC, PRISMA ONG and Hospital Universitario Japones approved the study. Although placenta is a discarded tissue at the end of the delivery, we requested a signed consent included in the cohort informed consent from the participants to collect and use placental and cord specimens for research.

IV.4. Results

Images were obtained and rated as described before. Examples of images and scoring are shown in Figure 9.

Placental specimens from the three study groups (M-B-, M+B-, and M+B+) were found to be similar regarding type of delivery, delivery incidents and macroscopic characteristics of the placental organ as shown in Table 2. Data regarding syphilis infection status of some patients was also obtained and showed no statistical difference between the study groups.

Figure 9. Examples of placental villi fields and scoring according to intensity



Characteristics of the newborns and mothers are summarized in Table 3. Although there is no statistical similarity on the median gestational age, all the deliveries occurred on gestational age more than 36 weeks, which is considered an adequate gestational age for a term delivery. Similarly, median maternal age was found to be significantly lower when comparing M-B- group to the groups where Chagas disease was diagnosed.

Table 2. Characteristics of delivery and placental specimens at the two sites of the study.

SITE		Hospital Japones n (%)/median (IQR)			Hospital Camiri n (%)/median (IQR)			Combined Sites n (%)/median (IQR)			Overall p for group comp
Study group		**M-B- (7)**	**M+B- (14)**	**M+B+ (13)**	**M-B- (18)**	**M+B- (14)**	**M+B+ (11)**	**M-B- (25)**	**M+B- (28)**	**M+B+ (24)**	
Delivery	**C-section**	4 (57%)	8 (57%)	7 (54%)	7 (39%)	6 (43%)	6 (55%)	11 (44%)	14 (50%)	13 (52%)	0.84
	Vaginal	3 (43%)	6 (43%)	6 (46%)	11 (61%)	8 (57%)	5 (45%)	14 (56%)	14 (50%)	11 (48%)	
Premature Membrane Rupture[A]		0	0	1 (8%)	2 (11%)	0	0	2 (8%)	0	1 (4%)	0.32
Placental weight (grams) [B]		550 (400-600)	550 (450-600)	520 (450-750)	720 (620-800)	662 (620-800)	700 (520-850)	650 (600-800)	620 (550-700)	650 (500-800)	0.39
Placental diameter (centimeters) [C]		19 (16-20)	18 (17.5-18.5)	18 (17-20)	19 (17-20)	18 (18-20)	19 (17-22)	19 (17-20)	18 (18-20)	19 (17-21)	0.38
Placental thickness (centimeters) [D]		1.6 (1.0-2.0)	2.0 (1.5-2.0)	1.5 (1.0-.3.0)	1.5 (1.0-2.0)	1.25 (1-1.5)	1.7 (1.2-2.5)	1.5 (1.0-2.0)	1.5 (1.0-2.0)	1.7 (1.1-2.3)	0.77
Median Umbilicus length (centimeters) [E]		50 (35-53)	47 (35-53)	48 (30-57)	48 (37-54)	50 (47-56)	48 (47-58)	49 (37-54)	48 (42-53)	48 (37-57	0.98
Positive prenatal VDRL serology [F]		0	1 (7%)	0	0	0	1 (9%)	0	1 (4%)	1 (4%)	0.43

A Premature Membrane Rupture refers to a patient who is beyond 37 weeks of gestation and has presented with rupture of membranes (ROM) prior to the onset of labor.
B Placental weight at term (after 36wks) is considered normal in the range from 315g to 540g (excluding the weight of the umbilicus). In the study the umbilicus was also weighted.
C Placental diameter in a maximum of 22cm is considered normal.
D Placental thickness in a maximum of 2.5cm
E Umbilicus length at term (after 36wks) is considered normal in the range of 47cm to 84cm.
F VDRL test is a screening test for syphilis and is done routinely at the pre-natal control visits. The majority of women presenting to the Hospital at the study did not have prenatal care and only went to the hospital for delivery.

Table 3. Newborn characteristics at delivery and maternal obstetrical data

SITE		Hospital Japones n (%)/median (IQR)			Hospital Camiri n (%)/median (IQR)			Combined Sites n (%)/median (IQR)			Overall p for group comp
Study group		**M-B- (7)**	**M+B- (14)**	**M+B+ (13)**	**M-B- (18)**	**M+B- (14)**	**M+B+ (11)**	**M-B- (25)**	**M+B- (28)**	**M+B+ (24)**	
Newborn Gender	**Male**	2 (29%)	6 (43%)	7 (54%)	8 (44%)	8 (57%)	6 (55%)	10 (40%)	14 (50%)	13 (54%)	0.56
	Female	5 (71%)	8 (57%)	6 (46%)	10 (56%)	6 (43%)	5 (45%)	15 (60%)	14 (50%)	11 (46%)	
[A] **Newborn weight (grams)**		3050 (2850-3250)	3100 (3100-3300)	3000 (2400-3650)	3500 (3300-3600)	3400 (3200-4000)	3200 (3000-3500)	3350 (3050-3550)	3250 (3100-3630)	3200 (2400-3650)	0.46
[B] **Newborn height (centimeters)**		49 (47-50)	50 (48-50)	49 (47-52)	50 (50-52)	53 (52-54)	52 (50-52)	50 (49-52)	50 (50-52)	50 (47-52)	0.79
[C] **Head Circumference (centimeters)**		33 (32-34)	33 (32-34)	32 (31-34)	34 (34-35)	34 (32-35)	35 (34-36)	34 (33-35)	34 (32-35)	33 (31-35)	0.43
[E] **1 minute APGAR score <=7**		1 (14%)	2 (14%)	1 (8%)	1 (0.6%)	2 (14%)	2 (18%)	2 (8%)	4 (14%)	3 (13%)	0.73
[F] **5 minute APGAR score <=9**		0	0	0	1 (0.6%)	2 (14%)	2 (18%)	2 (8%)	2 (7%)	1 (4%)	0.81
[D] **Gestational Age (weeks)**		39.5 (39.0-40.0)	40.0 (36.0-40.0)	37.5 (36.0-39.0)	39.5 (39.0-40.0)	39.0 (39.0-39.0)	39.0 (39.0-40.0)	39.5 (39-40)	39.0 (39.0-39.0)	39.0 (36.0-39.5)	0.04*
Maternal Age (years)		18 (21-27)	24 (18-28)	27 (22-33)	23 (20-26)	19 (18-24)	25 (23-34)	20 (18-24)	24 (21-30)	24 (21-30)	0.04*
Number of pregnancies		1 (0-2)	3 (2-4)	1 (0-3)	1 (1-2)	2 (2-4)	4 (2-5)	1 (1-2)	3 (2-4)	2 (1-5)	0.06

A Normal weight range from 2500g to 4500g in term neonates
B Normal height range from 45.7cm to 60cm in term neonates
C Normal head circumference range in male from 31 cm to 38cm and females from 30cm to 37cm
D Term pregnancies are considered after 36wk.
E Apgar score at 1 minute higher than 7 points out of 10 is considered normal.
F Apgar score at 5 minutes below 9 is considered abnormal.

Medians, inter quartile ranges (IQR)and p values * were calculated by one-way ANOVA analysis, adjusted by Bonferroni correction.

Median birth weight and height and median maternal age were found to be higher on the M+B+ group in the overall comparison. Median newborn height and weight were found to be statistically different in the M+B- group between hospitals.

The majority of *T. cruzi* vertical transmission cases in this study was diagnosed in cord blood by q-PCR at the cohort source study and was published earlier. (Bern *et al.* 2009, Rendell *et al.* 2015, Kaplinski *et al.* 2015) One out of 24 (4.2%) specimens from cases of congenital *T. cruzi* transmission included in this study was diagnosed by micromethod at birth, the current method for diagnosis in Bolivia. Nine out of a total of 25 cases (36%) were identified by this method over a 9-month follow-up period. (Table 4)

Table 4. Conventional and study diagnostic tests for *T. cruzi* vertical transmission cases included in the study [%]:

Diagnostic test	**Hospital Univ. Japones (14)**	**Hospital Camiri (11)**
Conventional Diagnosis *(positive test/available specimens)*		
Positive cord blood micromethod	1/14	0/9
Positive micromethod 1 month	3/10	2/5
Positive micromethod 3 months	1/8	1/4
Positive micromethod 6 months	1/8	
Positive serology at 6 months	2/2	2/2
Positive serology at 9 months	5/5	6/6
Study tests for diagnosis *(positive test/available specimens)*		
Positive cord blood q-PCR	9/14	8/11
Positive TESA-blot in cord blood	7/13	3/9
Positive umbilicus qPCR	11/13	6/10
Treated by the NCDCP	12/14	6/11
Lost to follow-up at the cohort study	2	5

% From a total of 77 specimens included in the 3 groups of the study, 35 specimens were found to be available from the specimens collected at Hospital Universitario Japones and a 42 were found to be available from the specimens collected at Hospital Camiri.

The agreement of the scoring between the readers was measured by un-weighted Kappa coefficient for inter reader percent agreement and was found to be 89%. Agreement within the same reader was assessed by the intra reader percent agreement and was found to be in 92%.

Successful methods for staining resulting from the combination of each step on the procedures are summarized in supplemental tables 1, 2 and 3 below.

IV.5. Discussion

Our study described successful techniques to identify structural proteins related to placental barrier integrity, including endothelial markers involved in possible endothelial dysfunction in placental conditions related to neonatal pathologies and immune related markers described earlier to be altered when infections occur. The use of TMA avoids selection bias when processing high number of specimens. When processing TMAs, cases and controls are allocated in the same slide ensuring same processing and blinding for the person performing the experiment. One of the main problems when processing tissues for histology is the consistency of the results since the tissues inadvertely may be processed in different conditions. Minimum changes in the concentration of reagents, temperature, incubation times and staining may produce different results in the same tissues. TMA reduces the person-time needed for processing, ensuring same treatment of the specimens by the same person at the same time.

A series of publications including *T. cruzi* vertical transmission cases performed until the 90's by Bittencourt in Brazil (Bittencourt *et al.* 1967, Bittencourt *et al.* 1972, Bittencourt *et al.* 1974, Bittencourt *et al.* 1978, Bittencourt *et al.* 1984, Bittencourt *et al.* 1992) reported abortions, premature births, retarded

growth and deformations in *T. cruzi*-infected neonates. These studies were also able to show *T. cruzi* parasites at the syncytiotrophoblast level in nests from placental specimens obtained from highly infected stillborn or abortions. All these findings however were no longer observed in later reports from Bolivia, (Salas *et al.* 2007, Bern *et al.* 2009, Kaplinski *et al.* 2015, Rendell *et al.* 2015) Argentina, (Freilij *et al.* 1995, Altcheh *et al.* 2005, Moya *et al.* 2005) or Chile (Apt *et al.* 2013) and were not evident in our study. More recent reports (Carlier and Truyens 2010, Carlier *et al.* 2005) and our study were not able to show *T. cruzi* parasites at the syncytiotrophoblast layer, relating this finding to the chronic state of the mother's infection at the moment of the delivery. (Carlier *et al.* 2012) Moreover, clinical manifestations of congenital Chagas disease were reported in Bittencourt's series and included meningoencephalitis and ocular involvement, comparing these findings to congenital *Toxoplasma* infection (Bittencourt *et al.* 1992). Clinical compromise in congenital toxoplasmosis is found in 10%-15% of diagnosed cases and in few congenital malaria cases suggesting transplacental infection late in pregnancy. (Carlier *et al.* 2012) Those symptomatic cases for both, *Toxoplasma* and malaria infections were smaller and developed fever, hepatosplenomegaly, jaundice, and prematurity (gestational age < 37 weeks). Severe cases of congenital malaria showed hemolytic anemia and thrombocytopenia. (Menendez *et al.* 2007) Seizures, brain calcifications and meningoencephalitis were mainly observed in severe congenital toxoplasmosis leading to psychomotor delay. (Remington *et al.* 2006) Some of these findings were observed only in severe congenital Chagas diseases in the past in Brazil by Bittencourt (Bittencourt *et al.* 1967, Bittencourt *et al.* 1972, Bittencourt *et al.* 1974, Bittencourt *et al.* 1978, Bittencourt *et al.* 1984, Bittencourt *et al.* 1992) and in few cases in Bolivia by Torrico in 2004. (Torrico *et al.* 2004, Carlier *et al.* 2012) Clinical findings from congenital Chagas disease cases included in our study (data not shown) and recent

reports from Bolivia (Bern *et al.* 2009, Rendell *et al.* 2015, and Kaplinski *et al.* 2015) described mainly asymptomatic presentations of the disease and no severe clinical findings have been reported recently. Reports from Argentina (Freilij *et al.* 1995, Altcheh *et al.* 2005, Moya *et al.* 2005) and Chile (Apt *et al.* 2013) described low frequency of hepatomegaly as the most severe clinical finding when assessing congenital cases. Although there are no recent reports for severe symptomatic presentation of congenital Chagas disease in Brazil as described until the 1990's, regional variation of *T. cruzi* strain involved in congenital Chagas disease would be an important factor to be studied.

A study of 22,689 pregnancies (Haines *et al.* 1991) showed that infants delivered from younger women tended to be lower weight and those from older women tended to have C-sections meanwhile Apgar scores were not affected by maternal age. Our results also do not show differences in Apgar scores at 1 and 5 minutes, birth weight and length and head circumference when comparing congenital cases to controls. As observed by Torrico in 2004 in Cochabamba (Torrico *et al.* 2004), our study found lower gestational age in congenital cases, although the gestational ages found in both studies remain in the *ad terminum* stage of pregnancy. However, a study in the hyperendemic area of Yacuiba in 2007 in Bolivia (42.2% prevalence in pregnant women) did not find statistical differences regarding newborn's head circumference, weight, height and gestational age when comparing congenitally infected newborns to controls. Moreover, they showed no increased risk for transmission in male compared to female newborns, maternal age older than 30 years old, multigesticity (more than 3 previous pregnancies) and C-section mode of delivery. (Salas *et al.* 2007) No differences in other measurements related to fetal growth

retardation or preterm delivery showed in our study may be evidence that chronic *T. cruzi* infection in pregnant women does not affect the normal growth of the fetus.

T. cruzi vertical transmission cases in our study do not show increased presentation of premature rupture of membranes and preterm delivery compared to controls. These pathological findings are associated with neonatal sepsis and intrauterine growth retardation commonly seen in the TORCH group (toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus), which are considered transplacental infections classically presenting as chronic villitis which includes alterations in the placental fetal vascular side. Previous studies in congenital toxoplasmosis, malaria and Chagas disease reported signs and symptoms associated with low birth weight (<2500 g), prematurity (gestational age < 37 weeks). (Carlier and Torrico, 2003; Freeman et al., 2005; Menendez and Mayor, 2007; Remington et al., 2006; Torrico et al., 2004) Moreover, premature rupture of membranes was described in congenital Chagas disease premature newborns and pneumonitis. (Torrico et al., 2004) A study including symptomatic CMV fetal infections in 18 cases showed that maternal infections were acquired before the 8th week of pregnancy in all cases. (Zavattoni *et al.* 2016) Our results showing mainly no differences in the placental morphology, delivery and neonatal characteristics may suggest the hypothesis that *T. cruzi* vertical transmission could be occurring later in pregnancy after constant exposure of the placental barrier to circulating parasites due to high parasitaemia in pregnant mothers but allowing a normal intrauterine fetal growth. Recent *in-vitro T. cruzi* vertical transmission studies including virulent *T. cruzi* strains and cultured trophoblast (Duaso *et al.* 2010, Duaso *et al.* 2011, Diaz-Lujan *et al.* 2016, and Liempi *et al.* 2016) described that *T. cruzi* vertical transmission could be associated with a placenta showing impaired trophoblast barrier activity.

Conventional diagnosis of *T. cruzi* infection at birth is still done by the micromethod in Chagas Disease' endemic countries (Carlier *et al.* 2011) and q-PCR is considered difficult to implement in rural and poor resources settings. (Salas *et al.* 2007, Rendell *et al.* 2015, Bern *et al.* 2009) Only 36% of the 25 congenital cases in our study were identified by micromethod over the 9 month follow-up time done by the cohort study, a diagnostic algorithm that is not performed when diagnosing congenital Chagas disease in the routine national program. Moreover, not all the cases included in our study were identified by this technique at multiple timepoints and some of the cases were diagnosed by serology and q-PCR instead. About 88% of cases included in this study would be correctly identified at birth if q-PCR would be available and implemented in those settings. Although positive q-PCR results in placental biopsies do not identify congenital cases, a high proportion of the congenital cases included in our study were found to be positive by this technique and were previously reported. (Bern *et al.* 2009) A previous study in Bolivia showed similar results regarding the reliability, robustness and simplicity of the PCR technique, identifying 18 of 18 cases of vertical *T. cruzi* transmission by PCR in newborn samples and obtaining similar results in their control samples. (Virreira *et al.* 2003) More efforts should be devoted to set up PCR techniques in the field and to develop field-friendly algorithms for diagnosis including molecular techniques in order to identify *T. cruzi* vertical transmission cases as early in life as possible.

Supplemental Table 1. Immunohistochemistry techniques found successful for structural marker's staining in placental specimens at the study

Protein	Antigen Retrieval Solution	Antigen Retrieval time (minutes)	Antigen retrieval method	3% Hydrogen Peroxide blocking solution	Hydrogen Peroxide incubation time (minutes)	Non specific binding Blocking solution	Primary antibody dilution	Blocking solution for Primary antobody	Primary antibody Incubation time	Blocking solution for Secondary antibody	Secondary antibody Incubation time	Horse Radish Peroxidase Dilution
Occludin	EDTA tween ph 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
PLAP	citrato tween ph 6	30 minutes	Heat induced Copling jar boiling	PBS	30	PBSt, Normal Goat Serum 10%, BSA 2.5%, non-fat milk 1%, Normal Horse Serum 10%	1 in 250	PBSt, Normal Goat Serum 10%, BSA 2.5%, Normal Horse Serum 10%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Horse Serum 10%, Normal Human Serum 10%	60 minutes	1 in 7500
Claudin 1	EDTA twen ph 9.5	20 minutes	Heat induced Microwave 80%	PBS	30	PBSt, Normal Goat Serum 10%, BSA 2%	1 in 25	PBSt, Normal Goat Serum 10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
Claudin 5	EDTA tween ph 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	120 minutes	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000
a- SMA	prot k 1/1000 Tris-EDTA buffer	15 minutes	enzyme method	methanol	15 RT	PBSt, 2% BSA, 10% Normal Goat Serum	1 in 200	PBSt, 2% BSA, 10% Normal Goat Serum	overnight	Conjugated anti Rabbit Secondary antibody, 10% Normal Human Serum	60 minutes	1 in 3000
Cytokeratin 7	EDTA tween ph 9,6	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, 2% BSA, 10% Normal Goat Serum	1 in 200	PBS, 2% BSA, 10% Normal Goat Serum	overnight	Conjugated anti Rabbit Secondary antibody, 10% Normal Human Serum	60 minutes	1 in 3000
Caspase - 3	prot k 1/1000 Tris-EDTA buffer	15 minutes	enzyme method	methanol	15 RT	PBSt, 2% BSA, 10% Normal Goat Serum	1 in 200	PBSt, 2% BSA, 10% Normal Goat Serum	overnight	Conjugated anti Rabbit Secondary antibody, 10% Normal Human Serum	60 minutes	1 in 3000
hCG-Beta	citrato tween ph 6 beaker	15 minutes	Heat induced beaker boiling	50% methanol 50% PBS	15	PBS, Normal Horse Serum 5%	1 in 200	PBS, Normal Horse Serum 1%	overnight	Conjugated anti Rabbit Secondary antibody	60 minutes	1 in 2000
Zonula Occludens 1	EDTA tween ph 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	120 minutes	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000

Supplemental Table 2. Immunohistochemistry techniques found successful for immune marker's staining in placental specimens at the study

Protein	Antigen Retrieval Solution	Antigen Retrieval time (minutes)	Antigen retrieval method	3% Hydrogen Peroxide blocking solution	Hydrogen Peroxide incubation time (minutes)	Non specific binding Blocking solution	Primary antibody dilution	Blocking solution for Primary antobody	Primary antibody Incubation time	Blocking solution for Secondary antibody	Secondary antibody Incubation time	Horse Radish Peroxidase Dilution
CD31	EDTA tween pH 9,6	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, 2% BSA, 10% Normal Goat Serum	1 in 200	PBS, 2% BSA, 10% Normal Goat Serum	overnight	Conjugated anti Rabbit Secondary antibody, 10% Normal Human Serum	60 minutes	1 in 3000
VEGF	EDTA tween pH 9,2	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, 2% BSA, 10% Normal Goat Serum	1 in 150	PBS, 2% BSA, 10% Normal Goat Serum	overnight	Conjugated anti Rabbit Secondary antibody, 10% Normal Human Serum	60 minutes	1 in 2000
von Willebrandt Factor	Sodium Citrate tween pH 6	30 minutes	Heat induced Copling jar boiling	PBS	30	PBS triton, Normal Goat Serum10%, BSA 2.5%, Non-fat milk 1%, Normal Horse Serum	1 in 250	PBS triton, Normal Goat Serum10%, BSA 2.5%, Normal Horse Serum 10%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Horse Serum 10%, Normal Human Serum 10%	60 minutes	1 in 7500
Caveolin-1	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2300
VE-Cadherin	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum10%, BSA 2.5%	120 minutes	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000
Placental Growth Factor	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum10%	60 minutes	1 in 3000
Claudin 1	EDTA twen pH 9.5	20 minutes	Heat induced Microwave 80%	PBS	30	PBSt, Normal Goat Serum10%, BSA 2%	1 in 25	PBSt, Normal Goat Serum10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
Occludin	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
Zonula Occludens 1	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum10%, BSA 2.5%	120 minutes	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000

Supplemental Table 3. Immunohistochemistry techniques found successful for endothelial marker's staining in placental specimens at the study

Protein	Antigen Retrieval Solution	Antigen Retrieval time (minutes)	Antigen retrieval method	3% Hydrogen Peroxide blocking solution	Hydrogen Peroxide incubation time (minutes)	Non specific binding Blocking solution	Primary antibody dilution	Blocking solution for Primary antobody	Primary antibody Incubation time	Blocking solution for Secondary antibody	Secondary antibody Incubation time	Horse Radish Peroxidase Dilution
CD3	EDTA tween pH 9.5	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 1000
CD4	EDTA tween pH 8.2	20 minutes	Heat induced Microwave 100%	PBS	30	PBS, Normal Goat Serum 5%, BSA 2%	1 in 50	PBS, Normal Goat Serum 5%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 1000
CD8	EDTA tween pH 9.18	30 minutes	Heat induced Copling jar boiling	PBS	30	PBS, Normal Goat Serum 5%, BSA 2%	1 in 100	PBS, Normal Goat Serum 5%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Goat Serum 5%	60 minutes	1 in 2000
CD14	EDTA tween pH 9.3	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2%	1 in 50	PBS, Normal Goat Serum 10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000
CD56	EDTA tween pH 9.3	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2%	1 in 50	PBS, Normal Goat Serum 10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000
CD163	EDTA twen pH 9.5	20 minutes	Heat induced Microwave 80%	PBS	30	PBSt, Normal Goat Serum 10%, BSA 2%	1 in 300	PBSt, Normal Goat Serum 10%, BSA 2%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
TLR2	EDTA tween pH 8.2	20 minutes	Heat induced Microwave 100%	PBS	30	PBS, Normal Rabbit Serum 10%, BSA 2%	1 in 50	PBS, Normal Rabbit Serum 10%, BSA 2%	overnight	Conjugated anti Goat Secondary antibody, Normal Rabbit Serum 5%	60 minutes	1 in 2000
TLR4	EDTA tween pH 8.2	20 minutes	Heat induced Microwave 100%	PBS	30	PBS, Normal Rabbit Serum 10%, BSA 2%	1 in 50	PBS, Normal Rabbit Serum 10%, BSA 2%	overnight	Conjugated anti Goat Secondary antibody, Normal Rabbit Serum 5%	60 minutes	1 in 2000
CAV	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2300
ZO1	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	same day	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000
OCC	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	overnight	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 2000
CLAU 5	EDTA tween pH 9	20 minutes	Heat induced Microwave 80%	PBS	30	PBS, Normal Goat Serum 10%, BSA 2.5%	1 in 100	PBS, Normal Goat Serum 10%, BSA 2.5%	same day	Conjugated anti Rabbit Secondary antibody, Normal Human Serum 10%	60 minutes	1 in 3000

Chapter Five – Endothelial Features of Human Placentas from Full-term Pregnancies Resulting in Congenital *T. cruzi* transmission In Bolivia

V.1. Abstract

Background

It is estimated that 14,400 newborns are at risk of congenital *T. cruzi* infection each year. *T. cruzi* vertical transmission may be the result from endothelial barrier dysfunction produced by decreased expression of angiogenesis factors secondary to the anti-angiogenic conditions produced by *T. cruzi* shown in *in-vitro* studies. (Ramirez *et al.* 2012) Defective endothelial barrier leading to vertical *T. cruzi* transmission may be associated with leaky endothelial tight junctions. This study aims to assess the expression of angiogenic factors and structural endothelial tight junction proteins in placental specimens obtained from a population at high risk for vertical *T. cruzi* transmission.

Methods

Using immunohistochemistry, we identified endothelial tight junction proteins (Caveolin-1, Claudin-1, VE-Cadherin and Occludin) and angiogenesis (PlGF, VEGFC, vWF and CD31) markers at the syncytiotrophoblast, cytotrophoblast and fetal blood vessels from placental specimens obtained from mothers enrolled in a cohort study of congenital Chagas disease in Santa Cruz, Bolivia. (Bern *et al.* 2009)

Results

CD31 and Fibrinopeptide A (FPA) markers were expressed exclusively at the blood vessels layer of the placental specimens. All the other markers were expressed in all the placental layers. Up-regulation of VE- Cadherin and Placental Growth Factor (PlGF) markers at the syncytiotrophoblast layer as well as for Occludin and Claudin-1 expression at the blood vessel layer were detected in cases when transmission to the offspring occurred from those mothers that were diagnosed with Chagas disease. Decreased expression of VEGFC and Claudin-1 at the syncytiotrophoblast layer, Occludin and PlGF at the cytotrophoblast cells and PlGF at the blood vessels was also detected in the placenta of those mothers that transmitted the disease to their offspring.

Conclusions

Our findings showed altered expression of endothelial angiogenesis and tight junction protein markers may serve as a diagnostic tool to detect those neonates at higher risk for vertical *T. cruzi* transmission. We speculate a link between prolonged inflammation due to chronic exposure to *T. cruzi* antigens and alteration of angiogenesis within the placenta. Claudin 1 expression is decreased at the syncytiotrophoblast layer of placentas allowed vertical transmission, suggesting that structural disturbance of tight junctions in this placental layer.

V.2. Introduction

The *T. cruzi* vertical transmission has increasing epidemiological importance, as some cases have recently been detected in children from non-endemic regions. It is believed that infection can occur among 5–10% of infants born to infected mothers. (Burgos *et al.*, 2007, Bern *et al.*, 2009) As the number of infected and fertile women is estimated by the WHO to be

around 1.8 million, it is predicted that 14,400 newborns are at risk of infection each year (PAHO, 2006). Moreover, *T. cruzi* vertical transmission can be repeated during each pregnancy of currently infected women and from one generation to another, indicating a long-term risk of parasitic transmission. Congenitally acquired *T. cruzi* infection is responsible for many cases of Chagas disease in non-endemic countries. (Torrico *et al.* 2004, Carlier *et al.* 2011)

The human placenta is a highly-vascularized organ, and by the end of the 40th week of gestation it is thought to develop a capillary network that is 550 km in length and 15 m^2 in surface area. Intrauterine fetal growth and poor obstetric outcomes are thought to be closely related to the development of new vessels and subsequent angiogenesis. (Burton *et al.* 2009)

Research in angiogenesis in congenital infections and placental endothelial cells was previously studied mainly to identify biomarkers for malaria placental infection (Ruizendaal *et al.* 2015) and for pre-eclampsia. (Hupertz, 2015) However, *T. cruzi* (Ramirez *et al.* 2012) and *Toxoplasma gondii* (Hunter *et al.* 2001) were shown to inhibit the expression of angiogenic factors in studies evaluating the relationship between tumor growth and endothelial dysfunction. The PlGF/VEGF axis has a central role in the activation and maintenance of the inflammatory switch associated with neo-angiogenesis. (Clark *et al.* 1998) Injury to blood vessels that disrupt the endothelial cell lining results in rapid platelet aggregation and stabilization on the exposed sub-endothelium, which is mediated by the von Willebrand factor (vWF). (Page *et al.* 2013, Lenting *et al.* 2014)

No previous studies have described the relationship between angiogenic factors, adherens and tight junction proteins in maintaining the integrity of the endothelial barrier in vertical *T. cruzi* transmission. VE-cadherin is the major endothelial molecule in the group of adherens junctions and is in close relationship to Occludin, both being used as main indicators of disturbed endothelial barrier *in vitro*. (Dye *et al.* 2001) Decreased VE-Cadherin was shown in malaria vascular endothelial dysfunction (Gillrie *et al.* 2007), and was also associated with embryonic death, showing severe defects during fetal angiogenesis. (Gavard *et al.* 2008) Increased expression of Caveolin-1 was found in previous hypoxia studies (Jiang *et al.* 2014) was also link to Occludin expression during bacterial infection leading to ischemia of epithelial barriers. (Marchiando *et al.* 2010)

T. cruzi vertical transmission is the result of endothelial barrier dysfunction produced by decreased expression of angiogenesis factors due to the antiangiogenic condition previously described for *T. cruzi*. Moreover, impaired angiogenesis expression may result in a defective endothelial barrier, which is associated with leaky endothelial tight junctions. These altered functions may be observed at the different layers of the placental barrier where endothelial expression is present.

The aim of this study is to assess the expression of angiogenic factors (PlGF, VEGFC, vWF and CD31) and endothelial tight junction proteins (Caveolin-1, Claudin-1, VE-Cadherin and Occludin) as marker of endothelial dysfunction at 3 histological layers the syncytiotrophoblast, the cytotrophoblast and the fetal blood vessels from placental specimens from cases of vertical transmission of *T. cruzi*.

V.3. Methods

V.3.1. Research Design

We nested data from a retrospective case-control study with placental specimens collected during a congenital Chagas disease cohort study performed at Hospital Universitario Japones and Hospital Camiri in Santa Cruz, Bolivia from 2007 to 2013. Specimens included placental tissue from normal deliveries and C-sections. (SEE CHAPTER FOUR)

Our nested study was designed to include placental specimens from 3 different groups: (1) Chagas disease seronegative mothers with uninfected newborns (M-B-), (2) Chagas disease seropositive mothers with uninfected newborns (M+B-) and (3) Chagas disease seropositive mothers with infected newborns (M+B+).

V.3.2. Sample collection and processing:

All the procedures regarding sample collection, diagnostics and tissue processing and IHC techniques are described elsewhere. (SEE CHAPTER FOUR)

V.3.3. Procedures

Expression of IHC endothelial markers was observed at the placental villi level as described previously. (SEE CHAPTER FOUR) Placental villi are covered by two layers of trophoblasts: (1) syncytiotrophoblast, the outer layer which prevents intercellular penetration and that is interposed between the maternal blood and fetal tissue during pregnancy, and (2) cytrotrophoblast (Langerhans cells), the inner layer that also covers

extra villous tissues such as chorionic plate. (Moya *et al.* 1979; Abrahams and Mor 2005; Carlier 2005, Romero *et al.* 2007, Carlier *et al.* 2011; Mor and Cardenas, 2010)

Briefly, angiogenesis markers such as Vascular Endothelial Growth Factor –C (VEGFC) and Placental Growth Factor (PlGF) were assessed using IHC techniques (SEE CHAPTER FOUR) to evaluate the effect of vascular permeability promoters in infected placental tissue. The endothelial tight junction proteins markers, vascular endothelial Cadherin (VE-Cadherin), Claudin-1 and Occludin are present at the placental villi structures, providing information regarding the paracellular junctions (VE-Cadherin), and cell-to-cell contact integrity of the endothelial barrier, present at the syncytiotrophoblast and at the endothelium of fetal blood vessels. (Gavard *et al.* 2008) Caveolin-1 has been proposed to be responsible for transcytosis in endothelial cells at the caveolae. (Minshal *et al.* 2005) Von Willebrand Factor (vWF) and CD31 (PECAM-1) are two known endothelial cell markers useful to identify endothelial cells within the blood vessels and also to identify cells with endothelial expression as the syncytiotrophoblast. (Ribes *et al.* 1987)

V.3.4. Statistical Analysis

A. Sample Size and Power Calculations

At the time of this study, no previous information related to systematic evaluation of placental specimens from patients was identified. The majority of information regarding endothelial involvement in *T. cruzi* vertical transmission was obtained from in vitro studies. We relied on a previously reported 7% *T. cruzi* vertical transmission rate in Santa Cruz de la Sierra (Bern *et al.* 2009) and included controls in a rate of 1:1:1 (cases vs. positive controls vs. negative controls).

B. Data Analysis:

Multinomial logistic regression was performed to analyze the association between the intensity observed by readers in each marker and the risk of being an M+B+ case. Models were adjusted for diffuse staining vs. local staining results. Staining reference was assigned to the M-B- group.

In order to evaluate the agreement of histological readings between readers, we performed an un-weighted kappa analysis as a measurement of inter-observer reproducibility. Summed inter-observer reproducibility of the study, and kappa values associated with each diagnostic category were calculated in the case and control material. Additionally, we performed a kappa analysis in order to evaluate the percent agreement of three readings of the same slide for each reader.

All data analysis was conducted using Stata/IC 10.1 (Stata Corp., College Station, TX 2009). A p-value of ≤ 0.05 was considered significant and confidence intervals (CIs) were calculated at 95%.

V.4. Results

CD31 and Fibrinopeptide A (FPA) markers showed expression exclusively at the blood vessel layer of the placental specimens. All other markers showed expression in all placental layers. Table 5 shows a descriptive view of localization and expression for endothelial markers that were deemed to be significant predictors during multinomial logistic regression for all the group comparisons. Von Willebrand Factor did not show a

Table 5. Localization and intensity of staining for endothelial markers (deemed to be significant predictors during multinomial logistic regression).

M+B+ vs. M+B-			
Marker	**ST**	**CT**	**BV**
VE-Cadherin	↑↑↑↑	NS	NS
Occludin	↓↓	↓↓↓↓	↑↑↑↑
von Willebrand Factor	NS	NS	NS
PlGF	↑↑↑↑	↓↓↓↓	↓↓↓↓
VEGFC	↓↓↓↓	NS	NS
Caveolin 1	NS	NS	NS
Claudin-1	↓↓↓↓	NS	↑↑↑↑
FPA	NA	NA	↑↑
CD31	NA	NA	NS

. M-B-			
Marker	**ST**	**CT**	**BV**
VE-Cadherin	↑↑↑↑	NS	↑↑↑↑
Occludin	↑↑	↓↓↓↓	↑↑↑↑
von Willebrand Factor	↓↓↓↓	NS	↑↑↑↑
PlGF	NS	NS	↓↓↓↓
VEGFC	↑↑↑↑	↑↑↑↑	↓↓↓↓
Caveolin 1	↑↑↑↑	NS	NS
Claudin-1	NS	NS	NS
FPA	NA	NA	↓↓↓↓
CD31	NA	NA	↓↓

M+B-vs. M-B-			
Marker	**ST**	**CT**	**BV**
VE-Cadherin	NS	NS	↑↑↑↑
Occludin	↑↑↑↑	NS	NS
von Willebrand Factor	↓↓↓↓	NS	↑↑↑↑
PlGF	↓↓↓↓	↑↑↑↑	↑↑↑↑
VEGFC	↑↑↑↑	↑↑	↓↓↓↓
Caveolin 1	↑↑↑↑	NS	NS
Claudin-1	↑↑↑↑	NS	↓↓↓↓
FPA	NA	NA	↓↓↓↓
CD31	NA	NA	NS

ST= Syncytiotrophoblast CT= Cytotrophoblast BV= Blood vessels
↑↑↑↑ Statistically significant increased expression (P<=0.05)
↓↓↓↓ Statistically significant decreased expression (P<=0.05)
NS Non statistically significant
↑↑ Non significant increased expression (P<=0.10)
↓↓ Non significant decreased expression. (P<=0.10)
NA No staining observed

significant difference when comparing the syncytiotrophoblast layer from the *T. cruzi* vertical transmission cases to the M+B- group.

Increased expression in endothelial markers: (Table 6 and Supplemental Table 4)

Table 6. Endothelial Markers found to have increased expression in *T. cruzi* vertical transmission cases and localization of staining

Blood vessels		
ENDOTHELIAL MARKER	Study Group comparison	RRR (p- value)
OCCLUDIN	. M+B-	2.53 (0.000)
CLAUDIN 1	. M+B-	1.72 (0.000)

Syncytiotrophoblast		
ENDOTHELIAL MARKER	Study Group comparison	RRR (p- value)
VE-CADHERIN	. M+B-	1.97 (0.000)
PLACENTAL GROWTH FACTOR	. M+B-	3.49 (0.000)

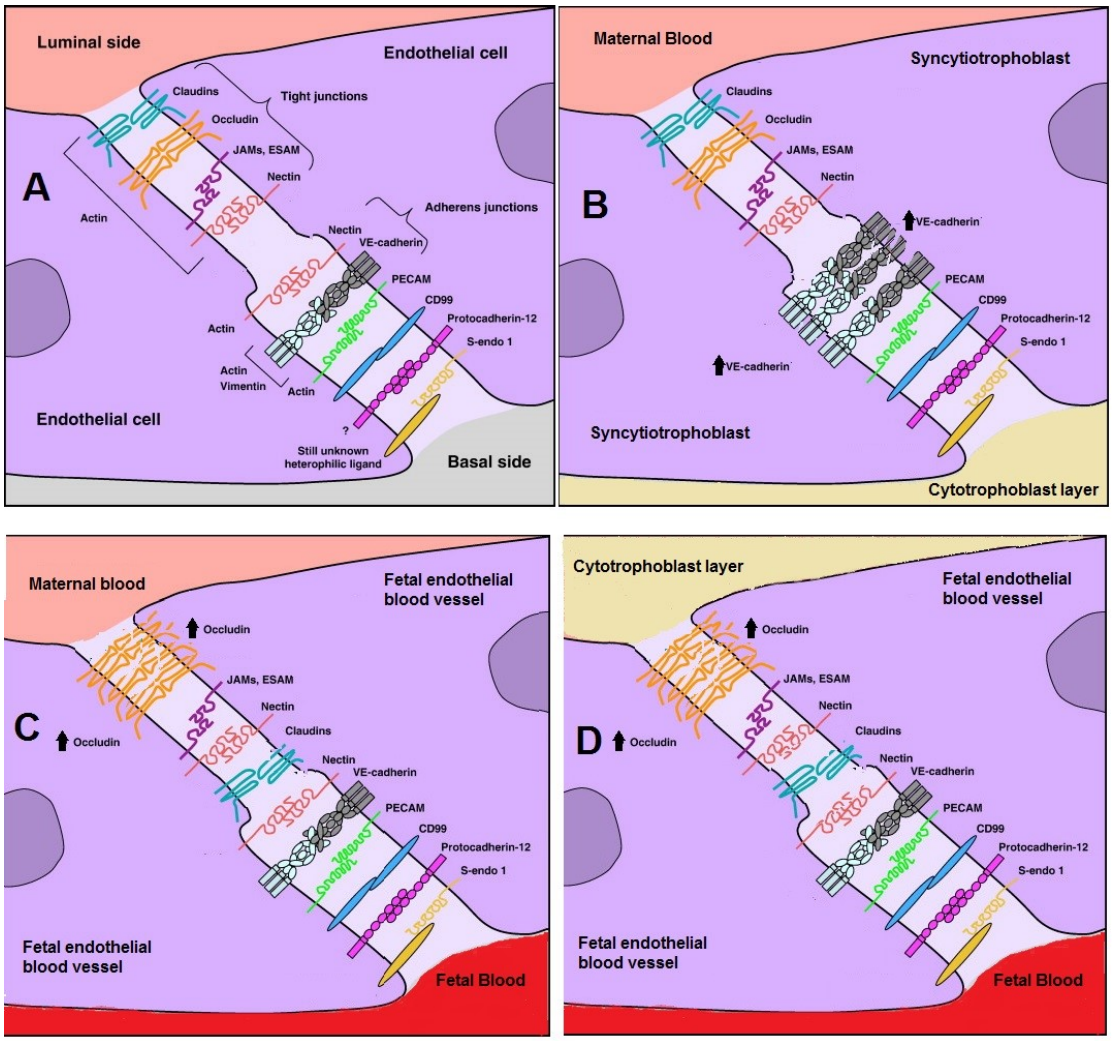
Significant increased expression was found in VE- Cadherin and Placental Growth Factor (PlGF) markers when comparing *T. cruzi* vertical transmission cases to M+B- specimens at the syncytiotrophoblast layer. When comparing the same groups, Caveolin 1 did not show significant increased expression at the syncytiotrophoblast layer.

When assessing the cytotrophoblast, VEGFC and Claudin-1 did not demonstrate significant up-regulation when comparing the M+B+ group to the M+B- group.

Significant increased expression was found for Occludin (Figure 10) and Claudin-1 at the blood vessel layer of the *T. cruzi* vertical transmission cases when compared to the

M+B- specimens. VE-Cadherin, VEGFC and FPA were found to be non-significantly increased at this layer when comparing the same groups.

Figure 10. Schematic of Endothelial cell-to-cell contact and tight junction proteins involved in endothelial function. (A) Shows a normal inter endothelial cleft (Modified from Waleez *et al.*, 2007). (B) Represents a schematic of increased VE-Cadherin expression at the syncytiotrophoblast of a *T. cruzi* vertical transmission case. (C) and (D) show increased Occludin expression found by this study when *T. cruzi* vertical transmission occurs. The mechanism involved in this phenomenon is not known but we hypothesized endothelial cells showing increasing levels of angiogenesis precursors as VEGF. (C) Shows the case of a fetal blood vessel in contact to the maternal blood stream which can be observed in the placental villi. (D) a schematic of a fetal blood vessel which is in contact to the cytotrophoblast layer, meaning a prior infection of this layer in order to observe the parasite gaining access to the fetal blood stream.



(Modified from Waleez *et al.*, 2007)

Decreased expression in endothelial markers: (Table 7 and Supplemental Table 5)

Table 7. Endothelial Markers found to have decreased expression in *T. cruzi* vertical transmission cases and localization of staining

Syncytiotrophoblast		
ENDOTHELIAL MARKER	Study Group comparison	RRR (p- value)
CLAUDIN 1	. M+B-	0.78 (0.021)
VEGFC	. M+B-	0.61 (0.010)

Cytotrophoblast		
ENDOTHELIAL MARKER	Study Group comparison	RRR (p- value)
OCCLUDIN	. M+B-	0.37 (0.003)
PLACENTAL GROWTH FACTOR	. M+B-	0.32 (0.000)

Blood vessels		
ENDOTHELIAL MARKER	Study Group comparison	RRR (p- value)
PLACENTAL GROWTH FACTOR	. M+B-	0.63 (0.014)

Significant differences were found for the level of production of VEGFC and Claudin-1 when comparing the syncytiotrophoblast layer from the *T. cruzi* vertical transmission cases to the M+B- group. Non-significant decreased expression was found for von Occludin when comparing the syncytiotrophoblast layer of the *T. cruzi* vertical transmission cases to the M+B- specimens.

Significant decreased expression of Occludin and PlGF was found on cytotrophoblast cells of *T. cruzi* vertical transmission cases compared to M+B- controls. Non-significant decreased expression was found for VE-Cadherin and Caveolin 1 and von Willebrand Factor when comparing staining intensity at the cytotrophoblast layer of M+B+ specimens to M+B- specimens.

Significant decreased expression was found for PlGF at the blood vessels of *T. cruzi* vertical transmission cases compared to the M+B- group. Non-significant decreased expression in blood vessel layer of *T. cruzi* vertical transmission cases compared to M+B- specimens was found for von Willebrand, Caveolin 1 and CD31 markers.

V.5. Discussion

This is the first study to report levels of CD31 and Caveolin-1 in placental tissue from patients infected with *T. cruzi* or within *T. cruzi* vertical transmission cases. Most markers in this study are mainly related to endothelial function and were found to be produced at roughly equal levels in the majority of the placental villi structures, confirming the fact that different placental villi cells show endothelial expression, although they are thought to have other functions (Wang *et al.* 2010).

Some of the mechanisms described for *T. cruzi* to invade endothelial cells are possibly related to *T. cruzi* vertical transmission. Decreased expression of VEGFC in the syncytiotrophoblast (which represents the first line of the placental barrier and is in close contact with the mother's circulating parasites during pregnancy) and PlGF at the cytotrophoblast and blood vessel layer (the ultimate barrier to gain access to the fetus) were found in specimens from infants infected by *T. cruzi*. *T. cruzi* calreticulin inhibits

angiogenesis and was shown to be in direct contact with endothelial cells, inhibiting their angiogenic expression. (Ramirez *et al.* 2012) Similarly, *Toxoplasma gondii* infection was found to be accompanied by strong suppression of angiogenesis, producing severe hypoxia and avascular necrosis. (Hunter *et al.* 2001) Moreover, *Toxoplasma gondii* proteins have shown to be related to decreased expression of CD31 marker in endothelial cells from tumors due to its anti-angiogenic effect. (Pyo *et al.* 2010) A mouse model also suggested that *T. cruzi* antigens are involved in inflammatory angiogenesis dependent on VEGF. (Guedes-da-Silva *et al.* 2015) The VEGF/PlGF axis provides endothelial paracellular permeability precursor expression and was associated with decreased expression of VE-Cadherin that favors permeability at the adherens junctions at the initial phase of the capillary formation. (Kevil *et al.* 1998) However, Chang *et al.* described the results of a rat model, which, after a progression phase, revealed that VEGF facilitates the tube formation and endothelial survival, increasing the levels of VE-Cadherin in a maturation phase. (Chang *et al.* 2005)

Our study found an increased production of PlGF and VE-Cadherin on the syncytiotrophoblast of placentas that resulted in congenital cases. These results may be explained by the chronic infection of the mother and chronic exposure of the placental barrier to circulating *T. cruzi* proteins that may produce a chronic angiogenesis maturation phase in vertical *T. cruzi* transmission.

This study found decreased expression of Occludin at the cytotrophoblast layer and Claudin 1 at the syncytiotrophoblast of *T. cruzi* vertical transmission cases. No previous studies related to endothelial tight junction proteins and *T. cruzi* vertical transmission cases have shown significant decreased expression when transmission occurs. Occludin was found to be decreased by VEGF *in vitro* (Harhaj *et al.* 2004) and also in placentas with

chorioamnionitis (Tosseta *et al.* 2014), increasing the paracellular permeability of the endothelial barrier through these mechanisms. Occludin was also found decreased in apoptotic endothelial cells of cerebral malaria cases, as evidence of leakier tight junctions and associated brain barrier disruption. (Bienvenu *et al.* 2010) Results showing decreased expression of Claudin 1 were mainly designed to describe weak tight junction activity in pre-eclampsia. Our findings however, support the hypothesis that leakier endothelial tight junctions at the syncytiotrophoblast and cytotrophoblast may be associated with *T. cruzi* vertical transmission.

Supplemental Table 4.- Endothelial Markers in all comparison groups and localization of staining (Increased in the. M+B- comparison)

Syncytiotrophoblast		
ENDOTHELIAL MARKER	Study Group	RRR (p-value)
Caveolin-1	M-B- (Ref)	
	M+B-	1.61 (0.003)
	M+B+	2.04 (0.000)
	. M+B-	1.27 (0.136)
VE-CADHERIN	M-B- (Ref)	
	M+B-	1.00 (0.983)
	M+B+	1.97 (0.000)
	. M+B-	1.97 (0.000)
PLACENTAL GROWTH FACTOR	M-B- (Ref)	
	M+B-	0.21(0.000)
	M+B+	0.73 (0.235)
	. M+B-	3.49 (0.000)

Cytotrophoblast		
ENDOTHELIAL MARKER	Study Group	RRR (p-value)
CLAUDIN 1	M-B- (Ref)	
	M+B-	0.65 (0.249)
	M+B+	1.11 (0.978)
	. M+B-	1.7 (0.978)
VEGFC	M-B- (Ref)	
	M+B-	2.09 (0.099)
	M+B+	2.87 (0.018)

Blood vessels		
ENDOTHELIAL MARKER	Study Group	RRR (p- value)
OCCLUDIN	M-B- (Ref)	
	M+B-	0.78 (0.105)
	M+B+	1.97 (0.000)
	. M+B-	2.53 (0.000)
CLAUDIN 1	M-B- (Ref)	
	M+B-	0.56 (0.000)
	M+B+	0.96 (0.729)
	. M+B-	1.72 (0.000)
FIBRINOPEPTIDE A	M-B- (Ref)	
	M+B-	0.64 (0.000)
	M+B+	0.78 (0.032)
	. M+B-	1.23 (0.052)
VEGFC	M-B- (Ref)	
	M+B-	0.17 (0.000)
	M+B+	0.22 (0.000)
	. M+B-	1.27 (0.299)
VE-CADHERIN	M-B- (Ref)	
	M+B-	2.03 (0.000)
	M+B+	2.06 (0.000)
	. M+B-	1.01 (0.949)

Supplemental Table 5. Endothelial Markers in all comparison groups and localization of staining (Decreased in the. M+B- comparison)

Syncytiotrophoblast		
ENDOTHELIAL MARKER	Study Group	RRR (p-value)
VON WILLEBRAND FACTOR	M-B- (Ref)	
	M+B-	0.72 (0.009)
	M+B+	0.72 (0.015)
	. M+B-	1.00 (0.972)
CLAUDIN 1	M-B- (Ref)	
	M+B-	1.43 (0.002)
	M+B+	1.12 (0.310)
	. M+B-	0.78 (0.021)
OCCLUDIN	M-B- (Ref)	
	M+B-	1.86 (0.000)
	M+B+	1.37 (0.077)
	. M+B-	0.73 (0.082)
VEGFC	M-B- (Ref)	
	M+B-	4.51 (0.000)
	M+B+	2.73 (0.000)
	. M+B-	0.61 (0.010)

Cytotrophoblast		
ENDOTHELIAL MARKER	Study Group	RRR (p-value)
OCCLUDIN	M-B- (Ref)	
	M+B-	1.07 (0.819)
	M+B+	0.39 (0.004)
	. M+B-	0.37 (0.003)
VE-CADHERIN	M-B- (Ref)	
	M+B-	0.76 (0.422)
	M+B+	0.65 (0.243)
	. M+B-	0.86 (0.673)
VON WILLEBRAND FACTOR	M-B- (Ref)	
	M+B-	1,13 (0.974)
	M+B+	1.91 (0.141)
	. M+B-	0.41 (0.973)
PLACENTAL GROWTH FACTOR	M-B- (Ref)	
	M+B-	3.15 (0.000)
	M+B+	1.01 (0.970)
	. M+B-	0.32 (0.000)
Caveolin-1	M-B- (Ref)	
	M+B-	1.40 (0.153)
	M+B+	1.02 (0.938)
	. M+B-	0.73 (0.162)

Blood vessels		
ENDOTHELIAL MARKER	Study Group	RRR (p-value)
CD31	M-B- (Ref)	
	M+B-	0.75 (0.124)
	M+B+	0.69 (0.056)
	. M+B-	0.94 (0.693)
PLACENTAL GROWTH FACTOR	M-B- (Ref)	
	M+B-	2.89 (0.000)
	M+B+	1.83 (0.004)
	. M+B-	0.63 (0.014)
Caveolin-1	M-B- (Ref)	
	M+B-	1.12 (0.538)
	M+B+	1.01 (0.942)
	. M+B-	0.91 (0.585)
VON WILLEBRAND FACTOR	M-B- (Ref)	
	M+B-	1.56 (0.000)
	M+B+	1.54 (0.000)
	. M+B-	0.98 (0.879)

Chapter Six – Immunological And Structural Features Of Human Full-Term Placentas that are Associated with *T. cruzi* vertical transmission In Bolivia

VI.1. Abstract

Background:

The relative importance of *T. cruzi* vertical transmission has increased becoming the major form of transmission. (Torrico *et al.* 2006; Carlier *et al.* 2011) To consider a microorganism a threat to the fetus, the cell layer of syncytiotrophoblast should show an interruption or the pathogen should gain access to the decidual compartment or to the placental villi. An immune response induced against *T. cruzi* during gestation has been associated with protection and pathogenesis.

Methods:

We nested a retrospective case-control study with placental specimens from a Congenital Chagas disease cohort study performed at Hospital Universitario Japones and Hospital Camiri in the department of Santa Cruz in Bolivia. Placental specimens were obtained from 3 different groups: (1) Chagas disease seronegative mothers with uninfected newborns (M-B-), (2) Chagas disease seropositive mothers with uninfected newborn (M+B-) and (3) Chagas disease seropositive mothers with infected newborn (M+B+). Immunohistochemistry techniques were performed to assess and identify expression of immune and structural placental proteins in each group and the differences in their expression when vertical *T. cruzi* transmission occurs. We hypothesize that *T.*

cruzi invasion of the syncytiotrophoblast and cytotrophoblast on its way to the fetal blood stream up-regulates the innate immune response via the activation of Pattern Recognition Receptors (PRRs) such as Toll-like receptor (TLR) 2 and 4, which may lead to apoptosis. Chronic inflammation may produce increased expression of some components of the innate immune response. Our aim is to study the expression of TLR-4 and its co-receptor CD14 and TLR-2 as markers of innate immune response, and changes in Hofbauer cells (CD163), Natural Killer cells (CD56) and CD3+, CD4+, and CD8+T cells in villi from placentas of mothers at risk for Chagas disease to established patterns of changes that may predict vertical *T. cruzi* transmission.

We also hypothesize that structural alteration of the placental barrier in vertical *T. cruzi* transmission is due to chronic inflammation. We assessed the structural integrity of the placental barrier in congenital *T. cruzi* transmission by measuring the expression of ZO1 and Claudin-5 as well as Cytokeratin 7, hCG-B and PLAP.

Results:

Expression in the major PRRs related to Chagas Disease was found altered on transmission cases when assessing placental specimens. Trends for increased expression of TLR-2 and decreased expression of TLR-4 were observed when comparing the transmission cases to the controls. Cytokeratin 7 expression was found to be significantly higher in the syncytiotrophoblast of M+B+ specimens, as well as increased hCG-B expression in the cytotrophoblast when compared to the control groups.

Conclusion:

Although the main expression of TLR-2 and TLR-4 may be found in the cytotrophoblast layer, our study showed trends in the expression surrounding blood vessels. Decreasing trends of activated Hofbauer cells at the placental cytotrophoblast of *T. cruzi* vertical transmission cases may represent an adaptation to a chronic condition of *T. cruzi* infection. Cytokeratin 7 expression was found to show higher expression on the syncytiotrophoblast of *T. cruzi* vertical transmission cases revealing compromise of the outer layer of the placental villi and may also be related to chronic exposure to circulating parasites.

VI.2. Introduction

The relative importance of vertical *T. cruzi* transmission has increased in recent years to become the major mode of disease transmission in certain areas. (Torrico *et al.* 2006; Carlier *et al.* 2011) Few resources have been devoted to efforts focused on preventing congenital infection or making an early diagnosis in congenitally infected infants. Diagnostic algorithms for *T. cruzi* vertical infection do not identify a proportion of congenitally infected children at birth. In order for a microorganism to infect a fetus, it must either cross the cell layer of syncytiotrophoblast, which should show an interruption in its endothelial lining, or the pathogen should gain access to the decidual compartment or to the placental villi. The risk of transmission is higher if placental barrier rupture occurs compared to minimal or no risk when there is no placental involvement. (Bittencourt, 1963; Carlier, 2005; Redline 2006; Schijman, 2006; Carlier et al. 2011; Mor and Cardenas, 2010)

The immune response induced against *T. cruzi* has been associated with protection and pathogenesis. Stimulation of TLR-2 at the cytotrophoblast is associated with

trophoblast apoptosis in the first quarter of pregnancy, leading to barrier disruption and facilitating fetal infection. (Abrahams and Mor 2005; Savino et al. 2007; Kayama and Takeda 2009; Palm and Medzhitov 2009, Munn et al. 1998; Thellin and Heinen 2003; Mor and Cardenas 2010) The cytotrophoblast cells are also able to activate TLR-4 via interactions with parasite-derived glycoinositol phospholipids (GIPLs) leading to activation of TRIF. (Oliveira *et al.* 2010; Palm and Medzhitov 2009)

Few *in vitro* studies have studied the role of structural proteins in vertical *T. cruzi* transmission. Sartori observed decreased PLAP expression associated with T. cruzi infection in cultured trophoblast. (Sartori *et al.* 2002) Placental alkaline phosphatase (PLAP) was previously identified by immunohistochemistry in the syncytiotrophoblast and microvilli membrane where it was thought to have an important role in maternal systemic immunity. (Kumpel *et al.* 2011) No previous studies assessed the expression of tight junction proteins in placenta from vertical *T. cruzi* transmission. Zonula Occludens 1 (ZO1) (Leach *et al.* 2002, Leach *et al.* 2000) and Claudin 5 (Lievano *et al.* 2010) are peripheral tight junctional molecules that were found to be integral proteins in placental tissues. Cytokeratin 7 was previously described as a marker for syncytiotrophoblast integrity in *Toxoplasma* infection (Xu *et al.* 2012), *Listeria* infection (Zeldovich *et al.* 2011) and Cytomegalovirus infection. (Maidiji *et al.* 2010) *T. cruzi* trypomastigotes were shown to induce hCG-B secretion in cultured BeWo cells, relating this finding to increased differentiation in the trophoblast cells as a protective reaction to the parasite. (Liempi *et al.* 2014)

As previously observed in congenital Chagas disease cases (Bittencourt et al. 1987, Bittencourt et al. 1992), malaria studies also found fibrin deposits at the inter-villous space (Ahmed *et al.* 2014) and the trophoblast. (Blazkowska *et al.* 2014)

T. cruzi vertical transmission may show altered innate immune response due to up-regulation of Pattern Recognition Receptors (PRRs) such as TLR-2 and TLR-4 as a result of *T. cruzi* invasion of the syncytiotrophoblast and cytotrophoblast on its way to the fetal blood stream. This increased expression may be associated with increased expression of placental macrophages, monocytes and Natural Killer cells. Structural impairment of the placental barrier as a result of inflammation in vertical *T. cruzi* transmission could be associated with leakier cell-cell contacts due to decreased expression of structural tight junction proteins as Zonula Occludens 1 (ZO1) and Claudin 5. Structural placental proteins at the syncytiotrophoblast like PLAP and Cytokeratin 7 could also be affected in vertical *T. cruzi* transmission. Meanwhile, Fibrin deposits may be found increased as a result of chronic inflammation. Increased expression of human Chorionic Gonadotrophin –Beta (hCG-B) as a result of increased trophoblastic differentiation due to inflammation could be also observed.

The aim of our study is to examine the expression of PRRs at the placental cells as well as the participation of immune cells at the placenta when vertical *T. cruzi* transmission occurs. We assessed the expression of TLR-4 and its co-receptor CD14 and TLR-2 at the placental cells as well as the expression of Hofbauer cells (CD163), Natural Killers cells (CD56) and CD3+, CD4+, and CD8+ cells in the placental villi.

This study is also aimed to assess the structural integrity of the placental barrier when vertical *T. cruzi* transmission occurs. We investigate the expression of ZO1 and Claudin-5 to assess tight junction proteins at the placental barrier cells as well as Cytokeratin 7 and PLAP to evaluate the structural expression of the syncytiotrophoblast at the placental barrier. Fibrin depositions were also assessed by the Masson's trichrome staining for

fibrin. Expression of hCG-B was also assessed in order to evaluate the differentiation of placental cells when *T. cruzi* vertical transmission occurs.

VI.3. Methods

VI.3.1. Research Design

We nested a retrospective case-control study with placental specimens from a Congenital Chagas Disease cohort study performed at Hospital Universitario Japones and Hospital Camiri in the department of Santa Cruz in Bolivia from 2007 to 2013, which collected placental tissue from normal deliveries and C-sections. (SEE CHAPTER FOUR)

Our nested study was designed to include placental specimens from 3 different groups: (1) Chagas disease seronegative mothers with uninfected newborns (M-B-), (2) Chagas disease seropositive mothers with uninfected newborn (M+B-) and (3) Chagas disease seropositive mothers with infected newborn (M+B+).

VI.3.2. Samples

All the procedures regarding sample collection, diagnostics and tissue processing and IHC techniques were described elsewhere. (SEE CHAPTER FOUR)

VI.3.3. Procedures

Expression of IHC structural markers and immune-related markers were observed at the placental villi level as described elsewhere. (SEE CHAPTER FOUR) Placental villi are covered by two layers of trophoblast: (1) syncytiotrophoblast, an outer layer which prevents intercellular penetration and that is interposed between the maternal blood and

fetal tissue during pregnancy, and (2) cytotrophoblast (Langerhans cells), the inner layer that also covers extra villous tissues such as chorionic plate. (Moya et al. 1979; Abrahams and Mor 2005; Carlier 2005, Romero et al. 2007, Carlier et al. 2011; Mor and Cardenas, 2010)

The expression of two major Pattern Recognition Receptors (PRRs) that are known to be involved in the immune response against *T. cruzi*, Toll Like Receptor (TLR) 2 and TLR-4 (Abrahams and Mor 2005) were identified through IHC techniques (SEE CHAPTER FOUR) in order to assess the degree of involvement of these receptors in the innate immune response during *T. cruzi* vertical transmission.

The expression of a set of immune markers including CD3, CD4, CD8, CD14, CD56 and CD163 was also assessed to describe the (1) cellular immune response (CD3, CD4, and CD8), (2) macrophage activation, especially in placental Hofbauer cells (CD163) and (3) Natural Killer cells activation (CD56) which is a marker also related to the NCAM (Neural cellular adhesion molecule) expressing cells.

Mechanical barrier structure was assessed in our study by the expression of proteins involved in the maintenance of the placental barrier. Expression of structural proteins surrounding blood vessels (Smooth Muscle Actin Alpha (alpha-SMA)) and syncytiotrophoblast (Cytokeratin 7); secreted proteins and hormones involved in the villi structure integrity (Human Chorionic Gonadotrophin Beta (hCG-B) and Placental Alkaline Phosphatase (PLAP) and structural tight junction proteins (Zonula Occludens-1(ZO1) and Claudin-1) was assessed as described elsewhere. (SEE CHAPTER FOUR).

A, Sample Size and Power Calculations

There is no previous information about systematic specimen evaluation in Chagas disease patients, and most of the information regarding placental involvement of *T. cruzi* vertical transmission was obtained from in vitro studies. We relied on a 7% *T. cruzi* vertical transmission in Santa Cruz de la Sierra previously reported (Bern *et al.* 2009) and included controls in a rate of 1:1:1 (cases vs. positive controls vs. negative controls), power of 80% and probability of 5% of type I error.

B. Data Analysis:

Multinomial logistic regression was performed to analyze the association between the observed staining intensity of each marker by the readers, and the risk of being an M+B+ case. Staining reference was assigned to the M-B- group. Models were adjusted for diffuse stainings and local staining results. To determine the degree of agreement of histological readings between examiners, we performed an un-weighted kappa analysis as a measurement of inter-observer reproducibility. Summed inter-observer reproducibility of the study, and kappa values to each diagnostic category were calculated in the case and control material. Similarly, we performed a kappa analysis to evaluate the percent agreement of 3 readings of the same slide for each reader.

All data analysis was conducted using Stata IC10.1 (Stata Corp., College Station, TX 2009). A p-value of ≤ 0.05 was considered significant and confidence intervals (CIs) were calculated at 95%.

Table 8. Localization and intensity of staining for Immune markers (deemed to be significant predictors during multinomial logistic regression).

. M+B-			
Marker	**ST**	**CT**	**BV**
TLR-2	↑↑↑↑	↓↓↓↓	↓↓↓↓
TLR-4	NS	NS	↑↑↑↑
CD3	NS	↓↓↓↓	↑↑↑↑
CD163	↓↓↓↓	NS	NA
CD14	↑↑↑↑	NS	NA
CD56	↑↑↑↑	NS	NA

. M-B-			
Marker	**ST**	**CT**	**BV**
TLR-2	NS	NS	↓↓↓↓
TLR-4	↑↑↑↑	NS	↑↑↑↑
CD3	NS	↓↓↓↓	↑↑↑↑
CD163	↓↓↓↓	NS	NA
CD14	↑↑↑↑	NS	NA
CD56	↑↑↑↑	NS	NA

M+B-vs. M-B-			
Marker	**ST**	**CT**	**BV**
TLR-2	↓↓↓↓	↑↑↑↑	NS
TLR-4	↑↑↑↑	NS	NS
CD3	NS	NS	NS
CD163	NS	↑↑	NA
CD14	NS	↑↑↑↑	NA
CD56	NS	↑↑↑↑	NA

ST= Syncytiotrophoblast CT= Cytotrophoblast BV= Blood vessels
↑↑↑↑ Statistically significant increased expression (P<=0.05)
↓↓↓↓ Statistically significant decreased expression (P<=0.05)
↑↑ Non significant increased expression (P<=0.10)
↓↓ Non significant decreased expression. (P<=0.10)

VI.4. Results

VI.4.1. Immunological markers:

All the markers showed expression staining at all layers of the placental specimens, except CD56, CD163 and CD14, which did not show expression at the blood vessel layer. Table 8 shows a descriptive view of localization and expression for immune markers (deemed to be significant predictors during multinomial logistic regression) for all the group comparisons.

Significantly increased expression was found for CD14, CD56 and TLR-2 at the syncytiotrophoblast as well as TLR-4 and CD3 in blood vessels when comparing the transmission group to the M+B- group. (Table 9)

Table 9. Immunological Markers found to have increased expression in *T. cruzi* vertical transmission cases and localization of staining

SYNCYTIOTROPHOBLAST		
Marker	**Study group comparison**	**RRR (p Value)**
CD14	. M+B-	1.92 (0.000)
CD56	. M+B-	1.32 (0.030)
TLR-2	. M+B-	1.69(0.001)

BLOOD VESSELS		
Marker	**Study group comparison**	**RRR (p Value)**
TLR-4	. M+B-	1.41 (0.007)
CD3	. M+B-	3.46 (0.000)

Non-significant increased expression was found for CD3 at the syncytiotrophoblast and for TLR-4 at the cytotrophoblast when comparing the same groups. (Supplemental Table 6)

Significantly decreased CD 163 expression was found at the syncytiotrophoblast. CD3 and TLR-2 were also significantly decreased at the cytotrophoblast and blood vessels when comparing the transmission group to the M+B- group. (Table 10) Non-significant decreased expression was found for TLR-4 at the syncytiotrophoblast layer and for CD163, CD56 and CD14 at the cytotrophoblast layer when comparing the same groups. (Supplemental Table 7)

Table 10. Immunological Markers found to have decreased expression in *T. cruzi* vertical transmission cases and localization of staining

SYNCYTIOTROPHOBLAST		
Marker	**Study group comparison**	**RRR (p Value)**
CD163	. M+B-	0.54 (0.002)

CYTOTROPHOBLAST		
Marker	**Study group comparison**	**RRR (p Value)**
CD3	. M+B-	0.32 (0.001)
TLR-2	. M+B-	0.72 (0.007)

BLOOD VESSELS		
Marker	**Study group comparison**	RRR (p Value)
TLR-2	. M+B-	0.57 (0.000)

Table 11. Localization and intensity of staining for immune markers (deemed to be significant predictors during multinomial logistic regression).

. M+B-			
Marker	**ST**	**CT**	**BV**
Claudin 5	↑↑↑↑	↓↓↓↓	NS
ZO1	↑↑↑↑	↓↓↓↓	NS
hCG-B	NS	↑↑↑↑	↓↓↓↓
Cytokeratin 7	↑↑↑↑	NA	NA
PLAP	↓↓↓↓	NA	NA
SMA a	NA	NA	↑↑↑↑
Fibrin (Masson)	NA	↓↓↓↓	NA

. M-B-			
Marker	**ST**	**CT**	**BV**
Claudin 5	↑↑	↓↓	↓↓↓↓
ZO1	↑↑↑↑	↓↓↓↓	NS
hCG-B	↓↓	↑↑↑↑	↓↓↓↓
Cytokeratin 7	↑↑↑↑	NA	NA
PLAP	↑↑	NA	NA
SMA a	NA	NA	NS
Fibrin (Masson)	NA	↑↑	NA

M+B-vs. M-B-			
Marker	**ST**	**CT**	**BV**
Claudin 5	NS	↑↑	↓↓↓↓
ZO1	↑↑	NS	NS
hCG-B	NS	↑↑↑↑	↓↓↓↓
Cytokeratin 7	NS	NA	NA
PLAP	↑↑↑↑	NA	NA
SMA a	NA	NA	↓↓
Fibrin (Masson)	NA	↑↑↑↑	NA

ST= Syncytiotrophoblast CT= Cytotrophoblast BV= Blood vessels
↑↑↑↑ Statistically significant increased expression (P<=0.05) ↑↑ Non significant increased expression (P<=0.10)
↓↓↓↓ Statistically significant decreased expression (P<=0.05) ↓↓ Non significant decreased expression. (P<=0.10)

VI.4.2. Structural Markers

All the markers showed expression staining at all layers of the placental specimens, except Cytokeratin 7 and Placental Alkaline phosphatase (PLAP), which showed staining only at the syncytiotrophoblast, and Smooth Muscle actine alpha (SMA-a), which only showed staining at the blood vessels layer. Table 11 shows a descriptive view of localization and expression for structural markers (deemed to be significant predictors during multinomial logistic regression) for all the group comparisons.

Significant increased expression was found for Claudin-5, Zonula Occludens 1 and Cytokeratin 7 at the syncytiotrophoblast layer when comparing *T. cruzi* vertical transmission cases to M+B- specimens. (Table 12)

Table 12. Structural Markers found to have increased expression in *T. cruzi* vertical cases and localization of staining.

SYNCYTIOTROPHOBLAST		
Marker	Study group comparison	RRR (p Value)
CLAUDIN 5	. M+B-	1.57 (0.002)
ZO1	. M+B-	2.60 (0.000)
Cytokeratin 7	. M+B-	2.56 (0.000)
CYTOTROPHOBLAST		
Marker	Study group comparison	RRR (p Value)
hCG-B	. M+B-	3.19 (0.000)
BLOOD VESSELS		
Marker	Study group comparison	RRR (p Value)
SMA	. M+B-	1.46 (0.004)

Human chorionic Gonadotrophin-Beta was significantly increased at the cytotrophoblast layer and Smooth Muscle Actin alpha was also found to be significantly increased at the blood vessel location when comparing the same groups. (Table 12 and Supplemental Table 8)

Significant decreased expression was found for PLAP at the syncytiotrophoblast layer. ZO1, Claudin 5 and fibrin (by Masson trichrome staining) were decreased at the cytotrophoblast layer when comparing *T. cruzi* vertical transmission cases to M+B- specimens. hCG-B was found to be significantly decreased at the blood vessel layer when comparing the same groups. (Table 13)

Table 13. Structural Markers found to have deceased expression in *T. cruzi* vertical transmission cases and localization of staining

SYNCYTIOTROPHOBLAST		
Marker	Study group	RRR (p Value)
PLAP	. M+B-	0.64 (0.000)
CYTOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CLAUDIN 5	. M+B-	0.48 (0.018)
ZO1	. M+B-	0.25 (0.000)
Masson trichrome staining for fibrin	. M+B-	0.49 (0.000)
BLOOD VESSELS		
Marker	Study group	RRR (p Value)
hCG-B	. M+B-	0.43 (0.000)

Non-significant decreased expression was found for hCG-B at the syncytiotrophoblast layer, and Claudin 5 was decreased at the blood vessel level when comparing the same groups. (Supplemental Table 9).

VI.5. Discussion

Our study is the first report of Claudin-5 and ZO1 expression in placental tissue from *T. cruzi*-infected patients and/or *T. cruzi* vertical transmission cases. This study identified trends of expression in the major pattern recognition receptors (PRRs) related to Chagas disease (TLR-2 and TLR-4) showing increased TLR-4 expression and decreased TLR-2 expression in villi blood vessels of congenital cases. Only a higher CD4 cell count and decreased macrophage (CD163) expression was found when transmission occurs.

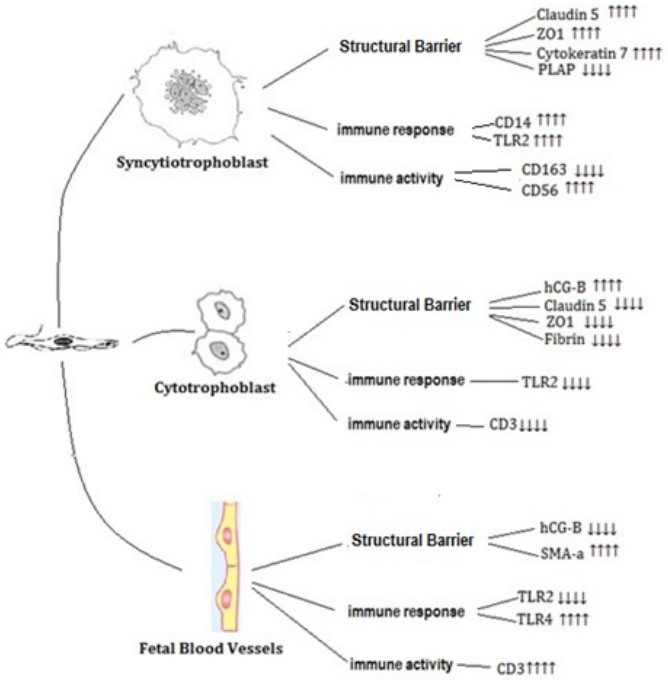
Structurally, Cytokeratin 7 expression was found to be significantly higher on the syncytiotrophoblast of M+B+ specimens and an increasing trend of hCG-B expression was found on the cytotrophoblast when comparing the three groups of the study. (Figure 11)

VI.5.1.Immunological Aspects:

This study found decreasing trends of TLR-2 expression at villi of fetal blood vessels and decreased expression at the cytotrophoblast layer when infection and transmission is present and related to maternal chronic Chagas disease. Meanwhile, studies in animals showed the important role of TLR-2 in response to acute infection with *T. cruzi*. (Bafica *et al.* 2006, Kayama *et al.* 2010) TLR-2 activation leads to a better immune response to *T. cruzi* (Pellegrini

et al. 2011), which we hypothesize is impaired when infection is present and when transmission occurs due to decreased expression of TLR-2. Similar to what is found in some chorioamnionitis studies (Rindsjo¨*et al.* 2007, Hayati *et al.* 2010), our study found decreased TLR-2 expression on infected specimens although none of the complications associated with this alteration, such as preterm birth and intrauterine growth restriction. (SEE CHAPTER FOUR)

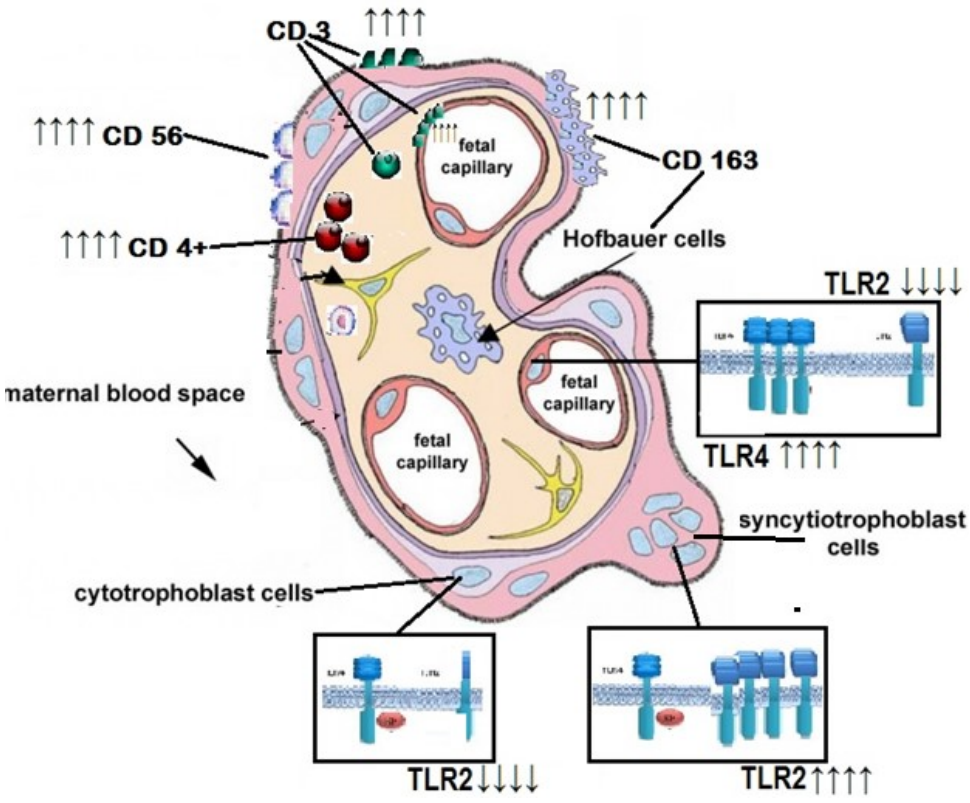
Figure 11. Placental barrier cell findings for Immunological and Structural markers. When *T. cruzi* invades the placental tissue, structural and immune reactions were found at the syncytiotrophoblast, cytotrophoblast and fetal blood vessels of the *T. cruzi* vertical transmission cases. A) At the syncytiotrophoblast increased expression of Claudin 5, Zonula Occludens 1 Cytokeratin 7 and Placental Alkaline Phosphatase was found showing altered structural reaction. The immune response reaction of these cells were found to have increased expression at the toll like receptors level, as well as CD14 co-receptor and PE-CAM(CD56) and macrophages' activity was found to have increased expression and decreased respectively. B) At the cytotrophoblast layer, this study found decreased tight junction proteins expression (ZO1 and Claudin 5) and fibrin deposits. Expression of hCG-B was found to have increased expression at this villi layer when *T. cruzi* vertical transmission occurs. The immune response showed weak TLR-2 expression as well as decreased CD3 activity. C) In contrast to cytotrophoblast, hCG-B was found to have decreased expression. However, Smooth Muscle Actin alpha (SMA-a) was found to have increased expression surrounding the blood vessels when transmission occurs. Decreased expression of TLR-2 and over expression of TLR-4 and CD3 was observed at this villi location.



Toxoplasma infection showed strong reactivity of TLR-2 and TLR-4 in the trophoblast of full term placentas, although expression of TLR-2 was found to be higher in viral and bacterial infections. (Hayati *et al.* 2010, Holmlund *et al.* 2002)

The capacity of *T. cruzi* to activate macrophages and initiate the response through an activation of TLR-2, which appears to assume different functions depending on the cell type was previously shown. (Gravina *et al.* 2013) CD163 expression, which is associated with Hofbauer cells (placental macrophages), was found to have decreased expression at the syncytiotrophoblast layer when infection and transmission were present and may be produced by the decreased expression of TLR-2 also observed in this study. (Figure 12)

Figure 12. Schematic of significant findings on the immunological markers at the placental villi of *T. cruzi* vertical transmission cases.



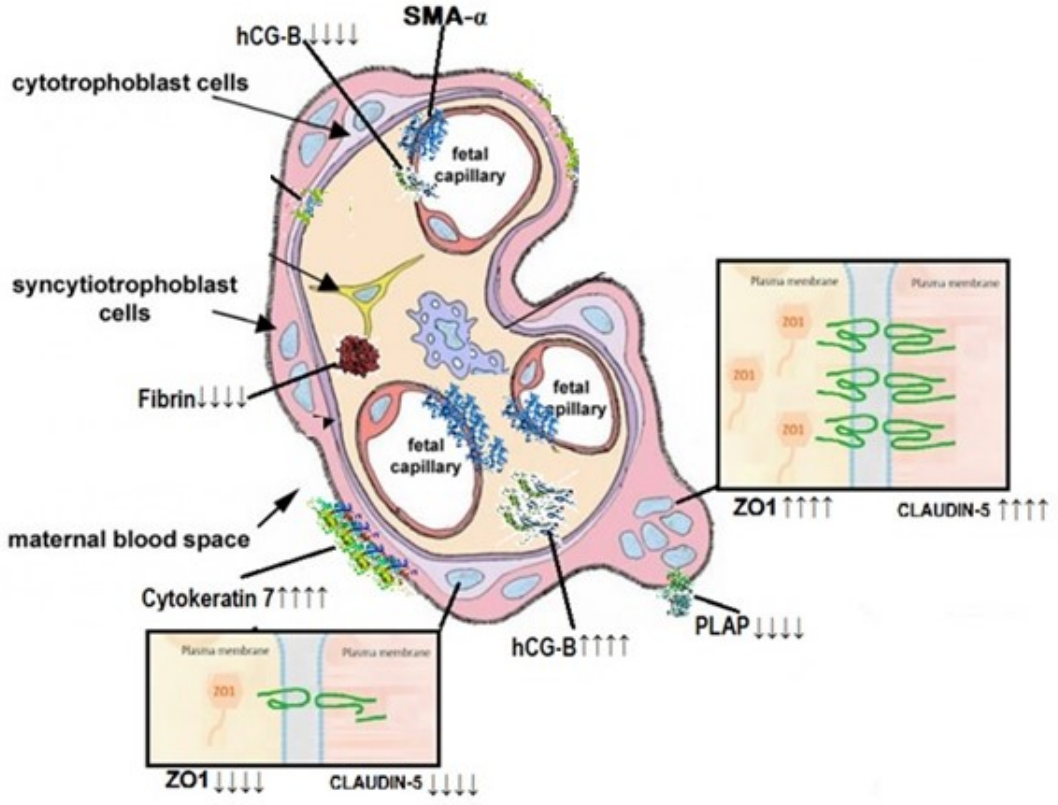
TLR-4 is critically involved in recognizing the GPI anchor of *T. cruzi* and previous studies reported that the expression of TLR-4 confers resistance to infection with *T. cruzi*. (Kayama *et al.* 2010). Our results showed an increasing trend of expression of TLR-4 at the fetal blood vessels of congenital cases. In *Toxoplasma* congenital infection, TLR-4 expression was increased at the maternal side of the placenta. (Beijar *et al.* 2006, Wujcicka *et al.* 2014) Our results found TLR-4 expression mainly located at the fetal side of the placenta in *T. cruzi* vertical transmission cases, suggesting an increased response close to the fetal compartment. CD14 expression was also increased in our study and this result confirms the cooperative role of CD14 to mediate the innate immune response with TLR-4 previously reported for *Toxoplasma* infection. (Debierre-Grockiego *et al.* 2007)

Recruitment of innate immune cells could be enhanced by stimulation of TLR-4 and since trophoblast cells express those receptors, they could modulate the maternal immune system in infective conditions. (Bernardi *et al.* 2012, Abrahams *et al.* 2005) Our study found increased count of CD4 cells at the cytotrophoblast layer in contrast to previous studies among T cells in the Chagasic villitis (Altemani *et al.* 2000), villitis of unknown etiology (VUE) and chorioamnionitis (Kim *et al.* 2008) that found CD8+ cells outnumbered CD4+ cells in all placentas. In our study, Natural Killer cells (CD56-positive cells) expression was found to be increased when transmission occurs although Altemani et al. in 2000 described poor CD56 expression in villitis from placental *T. cruzi* infected specimens. In *Toxoplasma* infection, natural killer cells were described as essential for early parasite control in animal models. (Denkers *et al.* 1993) Moreover, Natural Killers cells were shown to help CD8 T cells in absence of CD4 cells in *Toxoplasma* infection. (Combe *et al.* 2005)

VI.5.2. Structural Aspects:

Our study is the first report of Cytokeratin 7, alpha-SMA, FPA, ZO1 and Claudin-5 production levels in placental specimens from mothers infected with *T. cruzi* and *T. cruzi* vertical transmission cases. Similarly, our study is the first report showing trends in the production of hCG-B from specimens of *T. cruzi* infection and transmission. (Figure 13)

Figure 13. Schematic of significant findings on the structural markers at the placental villi of *T. cruzi* vertical transmission cases.



Increased expression of Cytokeratin 7 at the syncytiotrophoblast layer found in congenital cases may reflect a compromise on the structure of the first layer of the placental barrier and was also found to have increased expression at the same villi layer

in pre-eclampsia. (Buurma *et al.* 2012) This finding is, however, related to low birth weight and preterm deliveries in pre-eclampsia and other conditions, features that are not found in *T. cruzi* vertical transmission.

Placental alkaline phosphatase (PLAP) was previously identified by immunohistochemistry in the syncytiotrophoblast and microvilli membrane where it is thought to have an important role in maternal systemic immunity. (Kumpel *et al.* 2011) Increased PLAP expression was previously reported in cultured trophoblast (Priotto *et al.* 2009) and found in the tunica intima and media of fetal blood vessels from cytomegalovirus infection placental specimens. Our results however, showed decreased expression when transmission occurs. Most previous results showing increased PLAP expression were performed *in vitro* and included highly pathogenic *T. cruzi* strains so we believe that placental specimens may have a different response when chronic infection occurs

Studies in pre-eclampsia showed no significant variation in the expression of ZO–1 but decreased expression in Claudin 5. (Lievano *et al.* 2006) Meanwhile, our study found decreased expression of ZO1 at the cytotrophoblast and decreased expression of both ZO1 and Claudin 5 in the fetal blood vessels when *T. cruzi* infection is present. Impaired expression of these tight junction proteins in *T. cruzi* vertical transmission cases may be related to leaky tight junctions and disruption of the placental barrier at this placental villi structure. However, since no clinical alterations were found in our cases (SEE CHAPTER FOUR), we hypothesize that transmission may be occurring later in pregnancy and proximal to the delivery.

Supplemental Table 6. Immunological Markers in all comparison groups and localization of staining (Increased in the. M+B- comparison)

SYNCYTIOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CD14	M-B- (Ref)	
	M+B-	0.78 (0.173)
	M+B+	1.50 (0.010)
	. M+B-	1.92 (0.000)
CD56	M-B- (Ref)	
	M+B-	1.05 (0.739)
	M+B+	1.38 (0.015)
	. M+B-	1.32 (0.030)
TLR-2	M-B- (Ref)	
	M+B-	0.66 (0.005)
	M+B+	1.13 (0.379)
	. M+B-	1.69 (0.001)
CD3	M-B- (Ref)	
	M+B-	0.68 (0.172)
	M+B+	1.03 (0.924)
	. M+B-	1.51 (0.146)

CYTOTROPHOBLAST		
Marker	Study group	RRR (p Value)
TLR-4	M-B- (Ref)	
	M+B-	1.06 (0.579)
	M+B+	1.10 (0.334)
	. M+B-	1.04 (0.677)

BLOOD VESSELS		
Marker	Study group	RRR (p Value)
TLR-4	M-B- (Ref)	
	M+B-	1.11 (0.426)
	M+B+	1.57 (0.000)
	. M+B-	1.41 (0.007)
CD3	M-B- (Ref)	
	M+B-	0.86 (0.600)
	M+B+	2.98 (0.000)
	. M+B-	3.46 (0.000)

Cell count		
Marker	Study group	Cell count (p value)
CD4	M-B- (Ref)	11.990354 -
	M+B-	11.908571 (1.0000)
	M+B+	12.309242 (0.006)
	. M+B-	(0.001)
CD8	M-B- (Ref)	11.485834
	M+B-	11.352769 (0.322)
	M+B+	11.320087 (0.197)
	. M+B-	(1.000)

Supplemental Table 7. Immunological Markers in all comparison groups and localization of staining (Decreased in the. M+B- comparison)

SYNCYTIOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CD163	M-B- (Ref)	
	M+B-	0.78 (0.154)
	M+B+	0.42 (0.000)
	. M+B-	0.54 (0.002)
TLR-4	M-B- (Ref)	
	M+B-	1.44 (0.007)
	M+B+	1.31 (0.046)
	. M+B-	0.91 (0.475)

CYTOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CD3	M-B- (Ref)	
	M+B-	1.50 (0.214)
	M+B+	0.48 (0.031)
	. M+B-	0.32 (0.001)
CD163	M-B- (Ref)	
	M+B-	1.23 (0.099)
	M+B+	1.07 (0.665)
	. M+B-	0.86 (0.331)
CD56	M-B- (Ref)	
	M+B-	1.57 (0.005)
	M+B+	1.21 (0.266)
	. M+B-	0.76 (0.110)
TLR-2	M-B- (Ref)	
	M+B-	1.54 (0.000)
	M+B+	1.11 (0.373)
	. M+B-	0.72 (0.007)
CD14	M-B- (Ref)	
	M+B-	1.58 (0.006)
	M+B+	1.32 (0.101)
	. M+B-	0.84 (0.305)

BLOOD VESSELS		
Marker	Study group	RRR (p Value)
TLR-2	M-B- (Ref)	
	M+B-	0.97 (0.742)
	M+B+	0.55 (0.000)
	. M+B-	0.57 (0.000)

Supplemental Table 8.- Structural Markers in all comparison groups and localization of staining (increased in the. M+B- comparison)

SYNCYTIOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CLAUDIN 5	M-B- (Ref)	
	M+B-	0.85 (0.290)
	M+B+	1.33 (0.058)
	. M+B-	1.57 (0.002)
ZO1	M-B- (Ref)	
	M+B-	1.36 (0.081)
	M+B+	3.53 (0.000)
	. M+B-	2.60 (0.000)
Cytokeratin 7	M-B- (Ref)	
	M+B-	0.95 (0.761)
	M+B+	2.44 (0.000)
	. M+B-	2.56 (0.000)

CYTOTROPHOBLAST		
Marker	Study group	RRR (p Value)
hCG-B	M-B- (Ref)	
	M+B-	2.23 (0.000)
	M+B+	7.14 (0.000)
	. M+B-	3.19 (0.000)

BLOOD VESSELS		
Marker	Study group	RRR (p Value)
SMA	M-B- (Ref)	
	M+B-	0.80 (0.080)
	M+B+	1.17 (0.250)
	. M+B-	1.46 (0.004)
ZO1	M-B- (Ref)	
	M+B-	0.91 (0.661)
	M+B+	0.94 (0.778)
	. M+B-	1.03 (0.893)

Supplemental Table 9. Structural Markers in all comparison groups and localization of staining (Decreased in the. M+B- comparison)

SYNCYTIOTROPHOBLAST		
Marker	Study group	RRR (p Value)
hCG-B	M-B- (Ref)	
	M+B-	1.14 (0.382)
	M+B+	0.90 (0.536)
	. M+B-	0.79 (0.125)
PLAP	M-B- (Ref)	
	M+B-	1.60 (0.000)
	M+B+	1.02 (0.835)
	. M+B-	0.64 (0.000)

CYTOTROPHOBLAST		
Marker	Study group	RRR (p Value)
CLAUDIN 5	M-B- (Ref)	
	M+B-	1.89 (0.049)
	M+B+	0.91 (0.782)
	. M+B-	0.48 (0.018)
ZO1	M-B- (Ref)	
	M+B-	1.27(0.461)
	M+B+	0.32 (0.002)
	. M+B-	0.25 (0.000)
Masson trichrome staining for fibrin	M-B- (Ref)	
	M+B-	2.16 (0.000)
	M+B+	1.08 (0.706)
	. M+B-	0.49 (0.000)

BLOOD VESSELS		
Marker	Study group	RRR (p Value)
hCG-B	M-B- (Ref)	
	M+B-	0.52 (0.000)
	M+B+	0.22 (0.000)
	. M+B-	0.43 (0.000)
CLAUDIN 5	M-B- (Ref)	
	M+B-	0.69 (0.010)
	M+B+	0.61 (0.001)
	. M+B-	0.88 (0.348)

Chapter Seven – Integrated Discussion

Prevention of *T. cruzi* vertical transmission is not possible at present, but it can be detected early, and treatment of infected newborns is highly effective. Congenital *T. cruzi* infection is recognized as a major cause of transmission in endemic countries where relatively successful vector control and blood screening programs exist. The disease is also observed along the major migration routes leading from endemic into non-endemic countries. After eliminating transmission of *T. cruzi* infection by vectors and blood transfusions, a critical final step toward eliminating the disease will involve finding new tools to diagnose and provide timely treatment to infants with congenital *T. cruzi* infection at the primary health care level.

The appropriate diagnosis of congenital American trypanosomiasis by microscopy requires expert eyes. In countries where *Trypanosoma cruzi* is endemic, the superiority of PCR over microscopy has been demonstrated in several reports from well-designed studies in Argentina, Bolivia, and Paraguay. PCR assays for *T. cruzi* now have enough sensitivity and specificity to be considered the standard method for diagnosis in areas where *T. cruzi* is not endemic and where there are less experienced microbiologists.

In general, the true potential of real-time PCR will be recognized in situations for which PCR-based techniques have been promoted, such as congenital infections, monitoring parasitaemia during and after treatment, early detection of relapses after heart

transplantation and other immunosuppressive circumstances. To deal with more precise results and make them comparable between laboratories, the main constraint until now has been the lack of a universal reagent presenting accurately quantified *T. cruzi* DNA samples to be used as standards in all quantification assays. International standards for various infectious agents (human immunodeficiency virus, hepatitis C virus, etc.) are commercially available for molecular biology assays in order to standardize and compare methods and laboratory performances. In this sense, agencies providing such materials should be strongly encouraged to make properly characterized *T. cruzi* reagents available for this purpose.

Introduction of PCR for congenital Chagas disease diagnosis will not only improve our current knowledge on congenital disease epidemiology, but also favors a better diagnosis, and the ensuing specific treatment. Nevertheless, the lower positivity rate obtained at the time of birth, with respect to the ones recorded in subsequent samples, raises the need for improving its sensitivity.

Exhaustive protocols for antigen retrieval were tested in order to obtain successful staining for each marker and antibody selection was found to be critical for obtaining good results. The use of TMAs in IHC is a reliable and helpful method that could avoid costs and time when analyzing high number of samples in a short period of time. TMAs ensure same histological procedures and staining techniques for both cases and controls, avoiding bias in these technical steps. Our study was able to evaluate 77 placental specimens in only 7 slides, reducing costs and time-persons for processing the specimens by at least in 50%.

Previous studies performed in Bolivia reported preterm delivery as a risk factor for *T. cruzi* vertical transmission. This study does not report significant differences on placental pathology and neonatal measurements in the cases of *T. cruzi* vertical transmission. Although lower gestational age and older pregnant mothers were identified for the transmission group, the observed differences still belong to the full term gestational age and young age of pregnancy. It is known that placental growth mainly occurs at the 3rd trimester of pregnancy and pathologies including placental retarded growth or significant alterations, such as pre-eclampsia and placental malaria, linked these findings to fetal growth retardation and low birth weight. Our study did not find significant differences in birth weight, height and other parameters usually measured to assess neonatal health.

Our findings showed altered expression of endothelial angiogenesis markers and tight junction proteins in *T. cruzi* vertical transmission cases, which may be related to increased risk of transmission. Prolonged inflammation as a result of chronic Chagas infection may predispose the endothelial trophoblast layers and fetal blood endothelia to be in permanent contact with circulating parasites, due to the increased parasitemia described in pregnant mothers chronically infected with *T. cruzi*. Parasite antigens have been described to be involved in inflammatory angiogenesis and may be decreasing the expression of vascular permeability precursors such as VEGF and PlGF. These agents have been reported to be elevated in the final phase of angiogenesis induced by inflammation, losing their permeability induction effect due to increased tight junction proteins expression. Claudin 1, which is not associated with angiogenesis factors, was found to have decreased expression at the syncytiotrophoblast layer of *T. cruzi* vertical

transmission cases. This may be associated with poor tight junction proteins expression at this layer when transmission occurs.

Increased expression of TLR-4 and decreased expression of TLR-2 were found in blood vessels of vertical *T. cruzi* transmission specimens. Although main expression of these receptors was described previously on the cytotrophoblast layer when other infections were assessed, our study showed trends in the expression surrounding blood vessels in *T. cruzi* vertical transmission cases. Those blood vessels are in close contact with fetal circulation, and may be reflecting a reaction against pathogens already in the fetal blood stream. Although similar findings from other conditions are related to preterm birth and low birth weight, Chagas disease does not show these clinical features when *T. cruzi* vertical transmission occurs.

Decreasing trends of activated Hofbauer cells (CD163) at the placental cytotrophoblast of *T. cruzi* vertical transmission cases may be showing an adaptation to a chronic *T. cruzi* infection. *T. cruzi* infected mothers may be exposing the fetus to circulating parasites since implantation in the uterus. Cytokeratin 7 expression was found to show higher expression on the syncytiotrophoblast of *T. cruzi* vertical transmission cases revealing compromise of the outer layer of the placental villi and may be also related to chronic exposure to circulating parasites. Increased expression of hCG-B at the cytotrophoblast layer in the *T. cruzi* vertical transmission cases may be the product of constant production of this hormone due to constant inflammation of this layer by the infection.

VII.2. Limitations

The lack of a gold standard for vertical *T. cruzi* transmission diagnosis makes difficult the identification of cases in a timely manner. This case control study was nested into an ongoing Cohort Study were several diagnostic methods were added to the conventional diagnostic routine performed by the National Control Program in order to identify all the congenital cases as accurate and fast as possible. Active follow-up of participants was also added in the study and was performed until nine months after delivery due to the very low diagnosis rates observed at birth. However, in rural communities and poor resource settings, it is difficult to achieve high follow-up rates and 40% of participants from Camiri were lost to follow up and not treated although they were identified as positive by the study methods. Reasons for not continuing in the study included traveling to other cities and contact loss due to change of phone number, incorrect address and lack of resources for siblings care meanwhile the follow-up visit.

Our cord blood samples were obtained by a method that could introduce higher proportions of maternal blood at the moment of collection. However, other diagnostic tests such as q-PCR in umbilical tissue, TESA-blot and repeated micromethod at each visit were performed in order to assure the infectious status of the cohort participants. Measurement bias may be an issue in this study although all the nurses performing the study were continuously trained for collection methods and measurement techniques.

Obtaining placental measurements accurately may be difficult and could introduce measurement bias. We tried to avoid this issue by continuously training the

study personnel in charge of obtaining measurements and using adequate measurement devices.

Subjectivity in the image readings is an important issue when assessing immunohistochemical staining. In this study, two independent readers rated the images and inter-observer and intra-observer agreement was evaluated obtaining high agreement percentages (Kappa 89% and kappa 92% respectively). Image assessment was masked for the readers regarding their diagnostic condition and *T. cruzi* vertical transmission status was only revealed when the statistical analysis was performed.

Supplemental Information- Immunohistochemical techniques for assessing tissues

Immunohistochemical standard procedures (Kim *et al.* 2016) are summarized in Table 14.

Table 14. Summarized standard steps and commonly used protocols for immunohistochemical staining

Step	Protocol
Fixation	10% Neutral buffered formalin for 24 hr in room temperature
	Frozen section: cold acetone for 1 min
Embedding and sectioning	Paraffin embedding
	Mostly 4 μm
	Frozen sections: between 4 μm and 6 μm in thickness
Deparaffinization and hydration	60°C hot plate
Antigen (or epitope) retrieval	Heat induced epitope retrieval is most widely used
Blocking	Normal sera of same species of secondary antibody or premixed
	Vary from 30 min to overnight, from 4°C to room temperature
Add primary antibody	Antibody dilution by protein blocking solution or premixed Ab diluents
	Appropriate antibody selection and titration
Incubate	30–60 min, room temperature
Wash (TBS-T)	3 × 5 min
Add secondary antibody	-
Incubate	30–60 min, room temperature
Wash	3 × 5 min, TBS-T
Add substrate	250 μL of 1% DAB, and 250 μL of 0.3% hydrogen peroxide to 5 mL of PBS, 1–3 minutes, room temperature
Wash	3 × 5 min, DW
Counterstain	Hematoxylin, 1 min

TBS-T, Tris-buffered saline and Tween 20; DAB, diaminobenzidine; PBS, phosphate buffered saline; DW, dextrose 5% in distilled water.

From (Kim *et al.* 2016)

A. Tissue handling and fixation

Tissues obtained for IHC should be managed quickly since ischemia for delayed processing results in degradation of proteins and activation of tissue enzymes and autolysis. (Kumar *et al.* 2005) Fixation of tissues from surgical procedures is usually done with 10% neutral buffered formalin. The time for fixation is recommended to be no longer

than 24 hours in room temperature. (Wolff *et al.* 2007, Cross *et al.* 1990) The suggested tissue to fixative ratio varies from 1:1 to 1:20. Frozen sections also requires fixation in certain situations such as standardizing new antibodies. (Engell *et al.* 2011)

B. Antigen (or epitope) retrieval

Epitopes from formaldehyde-based fixation tissues are likely masked due to cross-linking of amino groups and methylene bridges. (Fox *et al.* 1985) Antigen retrieval is needed to unmask the epitope in most of the cases, and if optimized it may restore antigenicity to the sample. Heat induced epitope retrieval is the most common method for antigen retrieval and a variety of methods are used to apply heat to the samples including microwave ovens, pressure cookers, water baths and heating plates. Variation on the pH is also widely used and ranging from pH6 to pH 10. The best combination of methods should be empirically tested and comparing the staining results of each attempt. Some epitopes require enzymatic retrieval, which include trypsin of proteinase for 10 minutes at room temperature or controlled temperature. All reactions were terminated by rinsing with neutral phosphate buffered saline (PBS).

C. Protein blocking

Background staining could be avoided by protein blocking. This step is usually performed by adding 5%–10% normal serum from the same species of secondary antibody and other agents including protein buffers such as 0.1%–0.5% bovine serum albumin, gelatin, or nonfat dry milk. This process requires an incubation time varying from 30 minutes to overnight and temperatures ranging from 4°C to room temperature. (Vogt *et al.* 1987)

D. Endogenous enzyme blocking

Endogenous peroxidase activity is usually blocked by adding diluted hydrogen peroxide as 3%. Levamisole is used to block for endogenous alkaline phosphatase prevalent in frozen tissue. Endogenous biotin activity may be also found and should be blocked by adding avidin solution. All these blocking steps require empiric identification of incubation times and are usually performed at room temperature. (Gadulescu *et al.* 2007, Garba *et al.* 1985)

E. Antibody selection and validation

Antibody selection should be based on the purpose of the experiment and the information available from previous IHC results. Polyclonal antibodies bind to different epitopes in a single antigen. On the other hand, monoclonal antibodies react to a single epitope in an antigen. When the antibody is validated, it is important to select proper positive and negative control tissues. Optimization is necessary to tune antibody dilution, incubation times, and blocking for controlled laboratory conditions.

F. Detection system

The most common detection systems are the avidin-biotin complex method, labeled streptavidin biotin method, phosphatase anti-phosphatase method, polymer-based detection system, and tyramine amplification system. The use of manual IHC versus automated IHC machines is another important issue when assessing reproducibility and reliability of the results. (Hou *et al.* 2014)

G. Counter staining

Hematoxylin is the most commonly used counterstain, although various vital stains are now being used. This staining provides better discrimination of the IHC signal and has an important role for pathologists identifying the cell type and localization of the positive IHC results.

H. Quantification of the data

Resulting data from IHC experiments should be expressed in numerical values for statistical analysis.

I. Combinative semi quantitative scoring

The most commonly used method in biomarker research is the combinative semi quantitative scoring which incorporate both quantitative and qualitative assessments. The intensity of the staining is commonly scored from 0 to 3 (0, negative; 1+, weak positive; 2+, moderate positive; and 3+, strong positive). Although researchers can evaluate the IHC results both quantitatively and qualitatively with this method, too many variations can be created according to the combinations, resulting in different interpretations between researchers. Four or five score levels on average are recommended for the best sensitivity and reproducibility of the scoring system. (Shackelford *et al.* 2012, Thoolen *et al.* 2010)

J. Quantification using spectral image analysis

The optical signal from each chromogen can be isolated and assessed separately and quantitatively providing automated numerical values for the staining. (Taylor *et al.* 2006)

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CURRICULUM VITA

A. EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Universidad Catolica de Santa Maria de Arequipa, Peru	BA	2002	Medicine
Universidad Catolica de Santa Maria de Arequipa, Peru	MD	2002	Medicine
Universidad Ricardo Palma	Diploma	2006	Administration and Mgmt Health Services
Universidad Nacional de San Agustin	Diploma	2006	Hospital Epidemiology
Johns Hopkins School of Public Health, Baltimore, MD	MHS	2010	International Health

B. Positions and Honors

2011 - 2013 Professor at the Medicine Department – Infectious Diseases and Clinical Research – Medicine Research Institute - Universidad Catolica Boliviana San Pablo

2011 – Present Project title : "Molecular Biomarkers for Chagasic myocardiopathy"

- Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH

2009 – Present Project title : "Early detection of Congenital Chagas Disease in Bolivia"

- Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH

2009 – Present Project title: "Pupilometry and dysautonomia in Chagas Disease and Diabetes in Santa Cruz"

- Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH

2008 – Present	Project title: "Comparative study in megacolon biopsies of Chagas disease seropositive and seronegative patients in Santa Cruz"
	• Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH
2008 – Present	Project title: "Comparative study in uterine muscle biopsies of Chagas disease seropositive and seronegative patients in Santa Cruz"
	• Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH
2008 – Present	Project title: "Comparative study in skeletal muscle and fat biopsies of Chagas disease seropositive and seronegative patients in Santa Cruz"
	• Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH
2008 – Present	Project title: "Chagas Disease Treatment compliance and side effects in Arequipa – Peru"
	• Research Assistant, study coordinator, ABPRISMA, UPCH, JHSPH.
2006 - Present	Project title : "Congenital Chagas' disease in Santa Cruz Bolivia"
	• Research Assistant, study coordinator, ABPRISMA, UPCH.
2006- Present	Project title: "Community-based Epidemiologic Study of Chagas' disease in Arequipa, Peru"
	• Research Assistant, ABPRISMA, -UPCH
2004 - 2006	Ministry of Health, Network Nr 3 AREQUIPA, ACLAS Yanahuara- SACHACA
	• Clinician, Family doctor
2002 – 2003	Ministry of Health AREQUIPA Health Network Nr. 1 Camana - Caravelí, P.S. Juan Pablo Vizcardo Y Guzmán,
	• Clinician. Chief of the Health Post, in charge of A.R.I, A.D.I and Qualification Programs

C. Contributions to Science

Circulating Serum Markers and QRS Scar Score in Chagas Cardiomyopathy. Clark EH, Marks MA, Gilman RH, Fernandez AB, Crawford TC, Samuels AM, Hidron AI, **Galdos-**

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