Understanding the Role of *Leishmania* RNA Virus-1 (LRV-1) in the Pathogenesis of American Tegumentary Leishmaniasis (ATL)

by

Ruwandi Marianne Kariyawasam

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Institute of Medical Sciences
University of Toronto

Understanding the Role of *Leishmania* RNA Virus-1 (LRV-1) in the Pathogenesis of American Tegumentary Leishmaniasis (ATL)

Ruwandi Marianne Kariyawasam

Doctor of Philosophy

Institute of Medical Sciences
University of Toronto

2020

Abstract

American Tegumentary Leishmaniasis (ATL) comprises a spectrum of cutaneous leishmaniasis (CL) and mucosal or mucocutaneous leishmaniasis (ML/MCL) endemic to Latin America. *Leishmania* RNA Virus-1 (LRV-1) is a double stranded RNA virus identified in up to 25% of clinical isolates of the *Leishmania Viannia* subgenus. Approximately 1-15% of patients with healed CL will progress to ML/MCL on average 1-5 years later, the biological underpinnings of which could be related to LRV-1, given its ability to alter expression of certain proinflammatory cytokines and chemokines in both human and murine models. This doctoral thesis sets out to evaluate: 1) the prevalence of LRV-1 in ATL and its potential associations with clinical phenotype; 2) changes in proinflammatory biomarker expression from human macrophages infected with LRV-1 positive and negative strains of *Leishmania Viannia braziliensis* and *L. V. panamensis* and 3) virulence factor (VF) RNA transcript expression in previously infected human macrophages. LRV-1 was detected at a rate of 23%-26% and was not directly associated with clinical phenotype. Age was associated with clinical phenotype, whereby patients manifesting ML/MCL were, on average, 10 years older than patients manifesting localized CL (LCL). LRV-1 did not alter pro-inflammatory biomarker expression of TNF-α, IL-1β, IL-6, CXCL10 and SOD at 24- and 48- hours in strains of *L. V. panamensis*, regardless of isolate

source. However, clinical *L. V. braziliensis* demonstrated significantly lower levels of TNF-α, IL-1β, IL-6 and CXCL10 at 24- and 48- hours compared to the ATCC® *L. V. braziliensis* strain. A direct comparison of clinical *L. V. braziliensis* strains, revealed LRV-1 to increase TNF-α and decrease CXCL10 at 48-hours. Analysis of VF RNA transcripts of heat shock protein 23 (HSP23), HSP70, HSP83, HSP100, mannose phosphate isomerase (MPI), cysteine proteinase B (CPB) and zinc metalloproteinase (GP63) revealed no difference in expression by LRV-1 status, however a marked increase at 24- and 48- hours post-macrophage infection was observed. In conclusion, LRV-1 status and causative species potentially represent a combined marker of immune responses as observed by differences in *L. V. braziliensis* and *L. V. panamensis* populations, possibly predictive of clinical phenotype.

Acknowledgments

"To whom much is given, much will be required," is a quote that has been ingrained in me since the American Society of Tropical Medicine & Hygiene (ASTMH) 66th Annual Meeting I attended in November of 2016. My desire to help those who are particularly less fortunate is a guiding force in my career and thus pursuing a graduate degree in an area of research defined as 'Neglected Tropical Diseases (NTDs)' was only fitting. Over the years I've met with individuals who tirelessly work in this field, day in and day out, despite all the obstacles and hurdles they face. When I think about my family upbringing and current living situation being in two very different parts of the world, I am reminded how lucky I am to live in a country such as Canada, knowing full well the adversities my cousins my age face in obtaining something so simple as an education in Sri Lanka. For this reason, I remind myself on a daily basis to strive to contribute to society in ways to help those who can't necessarily help themselves. I hope the work undertaken in this doctoral thesis may be a small contribution to the overall global picture of Leishmaniasis.

First and foremost, I'd like to thank my outstanding PhD supervisor and mentor, Dr. Andrea Boggild, for believing in me and more importantly, providing me with endless opportunities to advance my career throughout my graduate degree. We've known each other for almost 7 years now and every day I am continuously amazed at her passion to help individuals facing adversity, whether as patients or as trainees at any stage of their career. To my committee members Dr. Sharmistha Mishra and Dr. Samir Patel, thank you for taking the time to join my Program Advisory Committee (PAC). I am immensely grateful for your expertise and guidance not only for my doctoral degree, but my career development as well. This thesis was written during the COVID19 pandemic, and I am undoubtedly grateful to my entire committee who in addition to working on the front lines, continued their mentorship during these unprecedented times. I would

also like to thank external faculty member Dr. Mario Ostrowski for serving as an SGS external faculty member in assisting with thesis revisions on such short notice.

To our collaborators in Peru, Dr. Alejandro Llanos-Cuentas and Dr. Braulio Mark Valencia-it has been a pleasure and an honour to watch you practice and dedicate your lives to helping the many patients at the 'Leishmania Clinic' at the Institute of Tropical Medicine 'Alexander von Humboldt,' in Lima, Peru. Your expertise and dedication are invaluable and a privilege to have witnessed. I would also like to thank Laura and Anita for assisting with the collection of samples from the biobank. Most importantly, I'd like to thank all the patients enrolled in these studies-when I conduct this research I'm reminded of the faces I encountered during a field expedition to the highlands of Peru, where our team was met with patients beyond grateful for our presence.

My graduate training was supported by a number of funding bodies which I would like to thank for providing me with assistance over the years. Thank you to the School of Graduate Studies at the University of Toronto, the Institute of Medical Sciences at University of Toronto, Dr. Arnie Aberman and the Queen Elizabeth II Graduate Scholarship in Science & Technology, The International Society of Travel Medicine, the American Society of Tropical Medicine & Hygiene and the Para Tryp Research Organization for supporting my graduate student training and conference attendances.

I would like to acknowledge key members affiliated with the Boggild Lab, both past and present. In particular, I'd like to thank Rachel Lau for your guidance and direction on the bench at Public Health Ontario (PHO). You have been a huge rock and I thank you from the bottom of my heart for answering my questions without any hesitation. I'd like to thank the wonderful department of Parasitology at PHO, who have been supportive and so kind as to share their expertise and knowledge on the clinical diagnostic side. I'd also like to thank Jerry Grewal, Dr. Andrew

Purssell and Avinash Mukkala for all their help and assistance during experiments that were pivotal during my graduate degree. To Michael and Stefanie Klowak, thank you for your friendship, guidance and support during the writing stages of my thesis. To the friends I've made at PHO, past and present, including Philip Banh, Taryn Athey, Joanna Spinato, Sarah Teatero, Kirby Cronin, Michael Li, Matthew Wong-Fung, Vithusha Ravirajan, Andrew Green, Christopher Hoy, Tanya Vodoviz and Kelsie Jagt; our countless morning coffee runs, lunches and adventures made my graduate degree time fly. To my friends I've made through the Institute of Medical Sciences (IMS) at University of Toronto, especially Hira Raheel and Jonathon Chio-thank you for your constant encouragement and support that made graduate school that much more enjoyable.

Last but not least, I would like to thank my immediate support system. To my wonderful parents, Gunasiri and Shalini Kariyawasam-your unconditional love has been a strong foundational pillar in my life and your countless sacrifices have allowed me to achieve my dreams thus far-thank you from the bottom of my heart. I want to thank my brother and my sister, Dilshan and Nilushi Kariyawasam-we are the three STEM muskateers. The struggles we faced individually were endured with ease thanks to all the laughs and words of wisdom shared between us three as we all took on graduate studies. To my fiancé, Jonathan Persad-this has been a wonderful journey and if it wasn't for your constant support, understanding, patience and love, I wouldn't have been as successful as I am today. I'm thrilled to begin a new chapter in my life, and there is no one else I'd rather have alongside me than you. Thank you for also bringing our dog, Domino, into my life, who has been a wonderful writing companion. To my best friend, Rosilda Xavier-you have been one of my biggest supporters from day one. I thank you from the bottom of my heart for being an inspiration in my life by pursuing and following your dreams no matter what the cost. To all my friends, specifically Hershi Sritharan, Geetha Gnanapragasm,

Jeffrey Leon and Matthew Kinley, I thank you all for the words and wisdom and support during my graduate training. The proverb ‘It takes a village to raise a child,’ has never more been true-it took an entire village to create the scientist I am today.

Table of Contents

Contents

List of Abbreviations

Atypical Cutaneous Leishmaniasis (ACL)

American Tegumentary Leishmaniasis (ATL)

American Type Cell Culture® (ATCC®)

Antigen Presenting Cells (APCs)

Bacillus Calmette-Guerin (BCG)

Basic Local Alignment Search Tool (BLAST)

Chemotactic Factor 10 (CXCL0)

Complement Receptor (CR)

Concanavalin-A (Con-A)

Cutaneous Leishmaniasis (CL)

Cysteine Peptidases (CPs)

Cysteine Proteinase B (CPB)

Delayed Type Hypersensitivity (DTH)

Diffuse Cutaneous Leishmaniasis (DCL)

Direct Agglutination Test (DAT)

Disseminated Leishmaniasis (DL)

Ethylenediaminetetraacetic Acid (EDTA)

Enzyme-Linked Immunosorbent Assay (ELISA/EIA)

Fetal Bovine Serum (FBS)

Filter Paper Lesion Impression (FPLI)

Food and Drug Administration (FDA)

Glycosyphophatidylinositol (GPI)

Glycoinositolphospholipids (GIPLs)

G-protein coupled receptors (GPCR)

Hand-Held Exothermic Crystallization Thermotherapy (HECT)

Heat Shock Protein (HSP)

Human Immunodeficiency Virus (HIV)

Indirect Fluorescent Antibody (IFA)

Infectious Diseases Society of America (IDSA)

Internal Transcriber Space (ITS)

Intravenous (IV)

Intramuscular (IM)

Interferon (IFN)

Interleukin (IL)

Internal Ribosomal Entry Site (IRES)

Kinetoplastid membrane protein-11 (kmp-11)

Leishmania Chemotactic Factor (LCF)

Localized Cutaneous Leishmaniasis (LCL)

Leishmania Elongation Factor 1a (EF-1a)

Leishmaniasis Recidiva Cutis (LRC)

Leishmania RNA Virus-1 (LRV-1)

Leishmanization (LZ)

Liposomal Amphotericin B (L-AmB)

Lipophosphoglycan (LPG)

Mannose Phosphate Isomerase (MPI)

Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF)

Matrix Metalloproteainase 9 (MMP-9)

Meglumine Antimomnaite (10% $Sb^{V)}$

Micro RNAs (miRNAs)

Million Years Ago (MYA)

Mitogen-Activated Protein (MAP)

Montenegro Skin Test (MST)

Mucocutaneous Leishmaniasis (MCL)

Mucosal Leishmaniasis (ML)

Multilocus Enzyme Electrophoresis (MLEE)

Multiplicity of Infection (MOI)

National Center f or Biotechnology Information (NCBI)

Neglected Tropical Disease (NTD)

New World Cutaneous Leishmaniasis (NWCL)

Nitric Oxide (NO)

Old World Cutaneous Leishmaniasis (OWCL)

Open Reading Frame (ORF)

Peripheral Blood Mononuclear Cell (PBMC)

Peritrophic Matric (PM)

Phorbol Myristate Acetate (PMA)

Phosphatidyl Choline (PC)

Phosphoprotein Phosphatases (PPPs)

Public Health Ontario Laboratory (PHOL)

Metal-Dependent Protein Phosphatases (PPMs)

Polymerase Chain Reaction (PCR)

Point of Care (POC)

Polymorphonuclear Neutrophil Granulocytes (PMN)

Post Kala Azar Dermal Leishmaniasis (PKDL)

Proteophosphoglycans (PPGs)

Randomized Controlled Trial (RCT)

Reactive Oxygen Species (ROS)

Restriction Fragment Length Polymorphism (RFLP)

Roswell Park Memorial Institute (RPMI)

Sanger Sequencing (SS)

Serine/Threonine Phosphatases (STPs)

Sodium Stibogluconate (8.1% $Sb^{V)}$

Superoxide Dismutase (SOD)

Telomere-Associated Mobile Elements (TATE)

T Helped Cell Type (Th)

Toll-Like Receptor (TLR)

Transmission-Blocking Vaccines (TBVs)

Tumor Necrosis Factor-alpha (TNF-α)

Untranslated Region (UTR)

Uracil-DNA glycosylase (UDG)

Virulence Factor (VF)

Visceral Leishmaniasis (VL)

Zinc Metalloproteinase (GP63)

List of Tables

List of Figures

Literature Review

Overview

American tegumentary leishmaniasis (ATL) comprises a discrete set of clinical presentations of leishmaniasis endemic to Latin America. Due to its endemicity in rural impoverished areas of Latin America, along with other sociopolitical factors, it remains a neglected tropical disease (NTD). The affected patient population continues to grow in case numbers and individuals at risk due to a multitude of factors including, but not limited to, climate-change induced expansion of vector ranges and urbanization facilitating greater host-vector interactions. In the better resourced areas of the world, such as Canada, there are comparatively few travel related cases, however, clinicians remain conflicted over treatment decisions due to the paucity of data surrounding the prognosis and treatment outcomes in ATL. Answering questions regarding the pathogenesis of ATL will not only inform Canadian travelers and migrants both before and after travel, but will also contribute to our understanding of optimal control strategies in affected endemic areas. The goal of this work is to generate a new body of knowledge surrounding the role of *Leishmania* RNA virus-1 (LRV-1), a viral parasite of the protozoan *Leishmania* parasite, in the overall pathogenesis of ATL. By addressing questions around the prevalence of LRV-1 in ATL, the potential association between LRV-1 and clinical phenotypes of ATL, the immune responses to LRV-1-containing strains of *Leishmania*, and the effect of LRV-1 on parasite virulence factor RNA transcript expression, we aim to illuminate the role of LRV-1 as a potential biomarker of ATL clinical course and outcome.

I. GENERAL BACKGROUND OF LEISHMANIASIS

Introduction

Leishmaniasis is a morbid and potentially fatal protozoan parasitic infection classified as a neglected tropical disease (NTD) given its widespread endemicity and ability to cause substantial disease in millions of people in the developing world (World Health Organization, 2010). Three main clinical manifestations can arise after infection with one of over 18 human species of *Leishmania*, divided by geographical origin, including: cutaneous leishmaniasis (CL), mucocutaneous (MCL) or mucosal leishmaniasis (ML), and visceral leishmaniasis (VL) (Reithinger et al., 2007). Species of *Leishmania* are categorized into two main groupings: the *Leishmania* complex and the *Viannia* sub-genus exclusive to the New World. Between these two groups, human infection can arise through zoonotic or anthroponotic cycles (Reithinger et al., 2007). In Latin America, clinical presentations of CL, MCL and/or ML comprise what is known as American Tegumentary Leishmaniasis (ATL). Mainly transmitted by the female *Lutzomyia* and *Phlebotomus* spp. sandflies, it is estimated there are 1.5-2 million new cases of leishmaniasis annually, translating into 70,000 deaths with a total "at risk" population of 350 million (Reithinger et al., 2007). These numbers are likely underestimated due to the significant number of cases in remote areas and the social stigma associated with the disease (Reithinger et al., 2007). An accurate understanding of disease epidemiology is necessary for control and prevention as the rate of infection continues to grow in endemic areas. Disease expansion due to human and environmental factors including climate change, deforestation, urbanization, and the migration of human and vector populations to and from endemic areas all contribute to sustained transmission of leishmaniasis (Reithinger et al., 2007).

History

A vector borne disease caused by a parasite of the order Trypanosomatida and genus *Leishmania*, the first known clinical description in 1756 by Alexander Russell known as Aleppo boil coincided with a conundrum of clinical nomenclature including kala-azar, white leprosy, pian bois and dum-dum fever dating back to pre-Incan artefacts depicting skin lesions and facial deformities consistent with leishmaniasis (Bañuls et al., 2007; Steverding, 2017). The exact causative agent was published in 1903 by William Leishman who stained the spleen of a British solider exhibiting fever, anemia and splenomegaly in India only to identify amastigotes of the parasite (Gibson, 1983; World Health Organization, 2010). Shortly after, Charles Donovan recognized similar symptoms in patients presenting with kala-azar and soon after, the linkage to the protozoan parasite brought forth the first identified species, *Leishmania donovani* (Steverding, 2017).

Origin and Evolution

The origin and evolution of the parasite has long been debated with three main hypotheses: Palearctic, Neotropical, and the Supercontinental origin being the main arguments (Steverding, 2017). The genus *Leishmania* evolved in the Mesozoic era 252-66 million years ago (MYA) with the existence of *Leishmania*-like species documented in preserved Dominican and Burmese amber (Steverding, 2017). The parasites were identified in the proboscis of the female *Palaeomyia burmitis* and *Lutzomyia adiketis* sandflies in both stages of the parasite: the promastigote stage in the sandfly midgut and the amastigote form post-vertebrate infection (Poinar, G Jr., Poinar, R., 2004; Steverding, 2017). The Palaearctic hypothesis suggests that *Leishmania* originated from the Palearctic region including Europe, Asia north of the Himalayas, northern parts of the Middle East, and the northern parts of the Sahara desert in Africa 65-56 MYA (Kerr, 2000). The Neotropical hypothesis, as suggested by Lainson and Shaw in 1987,

argues a Neotropical origin given the diversity of New World species encompassing the *Viannia* subgenus (Lainson R, Shaw JJ 1987). The Supercontinent hypothesis was brought upon in 2000 by Momen & Cupolilli suggesting that the separation of the supercontinent Gondwana including Antarctica, South America, Africa, Madagascar, Australia, the Arabian Peninsula and the Indian Subcontinent resulted in an African origin for Old World species, and multiple introductions in South America creating the diversity seen in the New World *Viannia* subgenus (Momen & Cupolillo, 2000). The compelling Supercontinent hypothesis is supported by molecular phylogenetic data highlighting the deviation of *Leishmania*-like trypanosomatids from *Leishmania* species identified in sloth- and porcupine-like animals and mammals, respectively (Harkins et al., 2016). Given the presence of Old World species in Ethiopia and Kenya, including *L. aethiopica, L. donovani, L. infantum, L.major* and *L. tropica*, it is hypothesized that these species originated from East Africa alongside the evolution of humans (Momen & Cupolillo, 2000). Interestingly, the New World species *L. mexicana* shares characteristics with *L. major* and upon the breakup of Gondwana, the geographic, climatic, and ecological factors gave rise to the species *L. amazonensis, L. venezuelensis,* and *L. waltoni* (Momen & Cupolillo, 2000). Given the molecular synonymy between *L. infantum* and *L. chagasi,* it is hypothesized that *L. chagasi* evolved from *L. infantum,* being brought to South America by European settlers including dogs 500 years ago (Momen & Cupolillo, 2000; Shaw et al., 2015). The *Viannia* subgenus including *L. Viannia (V.) braziliensis, L. V. guyanensis, L. V. lainsoni, L. V. panamensis*, and *L. V. peruviana* developed in South America upon the separation of Gondwana and accelerated in evolution due to climate change and a comprehensive range of geographic isolation and mammalian host organisms (Momen & Cupolillo, 2000).

Parasite Life Cycle

The life cycle of *Leishmania* spp. translates into specific clinical manifestations, and the unique bipartite states of the intracellular amastigote in the mammalian host and the flagellated promastigote parasite in the sandfly allow it to thrive regardless of environmental stresses. Leishmaniasis results from the bite of female sandflies belonging to the *Phlebotomus* spp., found in Europe, North Africa, the Middle East, and Asia, and the *Lutzomyia* spp., found in South America up to the southern USA (Dostalova A &Volf P, 2012). Recently, the documentation of a new sandfly species, *Psathyromyia elizabethdorvalae* in Brazil creates growing concern for the expanding vector list given similar breeding and habitat frequencies to other *Leishmania*-carrying sandflies (Brilhante AF et al., 2017).

The sandfly midgut contains a singular epithelium with microvilli lining whereas the foregut and hindgut are lined by chitin (Dostalova A &Volf P 2012). Confined to the digestive tract of the female sandflies, the parasite stage known as the promastigote exists in the midgut where the temperature ranges from 25-27°C in a basic environment (Dostalova A &Volf P 2012). New World species of the *Viannia* subgenus enter the hindgut prior to migrating to the midgut and are often referred to as peripylarian parasites (Lainson RD &Shaw JJ 1977). The remaining species of the *Leishmania* complex typically reside in the midgut and are referred to as suprapylarian parasites (Dostalova A &Volf P 2012). After the initial uptake of blood from an infected mammalian host, the immotile, round, and unflagellated amastigotes ranging from 3-5 μm begin a morphological transformation in the midgut of the sandfly whereby procyclic flagellated promastigotes are formed and multiply, prompted by the decrease in temperature and increase in pH within the sandfly (Rogers et al., 2002). Seventy-two hours later, the procyclic promastigotes slow replication and differentiate into long, non-dividing nectomonad promastigotes that move into the anterior midgut. These promastigotes develop into leptomonad promastigotes that attach

to the midgut by anchoring themselves using the peritrophic matrix (PM) that generally surrounds the food bolus (Rogers et al., 2002). Prior to transmission, the metacyclic promastigotes detach from the midgut and exit through the proboscis of the sandfly as a result of the filamentous proteophosphoglycan secreted that facilitates detachment and allows for the parasite to disseminate into the next vertebrate during blood feeding (Dostalava A &Volf P 2012).

Clinical Symptoms

Visceral Leishmaniasis (VL)

VL, also known as kala-azar, is a life-threatening disease primarily caused by members of the *Leishmania* complex including *L. donovani* and *L. chagasi/L. infantum* with more than 90% of cases found in Brazil, Ethiopia, India, Somalia, and Sudan with 0.2-0.4 million cases per year (World Health Organization, 2010; Savoia et al., 2015). Often initially asymptomatic, malnutrition and the subsequent immune suppression has deadly consequences, particularly in HIV infected patients (Savoia et al., 2015). The dissemination of *Leishania* parasites throughout the reticuloendothelial system produces symptoms including fever, weight loss, hepatosplenomegaly, anemia, thrombocytopenia, and hypergammaglobulinemia, which is often life threatening without treatment (World Health Organization, 2010). Animal reservoirs, particularly canine populations in which sexual and vertical transmission occur, facilitate transmission of VL in densely populated areas and contribute to increasing rates of VL observed in these countries (Savoia et al. , 2015). Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL characterized by maculopapular rash in patients who have previously recovered from VL caused by *L. donovani.* This complication is mainly seen in 50% and 5-10% of cases from India and Sudan, respectively (World Health Organization, 2010; Zijlstra et al., 2003). PKDL generally follows 0-6 months and 2-3 years post VL in Sudan and India,

respectively, and highlights the immunological pathogenesis of the parasite, as discussed subsequently (Zijlstra et al., 2003).

Cutaneous Leishmaniasis (CL)

CL is the most common form of leishmaniasis, with 90% of cases identified in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria (Reithinger et al., 2007). Often caused by members of the Old and New World *Leishmania* complex and *Viannia* subgenus, the first sign of infection is typically a small erythematous papule, occurring weeks to months after the initial sandfly bite (Reithinger et al., 2007). Over time, these skin sores change in appearance from papules, to nodules and ulcers over a period of 2 weeks to 6 months, generally self-healing with time. Variability in self-cure is often attributed to the infecting species whereby lesions produced by *L. major* cure within 2-6 months, whereas lesions produced by *L. mexicana* and the *Viannia* subgenus spontaneously cure from 3-9 months and 6-15 months, respectively (Reithinger et al., 2007). The ulcerative lesions have been known to harbor a spectrum of clinical manifestations including multifocal or disseminated leishmaniasis (DL) and diffuse CL (DCL) associated with macopapular skin lesions identified in two or more anatomical sites ranging from 10-300 in number, and nodular non-ulcerated lesions on the face, limbs and back of patients (Guimaraes et al., 2016; Reithinger et al., 2007). Lastly, atypical cutaneous leishmaniasis (ACL) is primarily identified in immunosuppressed persons and manifests as unusual crusted, lupoid, sporotrichoid, or verrucuous ulcers (Guimaraes et al., 2016) Lymphatic involvement can also be documented, occasionally even before lesion development, and contributes to a more severe, clinically recalcitrant form of CL (Reithinger et al., 2007). Self-healing is correlated to immunity against future infection, however, recurring trauma to the previously infected site has resulted in relapse of infection for reasons not yet known (Reithinger et al., 2007).

Mucocutaneous and Mucosal Leishmaniasis (MCL/ML)

Commonly identified in Latin America, ML/MCL is often a sequel of the initial CL infection, affecting the mucous membranes of the upper airway tract. There are approximately 35,000 annual cases of ML/MCL documented primarily in Brazil, Peru and Bolivia, although these numbers are grossly underestimated (Savoia et al., 2015). Predominantly caused by members of the *Viannia* sub-genus including *L. V. braziliensis, L. V. guyanensis* and less so the *L V. panamensis, L. amazonensis, L. infantum, L. tropica* and *L. major* species, the parasite metastasizes to mucous tissues through the lymphatic or haematogenous pathways. This leads to nasal inflammation or stuffiness at first, followed by the ulceration of nasopharyngeal mucosa and perforation of the septum (Reithinger et al., 2007; World Health Organization, 2010). Asymptomatic primary infection leading to ML/MCL development has also been documented as well as presentation after 20 years of the initial cutaneous lesion (Jara et al., 2016; Valencia et al., 2014; World Health Organization, 2010). Unlike CL, ML/MCL does not heal spontaneously and, as a consequence of frequent secondary bacterial infection that makes treatment more difficult, is potentially life-threatening (Reithinger et al., 2007). Although the reasons why patients develop MCL/ML is still unclear, it has been shown that the anterior nasal septum provides ideal conditions for the development of amastigotes, where lower temperatures impair macrophages from destroying parasites (Lessa et al., 2007). ML/MCL is often associated with an older patient population, given the large gap between healing of CL and presentation of ML/MCL. In a study assessing 327 patients with ML from 1995 and 2014, median age of patients was 38.5 years and a median time of 6 years between the onset of CL and diagnosis of ML (Cincura et al., 2017). This gap in presentation has been noted in a number of studies and reflects the natural course of infection in this patient population.

American Tegumentary Leishmaniasis (ATL)

American tegumentary leishmaniasis (ATL) is a subcategorization of CL and MCL/ML of New World origin identified in Central and South America. Chile remains the only country in South America with no reported cases of ATL (World Health Organization, 2010). With an annual incidence of 1-1.5 million cases, the disease is diagnosed in 3.3% of tourists visiting Latin America, yet is predominantly found in male agricultural workers, followed by students, domestic homemakers, and lastly, children (World Health Organization, 2010). New World *Leishmania* complex and members of the *Viannia* sub-genus are causative agents of ATL whereby *L. V. braziliensis* followed by *L. amazonensis, L. mexicana, L. V. guyanensis* and *L. V. panamensis* are the most prevalent, depending on geographic origin (World Health Organization, 2010). Given the complexity of species identification, particularly in under-resourced areas of the developing world, the overall prognosis of ATL by infecting species remains poorly understood, and limited to case reports and the collective anecdotal experiences of medical personnel with ATL-affected patients (Reithinger et al., 2007). In a study evaluating ATL from the Brazilian Amazon between 2010 and 2014, the highest prevalence of ATL was identified in men who were 20-40 years of age, of which 95% of cases were CL (Teles et al., 2019).

Transmission of ATL is often zoonotic unlike the anthroponotic nature of VL transmission in India and Africa, given the large number of vertebrates identified as reservoirs including: sloths, opossums, rice rats, agouti and rats (World Health Organization, 2010). A spectrum of clinical manifestations within American CL and ML exist as a result of the variety of causative species, whereby immunocompetent patients tend to exhibit LCL, whereas immunocompromised patients tend to exhibit unusual forms of CL including multifocal/disseminated CL, ACL and MCL/ML. Leishmaniasis recidiva cutis (LRC) is a rare form of ATL characterized by papular lesions in or around the healed scar produced by the *Viannia* subgenus, however in Brazil and Ecuador, the

causative species include: *L. amazonensis* and *L. V. pananensis* (World Health Organization, 2010). DCL is characterized by non-ulcerating nodular lesions as a result of a poor cell-mediated immune response (World Health Organization, 2010). Generally caused by *L. amazonensis* and *L. mexicana*, these lesions have the potential to ulcerate after trauma and can invade the nasal mucosa. Disseminated leishmaniasis (DL) is characterized by multiple lesions in two noncontiguous regions of the body, and in 29% of cases, mucosal involvement is found (Reithinger et al., 2007; World Health Organization, 2010). *L. V. naiffi* has been reported in certain areas of Brazil, demonstrating treatment failure and reduced spontaneous healing in a case series of 30 patients, where 8 (27%) were infected with *L. V. naiffi* (Fagundes-Silva et al., 2015).

Diagnosis of CL and MCL/ML

Correct diagnosis of leishmaniasis is important as the disease is clinically indistinguishable from many other co-endemic entities including mycobacterial and fungal infections (Elmahallawy et al., 2014; Martinez DY et al., 2018). Furthermore, infecting species weighs heavily on the course of infection, and thus guides treatment recommendations. Time of diagnosis is also an important factor to prevent dissemination of the disease as early as possible, and is a problem in both the developed and developing worlds. As resourced countries have access to reference laboratories, time of diagnosis may be delayed as a result of medical personnel being able to correctly identify the parasitic infection, whereas diagnosis in the developing world can be delayed due to limited diagnostic tools and basic access to healthcare. Moreover, secondarily infected dermal lesions require combination therapy to reduce the risk of potentially fatal secondary infections (Elmahallawy et al., 2014).

Parasitological Diagnostic Testing

Parasitological diagnoses including culture and microscopy remain the gold standard (Elmahallawy et al., 2014; World Health Organization, 2010). Microscopic examination of Giemsa-stained biopsy smears, aspirates, scrapings or impressions is the most common diagnostic tool used given the relatively inexpensive nature and availability in many health-care facilities in both endemic and non-endemic countries (Elmahallawy et al., 2014). The search for amastigotes visualized as round bodies 2-4 µm in diameter with visible nuclei and kinetoplasts can be rather difficult depending on the specimen (Elmahallawy et al., 2014). In South America,

the presence of amastigotes in Giemsa-stained lesions is sufficient to confirm the diagnosis, however, sensitivity varies. Data around intra-site sampling within the ulcer are conflicting, with some studies demonstrating higher parasite loads at the base of the ulcer with fewer parasites in the center or border (Elmahallawy et al., 2014), while other studies have found the inverse whereby median parasite loads were significantly higher in the base and center of the ulcer as opposed to the border (Suárez et al., 2015). Moreover, sensitivities decline from acute lesions (duration ≤ 3 months, to subacute (duration >3 to <12 months) and chronic lesions (duration ≥ 12 months) (Boggild et al., 2010). In addition, sensitivity has been found to vary by sampling method including cytology brushes, scrapings and biopsies (Suárez et al., 2015) Generally, positive cultures enable species identification, however, sensitivity is quite low and the culture process is time-consuming (with turnaround time of up to 30 days), thus, awaiting a positive culture can have potentially devastating effects for the patient (Boggild et al., 2010). Numerous aspirates can be taken from different lesions or portions of the ulcers for culture and the combination of microscopy can increase sensitivity, however, this causes much pain to the patient and poses a sharps risk to staff in the developing world (Boggild et al., 2010; Boggild et al., 2011; Valencia et al., 2012). Non-invasive diagnostic testing including the use of cytology

brushes and filter paper lesion impressions (FPLIs) for CL, and cytology brushes for ML/MCL, both of which have produced comparable sensitivities and specificities to invasively obtained specimens such as biopsies and aspirates, and should be considered as a very good alternative given the low cost, and low risk of harm for both patient and health care workers (Boggild et al., 2011; Boggild et al., 2010, 2011; Valencia et al., 2012).

Serological Diagnostic Testing

Serological diagnosis relies on the presence of a humoral response and host antibodies can be detected using enzyme-linked immunosorbent assay (ELISA/EIA), indirect fluorescent antibody (IFA), or direct agglutination test (DAT). The former two tests are restricted to use in well resourced areas with supported laboratory infrastructure, meanwhile the latter may be used in endemic settings, however, long incubation times are required (Elmahallawy et al., 2014). The utility of serology is confined to VL given low titer or undetectable levels due to a poor humoral response in CL and MCL/ML (Elmahallawy et al., 2014; World Health Organization, 2010). Diagnostic success rates are often higher for the diagnosis VL where the parasite load is quite high in splenic or bone marrow samples, and present in liver biopsies, lymph node and peripheral blood for microscopy or culture (Elmahallawy et al., 2014). Antigen detection including latex agglutination testing has been previously described for urine specimens, however, sensitivity is low and the test is particularly used for immunocompromised patients whose level of antibody production would be too low to detect on conventional serologic assays.

Molecular Diagnostic Testing

Molecular based detection assays have been used over the past decade extensively, however, they are limited to regions with substantial laboratory infrastructure, technical expertise, and funding for platform maintenance and consumables. PCR-based methods including real-time and

end-point PCRs along with restriction fragment length polymorphism (RFLP) analysis and Sanger sequencing (SS) improve sensitivity in specimens with low parasite load and can be used on a wide array of specimens including FPLIs (Boggild et al., 2010, Boggild et al., 2011) and cytology brushes (Boggild et al., 2011, Valencia et al., 2012, Jara et al., 2016). Current targets evaluated include internal transcribed spacer 1 (*its1), its2,* heat shock protein 70 (*hsp70),* cysteine proteainase b *(cpb)* and mannose phosphoate isomerase (*mpi)* (Valencia et al., 2012; Montalvo et al., 2012; de Almeida et al., 2011; Schonian G et al., 2003; Zhang et al., 2006). Multilocus enzyme electrophoresis (MLEE) is another method of molecular detection targeting multiple metabolic enzymes requiring cultured isolates, and is time consuming with species identification results reported months from initial culture submission (Reithinger & Dujardin, 2007). Proteomic analysis including Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry have also been shown to improve diagnostics however performed on cultured isolates rather than direct specimen (Cassagne et al., 2013). More recently the use of Whole-Genome Sequencing (WGS) has been evaluated as a diagnostic tool for species identification given its increasing cost-efficiency and ability to differentiate mixed and hybrid infections that also impair the current diagnostic algorithm (Lau et al., unpublished). However, these technologies are limited to areas with strong laboratory infrastructure and technical expertise. The rising issue of species misidentification has been documented in a comparative study of 16 laboratories across Europe and Asia (Tsokana & Athanasiou, 2015; Van der Auwera et al., 2016). Overall, there is a need for improvement for diagnostic testing, particularly the use of simpler diagnostic tools for field use that would be cost and time beneficial to the patient. Point-of-Care (POC) diagnostics may continue to be an ideal test in resource-limited settings however more work is required to appropriately diagnose leishmaniasis in many parts of the world where mimickers exist.

Treatment

Treatment for leishmaniasis in well-resourced settings is generally administered following confirmation of the diagnosis, however in many endemic parts of the world where laboratory infrastructure is poor or often lacking, empiric treatment may be given depending on severity of disease. Regardless, treatment is limited to expensive drugs with substantial toxic side effects. As such, supportive treatment and hospitalization is often required alongside supervision by medical personnel. For decades, pentavalent antimonials such as sodium stibogluconate (8.1% Sb^V) and meglumine antimoniate (10% Sb^V) have been the standard first-line treatments in many parts of the world, where the drugs are administered intramuscularly or intravenously (Ponte-Sucre et al., 2013; World Health Organization, 2010). However, numerous hepatic, cardiac, nephrologic, hematologic, and pancreatic adverse-effects have been observed leading to severe toxicity and death (World Health Organization, 2010). To date, the only US Food and Drug Administration (FDA)-approved medications for the treatment of leishmaniasis include intravenous liposomal amphotericin B (L-AmB) for VL, and oral miltefosine for CL, ML/MCL, and VL depending on the causative species (Aronson et al., 2016). Other drugs including paromomycin, pentamidine isethionate, and azoles including ketoconazole, fluconazole, and itraconazole have all been used with variable efficacy depending on infecting species and clinical manifestations (Aronson et al., 2016). In addition, the use of thermotherapy in South America has been increasingly popular in Peru where low-cost heat packs such as the hand-held exothermic crystallization thermotherapy for cutaneous leishmaniasis (HECT-CL) relies on sodium acetate inside to produce heat of 52°C for 3 minutes with cure rates up to 60%, which is comparable to antimonials, but more successful in cases of patient relapses (Valencia, Miller, Witzig, Boggild & Llanos-Cuentas, 2013). Preventative measures including vaccination and chemoprophylaxis are non-existent for travelers

or persons requiring blood transfusions, and the preventative measures to minimize sand fly bites mirror those used for the prevention of mosquito bites (Aronson et al., 2016).

Pentavalent Antimonials (Sb^V)

Compounds such as sodium stibogluconate and meglumine antimoniate are the first-line anti-leishmanial treatment regimens for over half a century (Herwaldt et al., 1999; Carvalho et al., 2019). These drugs are highly effective with similar mechanisms of action, however are limited by parenteral administration, requirement of long durations of therapy, high number of toxic side effects and cost (Herwaldt et al. 1999; Carvalho et al., 2019). Pentavalent antimonials exhibit their antileishmanial activity through the disruption of topoisomerases required for DNA replication (Carvalho et al., 2019). Moreover, when Sb^V is reduced to Sb^{III} within the amastigote through natural chemical reactions, the trypanothione reductase system is inhibited which prevents parasite survival and overall virulence (Carvalho et al., 2019). Furthermore, pentavalent antimonials modulate host immune responses by increasing the levels of circulating pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and TNF-α (Carvalho et al., 2019). The recommended dosage ranges from 10-20 mg/kg/day for a period of 20-30 days (Oliveira et al., 2011). To date, systemic pentavalent antimony is the first-line treatment for leishmaniasis, with an average cure rate of 77% for American CL (Galvao et al., 2017). In a systematic review assessing the adverse effects of treatment of New World CL, the most frequently reported clinical adverse events of 2900 patients were musculoskeletal pain, nausea, vomiting, diarrhea, abdominal pain, headache, anorexia, asthenia, fatigue, fever, exanthema, erythema and urticaria (Oliveira et al., 2011). A systematic review of meglumine antimoniate administration has shown that systemic meglumine antimoniate is more effective than intralesional meglumine antimoniate (Carvalho et al., 2019). This can be attributed to the differences in bioavailability for the oral,

intravenous (IV) and intramuscular (IM) routes, whereby the IV and IM routes have very high bioavailability of the drug compared to oral (Carvalho et al., 2019).

Amphotericin B (L-AmB)

L-AmB is a lipid-based formulation of an antifungal agent against many clinically relevant yeasts and moulds including *Candida* spp. and *Aspergillus* spp. (Moen et al., 2009). The antileishmanial mechanism relies on the drug-binding to the parasite ergosterol precursors such as lanosterol, thereby disrupting the parasite membrane (Croft et al., 2006). L-AmB is FDA-approved for the treatment of VL and administered intravenously over 2 hours every day for several infusions, with mild infusion reactions and occasional nephrotoxicity or thrombocytopenia documented (Aronson et al., 2016; World Health Organization, 2010). Lately, regional efficacies in response to L-AmB have been documented thus recommended treatment regimens have been outlined by organizations including the World Health Organization (World Health Organization, 2010). Guidelines indicate a daily dose of 3-5 mg/kg for 3-5 days for a total dosage of 15 mg/kg by infusion for the treatment of VL by *L. donovani* in Bangladesh, Bhutan, India, and Nepal (World Health Organization, 2010). In contrast, VL caused by *L. donovani* in Yemen and East Africa including Ethiopia, Eritrea, Kenya, Somalia, Sudan, and Uganda necessitates a dosage of 3-5 mg/kg given over 6-10 days for a total dosage of 30 mg/kg (World Health Organization, 2010). Lighter dosages are used for PKDL occurring in East Africa and VL caused by *L. infantum* in the Mediterranean Basin, Middle East, Central Asia, and South Asia, whereby a 2.5 mg/kg dose for 20 days and 3-5 mg/kg dose over a 3-6 day period totaling 18-21 mg/kg are advised, respectively (World Health Organization, 2010).

L-AmB is recommended for the treatment of CL associated with increased risk for ML/MCL, however experimental use of L-AmB for the treatment of CL alone or in combination with other

drugs has been shown through limited individual case reports and series where no standard dosage regimens have been established (Aronson et al., 2016). An option favoured by most tropical medicine experts is that which is outlined by Schwartz and colleagues (Solomon et al., 2013), consisting of 6 doses of 3 mg/kg/d, with additional weekly doses out to 7 weeks as needed. Varying species-specific effects have been observed in Israel, Brazil, and other parts of Latin America (Balasegaram et al., 2012). Similarly, sparse data surround the efficacy of L-AmB for treatment of ML/MCL with cute rates of 51-88% using a 2-3 mg/kg dose regimen up to 40-60 mg/kg total dosage as outlined by WHO (Aronson et al, 2016; Balasegaram et al., 2012). The WHO recommends, however, the use of L-AmB for relapse of CL whereby a 3 mg/kg dosage by infusion is recommended up to 20-40 mg/kg total (Aronson et al., 2016; World Health Organization, 2010).

Miltefosine

Miltefosine, licensed and marketed under the brand name Impavido in the United States, is an anti-cancer agent with efficacy against protozoan parasites including *Trypanosoma* spp., *Entamoeba histolytica* and *Acanthamoeba* spp. (Sundar & Olliaro, 2007). Miltefosine is an alkyl-lysophospholipid effective against both promastigotes and amastigotes, inducing apoptosis through the inhibition of the phosphatidyl choline (PC) synthesis, which is required for maintaining cell membrane integrity and involved in signaling molecules (Sundar & Olliaro, 2007). Miltefosine has been associated with numerous gastrointestinal side-effects, renal, and hepatic toxicities, and cannot be prescribed to pregnant women or women planning imminent conception due to teratogenic effects (World Health Organization, 2010). Currently, oral miltefosine is FDA-approved for the treatment of CL, ML/MCL and VL caused by particular species (Aronson et al., 2016). For treatment of VL, a 94% cure rate with miltefosine has been observed particularly in the Indian subcontinent (World Health Organization, 2010). In the New

World, it is FDA-approved for CL caused by all *Viannia* species and ML/MCL caused by *L. V. braziliensis* (Aronson et al., 2016). Anecdotally, miltefosine is becoming increasingly favoured for CL imported to North American and Europe from Central America due to lower cure rates among those treated with L-AmB for CL due to *L. V. panamensis*. Consequently, it is FDA approved for the treatment of CL and ML/MCL due to *L. V. braziliensis* and *L. V. panamensis* in patients < 12 years in the US (Aronson et al., 2016; FDA, 2014).

Pentamidine

Similar to pentavalent antimonials, pentamidine has been used for the systemic treatment of CL due to *L. V. guyanensis* and *L. V. panamensis* in North America.However, limited use for ML/MCL (Aronson et al., 2016). Conflicting studies have shown comparable or superior results to pentavalent antimonials in trials conducted in Suriname and Colombia, however a consensus for high-dose vs low-dose schedules have yet to be established (Aronson et al., 2016).

Paromomycin

Paromomycin is an aminoglycoside antibiotic with limited use in local therapies including heat and cryotherapy for the treatment of CL in species not associated with ML/MCL (Aronson et al., 2016). The mechanism of action is typically via inhibition of protein synthesis through binding of the 16S rRNA of the 30S ribosome subunit in bacteria, however, this is not well described for *Leishamnia* spp. (Croft et al., 2006). Paromomycin preparations include 15% paromomycin coupled with either 12% methylbenzethonium chloride (MBCL) or 0.5% gentamicin cream (WR 279, 396), and is limited to use in CL caused by *L. major* and *L. tropica* or *L. V. panamensis* in Colombia and Panama (Aronson et al,. 2016). WR 279, 396 has demonstrated response rates of 81%-94% in *L. major* infection after a 20-day course of once-daily applications in phase 2 and phase 3 clinical trials (Aronson et al., 2016). Although not

commercially available or FDA-approved, WR279, 396 is available for for US military healthcare beneficiaries (Aronson et al., 2016)

Azoles

Azoles including ketoconazole and itraconazole inhibit the C14α-demethylase with varying efficacies whereby *L. V. braziliensis* is relatively insensitive to ketoconazole, while *L. mexicana* is not (Croft et al., 2006). Oral azoles including ketonazole and fluconazole are also considered systemic treatment options available in North America for the treatment of CL, with fewer adverse events compared to L-AmB or miltefosine (Aronson et al, 2016). Fluconazole has currently entered clinical trials and has proven efficacious in very high dosages, particularly against *L. major* and *L. V. braziliensis* (200 mg daily for 6 weeks), however is not FDA-approved (Aronson et al., 2016; Croft et al., 2006; Sousa et al. 2011). Similarly, ketoconazole has been shown modest efficacies against *L. mexicana* and *L. V. panamensis* with a regimen of 600 mg daily for 28 days (Aronson et al., 2016).Further work is required given limited data surrounding the required blood serum levels needed to treat CL and MCL/ML infections with azoles (Finch RG, 2010). A recent systematic review of azole therapy for ATL showed an overall efficacy of 64% (CI 95%: 57%-70%) for treatment with fluconazole, ketoconazole and itroconazole, with varying efficacies by species (Gavao et al., 2017). Data surrounding itraconazole, posaconazole and voriconazole are not yet available (Aronson et al., 2016).

Cryotherapy and Thermotherapy

Thermotherapy delivers high temperature heat to lesions for several minutes, generally 50°C for 30 seconds (Ameen et al., 2010). Large studies including an RCT for the treatment of *L. tropica* CL in Afghanistan and Colombia have shown comparable cure rates to intralesional antimonials, however with fewer treatment sessions required (Ameen et al., 2010).

Wound Care

Simple wound care has been favoured as a therapeutic intervention for CL due to *L. major*, *L. mexicana* or *L. tropica* given the spontaneous resolution reported in these species in the absence of secondary bacterial infections (Showler AJ & Boggild AK, 2015; Bailey & Lockwood 2007; Ben Salah et al., 1995; Dowlati, 1996). Single-lesions have demonstrated re-epithelialization weeks to months using cleaning and simple wound-dressing in *L. major*, *L. mexicana* and *L. tropica* (Showler AJ & Boggild AK, 2015; Bailey & Lockwood 2007; Ben Salah et al., 1995; Dowlati, 1996). In a study evaluating French travelers, washing of the ulcer and wound dressing resulted in complete re-epithelialization in 92% of patients (Morizot G et al., 2013).

Immunotherapy

With the success seen in cancer therapy, immunotherapy for CL is based on the protective immune responses required for complete cure (Ameen et al., 2010). Relapsing forms of leishmaniasis are commonly encountered, with PKDL and ML/MCL. It is thought that drug therapy may be useful in reducing parasite burden while immunotherapy may be used to sustain effector immune responses necessary to ensure eradication of disease (Ameen et al., 2010). In the 1990s, killed *L. V. braziliensis* promastigotes in combination with Bacillus Calmette-Guerin (BCG) have been shown to induce a Th1 immune response (Ameen et al., 2010). A trial in Iran has demonstrated BCG in combination with killed *L. major* promastigotes to treat 30 cases of PKDL, with a 100% cure rate (Ameen et al., 2010).

Imiquimod is an immunomodulatory agent activating toll-like receptor 7 (TLR7) and TLR8 to induce the production of many pro-inflammatory cytokines including IFN-γ, TNF-α and IL-12, aimed at providing a Th1 response (Miranda-Verastegui C et al., 2009; Miranda-Verastegui C et al., 2005). In addition, it has been shown to stimulate nitric-oxide production in macrophages to

further killing of *Leishmania* amastigotes (Ameen et al., 2010). In a placebo-controlled double blinded study in Peru, a higher cure rate was observed with topical imiquimod compared to a standard 20-day course of parenteral antimonials (Ameen et al., 2010).

New Drug Development

New drugs are slowly being evaluated to replace the half a century old drug discovery pipeline for the treatment of leishmaniasis. Given its NTD classification, there are very small strides in new drug discovery, however there is a promising future for newly discovered plant compounds that have demonstrated efficacy against infection in a number of animal and *in vitro* models.

Despite the lack of new drug development, there have been a number of drugs used to treat other diseases with known anti-leishmanial properties. Azithromycin, a macrolide antibiotic, has demonstrated efficacy against *L. major* in Old World Cutaneous Leishmaniasis (OWCL), however sub-par efficacy in New World Cutaneous Leishmaniasis (NWCL) when compared to antimonial therapies (Ameen et al., 2010). Tamoxifen is an anti-estrogen cancer agent used for the treatment and prevention of breast cancer, and a number of *in vitro* studies demonstrating its efficacy in reduction of lesion size and parasite burden. Similarly, pyrazinamide, an anti-tuberculosis chemotherapy drug has similar *in vitro* efficacy. Overall, there is a significant need for novel drug development to combat this NTD.

Prevention of ATL

Successful control of leishmaniasis relies on a combined effort targeting the human host, the parasite, sandfly vectors, and the animal reservoirs. Despite the establishment of treatment guidelines for North American travelers to endemic countries, recommendations should be in place taking into account national and regional health care policies, particularly in the developing

world. In addition, there is substantial need for sufficient guidelines primarily regarding children, pregnant women, and immunosuppressed patients.

Currently there are no vaccines available for leishmaniasis, however, there have been significant developments regarding experimental animal models and strong immunity in patients recovering from infection. First-generation vaccine candidates are derived from whole killed parasites or extracts and include: Mayrink vaccine (*L. amazonensis* derived vaccine from Brazil), Convit vaccine (*L. mexicana* derived vaccine made in Venezuela given in combination with BCG) and Razi Institute vaccine (*L. major* vaccine given with BCG) (World Health Organization, 2010). Second-generation vaccine candidates are based on recombinant proteins, including Leish-111f + MPL-SE, an antigen-based vaccine efficacious in experimental animals and currently being evaluated in phase 1 and 2 clinical trials in Peru and India (Maspi, Abdoli, & Ghaffarifar, 2016). Prophylactic vaccines have yet to be evaluated, however, many genetically modified strains of *Leishmania* have shown protective responses in mice however much research is needed prior to engaging in clinical trials. The older practice of leishmanization (LZ), whereby young children were inoculated with exudate of an active lesion to develop a self-healing lesion offering protection against future protection, sets a precedent for immunologic memory and vaccination (Khamesipour et al., 2005). The inoculation of live virulent Old World *Leishmania* strains has shown to offer protection against further lesions on the face and other exposed areas of the body, in parts of the Middle East including Iran, Iraq and Uzbekistan. (Khamesipour et al., 2005; Nadim, Javadian, & Mohebali, 1997). The practice of LZ is restricted to parts of the world where strains of *Leishmania* cause mild self-healing clinical disease, and would never be practised in areas known to transmit strains associated with complications of cutaneous infection, such as the New World. Peptide vaccines for the prevention and treatment of leishmaniasis is promising, as they are easier to produce and are more stable than whole-attenuated organisms (De Brito et al.,

2018). These vaccines can generate specific immune responses and designed with multiple epitopes to be effective (De Brito et al., 2018).

At present, immunomodulators are not recommended for routine use given the unstable immune responses seen in patients presenting with leishmaniasis. Leishmanial antigens including ion transporters such as macrophage protein 1 (NRAMP1/SLC11A1) are being targeted as potential therapeutics given their role in controlling ion concentrations that are crucial for various processes involved in the immune responses of macrophages (Bezerra de Menezes, Guedes, Petersen, Fraga, & Veras, 2015).

Active case detection where health workers reach out to the community to screen and document cases of leishmaniasis could aid in the elimination strategy as seen by the successful campaigns in the Indian subcontinent. The house-to-house search, camp or incentive-based approaches have all shown to reduce disease transmission through early diagnosis and treatment and shortening the infectious period of the patients themselves. The majority of case detection in South America is passive (patients seeking care), however, surveillance measures should be implemented in these areas to inform public health policies that have wide-spread population effects. Vector control methods including chemicals such as insecticides or indoor residual spraying, environmental management through destruction or modification of sandfly breeding sites, and personal protective measures such as clothing and repellants, could all assist in control of leishmaniasis, however, the potential environmental effects of eliminated vector species warrant consideration.

The majority of *Leishmania* eradication efforts are predicated upon parasite-mammalian host interactions, however the parasite-vector interactions are now becoming increasingly studied. Potential vaccines including the use of vector-based transmission-blocking vaccines (TBVs)

focus on targeting developmental molecules of the parasite inside the sandfly midgut or targeting molecules expressed by salivary glands or midgut of the vector that have previously been identified in malaria control, and have potential in preventing leishmaniasis (Coutinho-Abreu & Ramalho-Ortigao, 2010). With the expanding geography and vector populations, it is imperative that concerted efforts are geared at the prevention and treatment of leishmaniasis.

II. LEISHMANIA IMMUNOLOGY

The host immune response plays a significant role in determining course of infection and treatment outcomes. The efficacy of antimonials has been attributed to the underlying host immune response, therefore the use of certain immunomodulating agents without understanding the patient's immune profile may contribute to the weak or refractory responses to antileishmanial therapy (Hartley et al.,, Kohl, Ronet, & Fasel, 2013). 'Immunophenotypes' could help serve as predictive and prognostic biomarkers of disease severity and treatment outcome, and could be of significant value given the high endemicity in populations concurrently exposed to severe malnutrition and other disease states (Hartley et al., 2013).

Following the sandfly bite, vasodilation occurs whereby the parasite may be killed in a number of ways: 1) leukocytes are recruited to kill the parasite; 2) promastigotes infect macrophages and are subsequently killed intracellularly; and 3) promastigotes infect polymorphonuclear neutrophil granulocytes (PMNs) to induce a signaling cascade resulting in the chemoattraction of macrophages, thus effectively killing the parasites (Roychoudhury K, Roy S, 2004) (Figure 1). PMNs are primary effector cells responding to acute inflammatory reactions, where destruction of the parasite occurs through proteolytic enzymes stored in granules and via the production of reactive oxygen species (ROS) (Laskay, Van Zandbergen, & Solbach, 2003). Interestingly, some PMNs have been referred to as Trojan horses whereby *L. major* parasites not killed by the

proteolytic enzymes use the granulocytes to multiply and silently deliver themselves to macrophages, capitalizing on *Leishmania* chemotactic factor (LCF) (Figure 1) (Laskay et al., 2003). PMNs are also involved in the recruitment of Th1 cytokines and chemokine contributing to the overall resistance which provides an escape mechanism for the parasite (Laskay et al., 2003).

Cytokines & Chemokines

Chemokines are short chemotactic cytokines divided into four classes based on cysteine residues: α and β chemokines, fractalkines, and lymphotactins (Roychoudhury K et al., 2004). Leukocyte recruitment is controlled by chemokines that interact with members of the 7-transmembrane-spanning G-protein coupled receptors (GPCRs) of targeted cells. Chemokines including CCL5 and CXCL10 have been implicated in viral infections such as HIV, as well *Leishmania* spp. (Steinke & Borish, 2006). Cytokines are derived from mononuclear phagocytic cells and other antigen-presenting cells (APCs) involved in inflammation and cellular infiltration (Steinke & Borish, 2006). Cytokine production can occur via: 1) antigen uptake by APCs which leads to processing and presentation to T-helper lymphocytes; or 2) monocyte triggering through the innate immune system (Steinke & Borish, 2006).

Th1/Th2

The control of *Leishmania* infection has been attributed to a dichotomous Th1/Th2 balance, particularly in mice (M.-A. Hartley, Ronet, Zangger, Beverley, & Fasel, 2012; Roberts et al., 2006). Classically, Th1 cytokines and chemokines are involved in cell-mediated immunity whereas Th2 cytokines and chemokines promote antibody responses (Borish & Steinke, 2003; Roberts et al., 2006). The $CD4^+$ Th1 response is characterized by IFN-γ, IL-12, IL-1β production whereas a Th2 response is characterized by production of IL-4, IL-5, IL-6, and IL-13 (M.-A.

Hartley et al., 2012; Roberts et al., 2006). In humans, this polarized paradigm is less clear with a mixed T-helper profile predominating in ML/MCL and a Th1 biased response in LCL (M.-A. Hartley et al., 2012; M. A. Hartley et al., 2013; Roberts et al., 2006; Diaz et al., 2002).

Inflammatory Cytokines and Chemokines

IFN-γ and TNF-α are key modulators of the Th1 response through stimulation of NO production in activated macrophages, and inhibition of Th2 and Th17 cells for clearance of pathogens (Figure 2A-2B) (M.-A. Hartley et al., 2012; Maspi et al., 2016). However, over-expression of these cytokines is implicated in autoimmune disease (Maspi et al., 2016; Tripathi, Singh, & Naik, 2007). IL-12 is an important cytokine in the differentiation of Th1-related cells through IFN-γ production, and two closely related cytokines, IL-23 and IL-27 (Maspi et al., 2016). Toll like receptor 9 (TLR9) signaling has been shown to be involved with early control of lesion development, through the induction of IL-12 (Weinkopff et al., 2013). In BALB/c mice deficient of IL-12, susceptibility to *Leishmania* infection is increased (Maspi et al., 2016). The coupling of IL-2 and IFN-γ activates lymphocytes to effectively kill the *Leishmania* parasite inside the macrophage vacuole (Maspi et al., 2016). IL-1, including subtypes α and β, regulates the differentiation of Th17 cells and reduces the pathogenic effects of *Leishmania* spp. by decreasing lesion size and nitric oxide (NO) production (Maspi et al., 2016). It has been shown that in patients infected with NO-resistant strains of *Leishmania* spp., including *L. amazonensis* and *L. braziliensis,* larger lesion size has been observed, due to the resistance in intracellular killing by 96 hours of infection, which confers a survival benefit for the parasite inside the macrophage vacuole (Giudice et al., 2006). Conversely, over-expression of IL-1 has been shown to contribute to leishmaniasis in humans by promoting overproduction of TNF-α, which could enable chronic infection and progression of disease (Maspi et al., 2016). IL-18 has been implicated in IFN-γ production and a Th1 response in BALB/c mice, however, this is dependent on the existing

cytokine milieu, whereby in the absence of IL-4, IL-18 can still promote a Th2 response and leads to exacerbated disease (Maspi et al., 2016). IL-15 like IL-18 is a pleiotropic cytokine facilitating IFN-γ and TNF-α production in conjunction with IL-5 and IL-13, involved in a Th2 response (Maspi et al., 2016). IL-8 is a proinflammatory cytokine secreted by macrophages in the tissue and remains a chemoattractant for neutrophils involved in killing of *L. V. braziliensis* and *L. amazonensis* (Maspi et al., 2016). IL-17 is implicated in tissue inflammation with both a Th1 and Th2 response where IL-17 deficient mice develop smaller and fewer lesions of leishmaniasis, however, increased IL-17 is correlated to immunopathology in not only C57BL/6 mice, but patients with ML (Bacellar et al., 2009; Maspi et al., 2016). Chemokines including CCL2, CCL3, CCL5, CCL7, CCL8, CXCL10, CCL13 and CCL17 are involved in recruiting leukocytes into the inflammatory sites with the help of particular cytokines, such as IL-2 and IFN-γ (Roychoudhury K et al, 2004). Specifically, CCL4, CCL5 and CXCL10 have been implicated in *Leishmania* spp. infection (M.-A. Hartley et al., 2012).

Concanavalin-A (Con-A) is a lectin isolated from the jack bean, that has been shown to increase the expression of inflammatory and anti-inflammatory markers important for the control of infection through a Th1 biased profile, but most importantly induces ROS production, which eliminates the parasites in the macrophage vacuole and is a suggested therapeutic intervention for infection (Thomazelli et al., 2018).

Anti-Inflammatory Cytokines and Chemokines

IL-4 is involved in the differentiation of Th0 cells into Th2 cells by upregulation of arginase, which inhibits antileishmanial activity in the macrophage and downregulates Il-12 production (Figure 2A-2B) (Roberts et al., 2006; Maspi et al., 2016). IL-4 has been found to counteract the effects of NO stimulation and release allowing the parasite to survive (Maspi et al., 2016).

Alongside IL-13, IL-4 decreases inflammatory responses through downregulation of IL-1, IL-6, TNF-α and Il-12. IL-13 blocks production of IL-12 and delays the Th1 response while limiting NO production particularly in *L. V. guyanensis* (Maspi et al., 2016). IL-6 is a pleiotropic cytokine demonstrating Th2 biased response in animal models, however, in conjunction with IL-17 and IL-10 it produces a Th1 type response (Maspi et al., 2016). IL-10 is known to suppress the immune responses against *Leishmania* spp. and associated with chronic lesions (Maspi et al., 2016). Similarly, IL-27 can inhibit IL-17 and IL-23 production of pro-inflammatory cytokines while controlling infection through the production of IFN-γ (Maspi et al., 2016). IL-10 has been shown to be increased in Th1 or Th2 biased responses by individuals infected with *L. V. braziliensis,* whereby suppression of IFN-γ and TNF-α facilitates parasitic persistence and regulation of tissue damage and wound healing by increasing production of inflammatory cytokines (Maspi et al., 2016).

Immunology Specific to CL

CL immunology is confined to Th1 mediators: IL-12, IFN-γ, TNF-α, lymphotoxin, and NO are all associated with a healing response (Figure 2A-2B) (M.-A. Hartley et al., 2012). IFN-γ deficient C57BL/6 mice are shown to be susceptible to infection with *L. major,* and infection with *L. amazonensis* has been shown to produce larger lesions, increased parasite burden, and elevations of IL-4 driven Th2 response in such mice (Tripathi et al., 2007). Increasing TNF-α in C57BL/6 mice has shown to decrease parasite burden and lesion size in CL, whereas neutralizing TNF-α receptors in mice leads to lesion development (Tripathi et al., 2007). Reduction in IL-2 has been associated with severe CL in humans by increasing IL-4 production (Maspi et al., 2016). IL-22 is known to have antimicrobial activity providing protection against tissue damage in CL (Maspi et al., 2016). Non-healing CL ulcers are supported by Th2 mediators: IL-4, IL-13 and arginine (Tripathi et al., 2007). In localized CL (LCL), patients produce a Th1 type response

based on analysis of peripheral blood mononuclear cells (PBMCs) when stimulated with *Leishmania* spp. antigen *in vitro.* Patients with severe forms of CL including DL, DCL, or ACL show a PBMC profile with low levels of IFN-γ and high levels of IL-4, IL-5, and TNF-α (Guimaraes et al., 201). In a study evaluating mRNA expression for Th1 and Treg mediators in early active CL disease, it was found that IFN-γ and TNF-α were high expressed, along with IL-4, IL-10 and TGF-β in combination with Foxp3 expression (Souza et al., 2016). Again, patients with severe forms of CL were attributed to an exacerbated Th1 response, as demonstrated by Nicodemo et al., 2012, whereby a case report of an otherwise healthy individual infected with severe CL, demonstrated higher serum levels of IL-1β, IL-8, TNF-α, IL-12 and IFN-γ with lower levels of IL-6 and NK cells (Nicodemo et al., 2012). The authors did note elevated expression of TLR3, attributable to the recognition of double-stranded RNA (Nicodemo et al., 2012). It is also believed that age contributes to the immunological response and clinical presentation of ATL, whereby older patients have been shown to present with less lymphadenopathy, more scars and bigger lesion sizes, correlated with higher levels of IL-10 and lower levels of IFN-γ (Carvalho et al., 2015).

Immunology Specific to MCL/ML

In MCL, a mixed T-helper profile occurs whereby increased TNF-α and IFN-γ expression is associated with severe disease (Carvalho et al., 2007). Th1 suppressing cytokines including IL-10 and transforming growth factor β (TGF-β) have been documented in MCL, and contributing to an intermediate phenotype with increased levels of TNF-α, CXCL10, and CCL4 resulting in localized tissue damage and secondary lesions associated with MCL (Figure 2A-2B) (Vargas-Inchaustegui et al., 2010; M.-A. Hartley et al., 2012). Metastatic MCL has been associated with diminished IL-10 and increased TGF-β and IFN-γ that has been shown to favour parasite survival in strains of *L. V. braziliensis* inoculated into TNF-α knockout mice (Hartley et al.,

2012). In PBMCs of patients with ML, high levels of IFN-γ and TNF-α were observed, coupled with suppressed IL-10 and TGF-β production(Gaze et al., 2006; Bacellar et al., 2002)). The hyperactive Th1 response in MCL could be attributed to the patient's immunity or other factors affiliated with the parasite including parasite load, virulence factors, or the potentially immunomodulating parasite of the parasite, *Leishmania* RNA virus 1 (LRV1). Low levels of Th2 cytokines have been found to accelerate wound healing (Maspi et al., 2016). Increased expression of IL-2, IL-4 and IL-5 has been documented in patients with MCL compared to LCL who both presented with a positive Montenegro skin test (MST) (Nogueira et al., 2008). Increased levels of IFN-γ and TNF-α in patients infected with *L. V. braziliensis* manifested a greater number of lesions, which was associated with increased tissue damage (Maspi et al., 2016). In a case report of a patient treated with anti-TNF-α for juvenile idiopathic arthritis who previously presented with VL, mucosal relapse was documented two years later which may speak to a range of TNF-α necessary to control infection, without furthering tissue damage (Jeziorski et al, 2015). Anti-TNF-α therapies have been evaluated in autoimmune diseases, including diseases maintainted by chronic inflammatory cytokine synthesis, however to a lesser extent in ATL (Blackwell et al.,1999). The addition of pentoxifylline, a TNF-α inhibitor, to antimony was shown to be effective in the treatment of ML (Lessa et al., 2007; Amato et al., 2003).

Th9, Th17, Thf, Tregs and other cells

The Th9, Th17, Thf, and Tregs sub-types have also been implicated in the pathogenesis of *Leishmania* infection (Figure 2A-2B) (M. A. Hartley et al., 2013). A Th9 response was originally thought to be part of the Th2 profile given its detrimental role in non-healing (Tripathi et al., 2007). Encompassing the production of IL-9, IL-10 and IL-4, Th9 is attributed to induction by TGF-β, IL-21, IFN-γ and β, IL-4 in susceptibility to infection, in mice (M. A. Hartley et al.,

2013). The Th17 response is responsible for neutrophil attraction and proteinase release, leading to tissue damage (Boaventura et al., 2010). Th17-related cytokines have been associated with tissue destruction and chronic inflammation, particularly in MCL with increases in IL-17α and IL-23 (Boaventura et al., 2010; Bacellar et al. 2009; Tripathi et al., 2007). However, in strains of *Viannia* known to cause CL, this has been detrimental particularly in forms other than LCL (Tripathi et al., 2007). The Thf response, known as the 'B-helper' follicular T-cell lineage is believed to be a separate lineage consisting of IL-4, given that the majority of IL-4 is produced in the lymph nodes in mice as opposed to the thymus, and IL 21 (Tripathi et al., 2007). In CL, this lineage is responsible for disease severity owing to parasite survival through antibody uptake (Tripathi et al., 2007). Tregs have been shown to be responsible for controlling aberrant immune responses, such as the production of IL-10 in CL infection with *L. V. guyanensis* (Tripathi et al., 2007; Ehrlich et al, 2019). Foxp3 is a marker of Tregs and have been found in varying levels across many different clinical phenotypes including CL, DCL and MCL, reflective of the immune responses in each of these manifestations (Carneiro et al., 2009; Ehrlich et al., 2019). In infections with *L. V. panamensis*, Tregs have been shown to play a beneficial role in controlling immune responses and may highlight a pivotal role in controlling infection compared to MCL/ML causing species like *L. V. braziliensis* and *L. V. guyanensis* (Carneiro et al., 2009). Deficiencies in CCL5 and CCR5 have shown to contribute to lower recruitment of Tregs in ML patients compared to those with CL, which also highlight the potential contribution of Tregs to controlling the infection (Barros et al., 2018). Langerhans cells may also differ in proportion across patients with ML and CL lesions, where a low density in patients with ML may reflect the migration of these cells from the epidermis to regional lymph nodes, which may account for the tissue-damaging state due to defective signaling (Diaz et al., 2002). Neutrophils are the primary component of inflammatory infiltrate in chronic CL and MCL. Species-specific differences in

neutrophil activation was observed between amastigotes of *L. V. braziliensis* and *L. amazonensis* infection, whereby neutrophils were able to kill *L. V. braziliensis* more effectively than *L. amazonensis* observed through monocyte oxidative burst, as a result of ROS-parasite clearance (Carlsen et al., 2015).

Animal Models Limit our Understanding of Human Pathology in CL

The majority of immunologic work surrounding *Leishmania* has been performed on mice and hamster models, particularly in Old World strains causing CL. Although there is a significant number of validated mouse antibodies and cytokines to easily evaluate the immunology of leishmaniasis, the lack of nasopharyngeal pathology in mice has favoured the hamster model (Hartley et al., 2012; Olivier, 2011). To add, there is variation the efficacy of mouse models depending on the clinical manifestations, where CL is believed to be better studied on mice than VL. It is found that C57BL/6, CBA and C3H/He strains of mice are naturally resistant to *Leishmania* infection whereas BALB/c mice remain susceptible which can heavily influence interpretation of immunologic data (Maspi et al., 2016). Strains of *L. V. braziliensis* have shown varying phenotypes where VL has appeared through infection targeting CL (Hartley et al., 2012; Maspi et al., 2016). Development of secondary lesions is rarely seen in mice models of MCL, however disease severity in the site of primary infection remains a surrogate for assessing pathogenicity (Hartley et al., 2012).

Asymptomatic vs. Recurrent Infections

In many endemic countries, individuals are often infected multiple times over a lifetime, introducing novel strains of similar species, which may contribute to delayed healing or worsened phenotype. In a study evaluating immunity in the context of continued exposure to *Leishmania Viannia* spp. infection, the development of disease is attributable to lack of stable

immunity, whereas the absence of disease may be attributed to non-exposure of infection or presence of stable immunity (Bosque et al., 2000). This study conducted by Bosque et al. found no demographic or occupational risk factors contributing to recurrent disease, however it was found that patients with recurrent disease mounted a lower delayed type hypersensitivity (DTH) response to the Leishmanin skin test (MST), and that the number of macrophages infected in patients with a history of recurrent disease was much higher than in asymptomatic patients (Bosque et al., 2000). This group highlights that macrophages from patients with a history of recurrent disease are more permissive to infection than in asymptomatic patients, despite clinical healing in previous lesions (Bosque et al., 2000). The exact reasoning behind the permissive nature of macrophages in patients with recurrent disease may highlight the difference in clinical outcome in patients (Bosque et al., 2000). In a similar study by Cooper et al., MCL patients had long durations of low levels of infected macrophages, who had a much more robust T cell reaction compared to patients with LCL infections (Cooper et al., 1994). Furthermore, in patients with no prior exposure to ATL, TGF-β was differentially expressed upon infection of PBMCs with *L. V. guyanensis* whereas in patients with a prior history of LCL, IL-10 was differentially expressed (Bourreau et al., 2007). Both IL-10 and TGF-β are involved in suppressing Th1 cytokines during human infection but seem to express differently depending on the infection status of the host (Bourreau et al., 2007). Another important aspect to consider is the role of sand fly salivary proteins and its influence on overall *Leishmania* spp. immunity. The active secretion of salivary proteins into the dermis facilitates blood feeding, avoiding hemostasis and inflammation (Gomes et al., 2012). Initial infection with *Leishmania* spp. promastigotes in combination with salivary proteins results in a robust immune response characterized by upregulated Th2 cytokines (Gomes et al., 2012). Multiple exposure to sand fly bites have shown

to induce immunity to these salivary proteins, represented by milder subsequent infections (Gomes et al., 2012).

III. LEISHMANIA VIRULENCE FACTORS

Kinetoplastida including *Leishmania* have unique gene expression controlling mechanisms to further their capacity to cause disease that are not essential for their viability. Virulence factors (VFs) are endogenous molecules known to affect the fitness and stress tolerance of an organism, the host, and host cell invasion, and contribute to the modulation and evasion of the host immune response in order to propel infection (Bifeld & Clos, 2015). There is only minimal variation in transcription control, therefore expression of these genes are reliant on regulated translation for gene expression (Bifeld et al., 2015). Extensive research has surrounded the role of VFs in VL-causing species whereby the amastigote-specific A2 protein family is implicated in protection against temperature stress and allows the parasite to persist through high temperatures including the host cell fever (Bifeld & Clos, 2015, McCall et al., 2012). In *L. major,* the A2 protein exists as a non-expressed pseudogene, co-localizing with HSP23, and has been shown to enhance expression following heat-shock following 24 hours after the promastigote has differentiated into the amastigote stage (McCall et al., 2010). In New World strains causing ATL, VFs have not been described as extensively as in *L. major* or *L. donovani,* however, many concepts of stress tolerance in Old World species could be adapted to strains from the New World.

VFss endogenous to *Leishmania* spp. include, but are not restricted to: molecular chaperones such as heat-shock proteins (HSPs), cysteine peptidases, phosphatases, surface proteases and proteinases. Prior to modulating the host immune response, the parasite must achieve host cell invasion (Bifeld & Clos, 2015). One of the ways this is achieved is through complement-mediated recognition. This mechanism allows phagocytosis of the parasite through the activation

of precursor enzymes and proteins in the blood when bound to the parasite through the formation of the membrane attack complex (Bifeld & Clos, 2015).

Zinc Metallopeptidase GP63 (GP63)

Zinc metallopeptidase GP63 (GP63) is an abundant surface molecule involved in enhancing the interaction between the parasite and macrophages through fibronectin receptors and complement-mediated recognition (Bifeld & Clos, 2015). In addition, it cleaves the complement protein C3b into the inactive iC3b state (Olivier, Atayde, Isnard, Hassani, & Shio, 2012). This allows macrophages to phagocytose the parasite through complement receptor 3 (CR3) and CR1, and allows the parasite to survive the phagolysosome when the receptors are cross-linked to prevent oxidative burst of the cell (Olivier et al., 2012). GP63 is also involved in downregulating Mitogen-activated protein (MAP) kinase and Janus kinase signaling in macrophages, allowing the parasite to proliferate (Olivier et al., 2012). GP63 is also implicated in inactivating pro-inflammatory transcription factors including AP-1 and NF-κB, preventing an inflammatory response in the cell by allowing the transfer of exosomes to the cytosol of the host cell for successful infection (Bifeld & Clos, 2015). In addition, GP63 suppresses the presentation of antigens to the MHC class I molecules to prevent the fusion of the phagosomes and lysosomes, thus preventing assembly of the NADPH oxidase complex, and inhibiting the degradation of phagolysosomes (Bifeld & Clos, 2015). GP63 has also been shown to inhibit IL-1β production through cleavage of the NLRP3 inflammasome, a multiprotein intracellular receptor platform that produces IL-1β, which can contribute to poor immunologic control (Isnard et al., 2012; Shio et al., 2015; Moreira e al., 2017). Differential expression of GP63 has been shown in both life-cycle stages and exosome vesicles of *Leishmania* spp., whereby GP63 is highly expressed during the metacyclic stage, rather than the stationary and logarithmic stages (Isnard et al. 2012; Marshall et al., 2018).

Lipophosphoglycans (LPGs)

Three main classes of glycosyphophatidylinositol (GPI)-anchorered molecules exist, including lipophosphogycans (LPGs), glycoinositolphospholipids (GIPLs) and proteophosphoglycans (PPGs) (Spath et al., 2000; Madeira da Silva et al., 2009). GPIs are essential for attaching to the sandfly midgut, and to appropriate differentiation and growth prior to transmission during a blood meal (Bifeld & Clos, 2015; Rogers et al., 2002). In the human host, LPG is shielded preventing the complement membrane attack complex (C5b-C9) from inserting into the promastigote membrane and thereby preventing parasite killing (Madeira da Silva et al., 2009; Bifeld & Clos, 2015). In the case of phagocytosis, the LPG molecules prevent fusion of the phagosome-endosome by disrupting the lipids on the membrane which hinders the phagolysosome from achieving an acidic, antileishmanial environment (Bifeld & Clos, 2015). Evasion of phagosome-endosome fusion has been documented in other intracellular pathogens including *Mycobacterium tuberculosis* (Bifeld & Clos, 2015). LPG-mediated phagosome fusion evasion is necessary for the survival of the parasite inside neutrophils whereby an inflammatory immune response is avoided (Bifeld & Clos, 2015). NK cells and macrophages express TLR2 and TLR9, which interacts with LPG to deactivate macrophages through the induction of IL-10 and TGF-β, producing a Th2 biased response (Srivastava et al., 2013). Upon differentiation from the promastigote stage to the amastigote stage, LPG is expressed in lesser quantity thus highlighting the role of LPG in achieving infection early on (Bifeld & Clos, 2015).

Kinetoplastid membrane protein-11 (kmp-11)

Kinetoplastid membrane protein-11 (kmp-11) is highly expressed during the amastigote stage highlighting its role in effectively modulating the host immune response (Silva-Almeida, Pereira, Ribeiro-Guimarães, & Alves, 2012). Kmp-11 has been shown to reduce inducible nitric oxide synthase (iNOS) activity in macrophages because of the structural analogue of L-arginine, N^G-

methyl-L-arginine, which inhibits arginase to control intracellular parasite growth and exacerbate parasite proliferation (Bifeld & Clos, 2015). Moreover, *Leishmania*-infected macrophages allow the synthesis of polyamines, essential metabolites of eukaryotes, by inducing arginase-I to hydrolyze L-arginine to L-orthinithine to further exacerbate disease (Bifeld & Clos, 2015).

Cysteine Peptidases (CPs)

Cysteine peptidases (CPs) are important enzymes expressed in all kinetoplastids, but more importantly during the amastigote stage where the *Leishmania* parasite resides inside lysosomes. The C1 family of papain-like cysteine peptidases consists of Clan CA, including CPA, CPB and CPC (Mottram, Coombs, & Alexander, 2004). CPB has been extensively studied in regards to the Th2 immune response with significant increases in IL-4 and inhibition of IL-12 responsible for the cleavage of NF-κB and IκB whereby CPB null mutants in BALB/c mice exhibited a strong Th1 response in addition to the internalization of MHC class II molecules due to infections of *L. mexicana* and *L. major* (Mottram et al., 2004; Silva-Almeida et al., 2012). Calpains belong to the Clan CA family, belonging to intracellular Ca^{2+}-dependent CPs and are responsible for modulating cellular processes such as cytoskeletal rearrangement, signal transduction pathways and apoptosis (Ennes-Vidal et al., 2019). Increased expression of these proteins has been associated with drug resistance and PKDL (Ennes-Vidal et al, 2019). In *L. V. braziliensis,* 34 different calpain-related genes on 13 different chromosomes were identified, some of which were differentially expressed between procyclic and metacyclic promastigotes (Ennes-Vidal et al., 2019).

Heat Shock Proteins (HSPs) and associated molecules

Heat shock proteins (HSPs) provide thermotolerance inside the mammalian host. HSP23 is preferentially expressed during the amastigote stage and is essential for stress tolerance

particularly in *L. donovani* (Bifeld & Clos, 2015; Hombach, Ommen, MacDonald, & Clos, 2014). Pentavalent animonials are effective at killing *Leishmania* parasites through the loss of HSP23 (Bifeld & Clos, 2015; Hombach et al., 2014). HSP70 is the most conserved protein in eukaryotes and in *Leishmania* spp., and is correlated to cell survival by protecting against protein denaturation (Drini et al., 2016; Holakuyee, Mandavi, Hassan, & Abolhassani, 2012). In *L. major*, HSP70 upregulation has been associated with a Th2 response and shown to be increased in strains of *L. V. braziliensis* of ML patients. In conjunction with HSP90 and co-chaperone cyclophilin 40 (CyP40), HSP70 has been shown to avoid environmental and chemical stresses (Drini et al., 2016). HSP100 was identified as the first HSP VF implicated in protein misfolding and aggregation (Krobitsch et al., 1998). It is essential for intracellular survival by acting as an antagonist to the amastigote-to-promastigote stage reversion. HSP100 is associated with a Th1 biased response with increases in CD^{4+} cells and IFN-γ (Krobitsch et al., 1998).

Type 1 chaperonins, such as CPN60 and CP10 assist in the folding and refolding of newly synthesized proteins along with HSP23 and HSP100 (Colineau et al., 2017). Quantification of CP10 in *L. donovani* has shown reduced amounts correlated to increased infection rates, implying that CPN10 is associated with restricted uptake of promastigotes, whereby to establish long term infections, silent entry into the human hosts via macrophages (Colineau et al., 2017). Moreover, *P46* is a 46 kD protein exported from the amastigote to the cytosol of the macrophage and shown to act independently of HSP100, however in combination, boosts cellular survival in knockout experiments (Bifeld et al, 2015).

Other Proteins and Host Factors

Viannia specific genes have been identified, absent in other *Leishmania* spp. including 22 genes through WGS (Coughlan et al., 2018). Three of these gene groups are involved with the RNAi

pathway, unique to members of the *Viannia* subgenus; as well as telomere-associated mobile elements (TATE) DNA transposons unique to this subgenus (Coughlan et al., 2018). c-Myc is a regulator gene that codes for transcription factors, highly involved in the formation of cancer. c-Myc silencing upregulates microRNA (miRNA) expression controlling *Leishmania* infection, however during infection with *L. donovani,* c-Myc expression in macrophages is hijacked and upregulated, silencing c-Myc-dependent miRNAs which is essential for parasite survival (Colineau et al., 2018). *Leishmania* phosphatases such as serine/threonine phosphatases (STPs) have been linked to resistance against antibiotics such as paromomycin while others are involved in sensitivity to Sb^V (Soulat et al., 2017).

Other host factors involved in mounting an immune response may be important therapeutic targets for controlling infection. Matrix metalloproteainase 9 (MMP-9) is involved in cell migration, and important for disruption of cell focal adherence. Increased expression of MMP-9 has been observed in patients with ML, correlated to the disruption of cell adherence allowing for migration and parasite persistence (Maretti-Mira et al., 2011). Host phosphatases such as phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and aspartate-based phosphatases (F cell production phosphatase) are involved in producing antileishmanial molecules such as cytokines and ROS to control infection, some of which are activated by other molecules such as GP63 and *Leishmania elongation factor 1α* (*EF-1α)* (Soulat et al., 2017).

IV: *LEISHMANIA* RNA VIRUS-1 (LRV-1)

Leishmania RNA Virus (LRV) is classified as a Group III dsRNA virus belonging to the family *Totiviridae* and genus *Leishmaniavirus* (Gupta & Deep, 2007)). Since the discovery of many virus like particles (VLPs) in unicellular eukaryotes in the 1960s, isolation of viruses from other

living organisms has occurred on many occasions (Gupta et al., 2007). *Totiviridae* is a family of viruses with an unsegmented genome, infecting protozoa and fungi. Three genera of *Totiviridae* exist including *Totivirus, Giardiavirus,* and *Leishmaniavirus* (Gupta A, 2007). Phenotypic alteration of the host has been documented in members of the *Totiviridae* family including the L-A virus of the *Saccharomyces cerevisiae* linked to a toxin absent in strains uninfected with the virus (Gupta et al., 2007). In *Trichomonas vaginalis,* the virus TVV produces phenotypic variation of the surface protein immunogen P 270 (Gupta A, 2007). In leishmaniasis, the same trend remains where LRV confers a state of hypervirulence in this host parasite, however much of the mechanistic effects on the virus itself is unknown (Gupta A, 2007).

Genome

Containing a 5284 nucleotide sequence, the genome encodes two large and one small open reading frames (ORFs) on the plus-strand of the virus (Brettmann et al., 2016; Grybchuk et al., 2017) (Figure 3). Icosahedral in structure with a non-enveloped capsid, this non-segmented virus has a diameter of approximately 40 nm and replicates in eukaryotic cytoplasm through cleavage of the RNA dependent RNA polymerase (RPRP) protein by a *Leishmania* cysteine protease (Cadd et al., 1994; Gupta et al., 2007; Mysuria 2018). LRV can be controlled through the *Leishmania* RNA-interference (RNAi) pathway, which converts dsRNA into short interfering RNAs (siRNAs), which degrades the complementary mRNA strand (Brettman et al., 2016). LRV has been introduced into uninfected strains, however the virus is not able to persist (Armstrong et al., 1993). Uninfected strains without RNAi activity may explain why stable infection with LRV is so difficult to achieve (Mysuria, 2018). Furthermore, with the advent of molecular techniques, sequencing of the virus has shown species and geographical variation (Cantanhede et al., 2018).

ORF2 exhibits a 36% sequence homology with the capsid protein of LRV1-4 (Scheffter et al., 1995). Meanwhile, the ORF3 encodes the RDRP exhibiting a 43% sequence identity to LRV-1-4 (Scheffter et al., 1995). ORF3 is highly conserved between isolates containing the RNA-dependent RNA polymerase (RDRP) (Lee et al., 1996; Gupta et al., 2007). The small ORF at the 5' terminus is not well conserved and implicated in the establishment of persistent infection and encoding toxins (Gupta et al., 2007). ORF2 encodes the major non-enveloped capsid protein which overlaps with ORF3 generating +1 ribosomal frameshifts, or tRNA slippage producing a Gag-Pol-type fusion protein, previously observed in *Giardiavirus* and L-A virus (Lee et al., 1996; Cadd et al., 1994; Scheffter, Ro, Chung, & Patterson, 1995; Grybchu et al., 2017) . The 5' untranslated region (UTR) spanning the first 450 nucleotides of the plus-strand is believed to contain conserved regulatory sequence elements such as the internal ribosomal entry site (IRES), as there is much sequence homology between LRV-1 of different strains (Widmer et al., 1995; Zamora et al., 2000). The IRES is used to initiate translation when a hypermethylated structure containing the initiation codon AUG is missing (Zamora et al., 2000).

Strains and Sub-types

There are two main types of LRV known to infect strains of *Leishmania:* LRV-1 and LRV-2 have 90% homology to one another, with New World and Old World origin, respectively (Zangger et al., 2014). Given the geographic distribution of these viruses in a variety of species of *Leishmania,* it is hypothesized LRV-1 diverged from the Old World LRV-2 along with the divergence of the species during the separation of Gondwana (Steverding, 2017; Zangger et al., 2014). Given that *Leishmania* spp. reproduction is asexual, genetic recombination of LRV does not exist, therefore phylogenetic analyses indicating evolution is quite accurate (Gupta et al., 2007). Restriction fragment length polymorphisms (RFLP) analysis of LRV isolates revealed the

co-evolution between LRV-1 and LRV-2 supporting the notion of LRV being an ancient virus (Gupta et al., 2007). LRV was thought to have arisen during laboratory manipulation *in vitro,* since many parasites containing the virus grow readily in culture compared to uninfected parasites. This hypothesis was disproven when LRV-1 was initially detected in the nonhuman parasite species, *L. heritigi* in 1974 and first detected in a human isolate of *L. V. braziliensis* in 1988 (Ginouvès et al., 2016; Tarr et al., 1988).

LRV-1 in the New World has 14 subtypes (LRV-1-1 – LRV-1-14) predominantly isolated from the Amazon basin (Saiz et al., 1998; Ginouvès et al., 2016; M.-A. Hartley et al., 2012; Salinas, Zamora, Stuart, & Saravia, 1996; Saberi et al., 2019). LRV-2 was first identified in the Old World strain, *L. major,* displaying an immunologically distinct profile from the LRV-1-1 and LRV-1-4 subtypes predominantly found in Latin America whereby the tRNA slippage is not maintained therefore the capsid and RDRP genes do not overlap (Scheffter et al., 1995) (Figure 3). Since then, LRV-2 has been identified in other Old World species including *L. aethiopica, L. tropica* and *L. infantum* however not in *L. donovani,* a species known to cause VL **(**Hajjaran et al., 2016; Nalcaci et al., 2019; Kleschenko et al., 2019). It is important to note that in a subset of *L. donovani* samples from VL patients, a non-LRV was identified, belonging to *Leptomonas seymouri* also identified in these patients (Sukla et al., 2017).

LRV-1 Pathogenesis

The role of LRV-1 in the pathogenesis of ATL has long been evaluated given the possible association between severity of disease and clinical outcome in patients where LRV-1 has been detected (Bourreau et al., 2016; de Oliveira Ramos Pereira et al., 2013; Ginouvès et al., 2016; Macedo et al., 2016; B. Valencia et al., 2014). Numerous studies have attempted to evaluate the prevalence of LRV-1 and its immunomodulatory effects on the parasite and the host (Bourreau et

al., 2016; Ives et al., 2011). Since the detection of LRV-1 in a patient with cutaneous satellite lesions and lymphatic involvement after visiting Suriname, the notion that the virus in the parasite might be causing more severe disease has been the focus of evaluation in ATL over the past few decades (Tarr et al., 1988). The passaging of this same strain through a hamster model produced a mucocutaneous phenotype similar to the mucosal presentation in humans (Tarr et al., 1988).

In 2011 the use of a metastatic LRV-1-containing human *L. V. guyanensis* strain infected into mice and hamsters confirmed the severe phenotype by modulation of the host macrophage response whereby significant alterations to chemokines and cytokines CCL5, CXCL10, TNF-α and IL-6 were observed over a period of 6 hours (Ives et al., 2011). In addition, the mechanism by which LRV-1 manipulates the host immune response was examined whereby knockouts of toll-like receptor 3 (TLR3) showed a significant decrease in footpad swelling and parasite burden in comparison to wild-type mice (Ives et al., 2011). TLRs recognize nucleic acid motifs, whereby TLR3 is implicated in dsRNA recognition. Mechanistic studies have established the upregulation of miR-155 to promote parasite persistence mediated by macrophage survival through akt activation with miR-155 inhibition reduced the severity of disease in LRV-1 models (Eren et al, 2016; Conceicao-Silva et al., 2018; Borges et al., 2018). This is also linked to an exacerbated IFN-γ response as observed by LRV1 and exogenous viral coinfections in macrophages infected with parasites containing LRV1 (Rossi et al., 2017). Furthermore, despite being a non-enveloped dsRNA virus, LRV1 uses the host exosome as a mechanism to protect the virus from degrading enzymes and other extracellular responses, but importantly a requirement to be recognized by TLR3 to maximize infectivity (Atayde et al., 2019).

LRV-1 Prevalence

In South America, LRV-1 was specifically detected in areas in and around the Amazon basin, specifically Brazil, Bolivia, Peru and French Guiana (Guilbride et al., 1992; Salinas et al., 1996). It is believed that the increase in prevalence in areas of Central America including Costa Rica is a result of environmental consequences leading to shifting vector ranges and human populations (Salinas et al., 1996). LRV-1 has historically been documented in isolates of *L. V. braziliensis,* and *L. V. guyanensis,* but has now expanded to other species in Latin America including *L. amazonensis, L. V. lainsoni, L. mexicana* and the newly identified *L. V. naiffi*, a species traditionally identified in armadillo (Vieira-Goncalves et al., 2018). The prevalence of LRV-1 has geographic variation with rates of 25%-47% in strains of *L. V. braziliensis* (Adaui et al., 2016; Bourreau et al., 2016; de Oliveira Ramos Pereira et al., 2013; Ginouvès et al., 2016; Ito et al., 2015; Macedo et al., 2016; B. Valencia et al., 2014). In French Guiana, LRV-1 has been associated with treatment failure of Sb^V with strains of *L. amazonensis* and *L. V. lainsoni* reportedly carrying the virus, a finding not previously reported (Ginouvès et al., 2016). The lack of LRV-1 detection in other species could be a reflection of the poorly selected targets for speciation that are not different enough to produce species-specific results. Similarly, LRV-1 has been associated with treatment failure in Peru and Bolivia (Adaui et al., 2016). Brazil has historically provided conflicting results of geographic nature whereby areas other than the Amazon have demonstrated low detection rates of LRV-1, particularly in Rio de Janeiro and Southeastern Brazil (Adaui et al., 2016; Ito et al., 2015; Macedo et al., 2016).

LRV-1 Antiviral Targets

A wide number of studies in Latin America set out to evaluate the prevalence and target LRV-1 as a predictive biomarker of therapeutic potential (Adaui et al., 2016; Bourreau et al., 2016;

Cantanhede et al., 2015). The discovery of active, endogenous RNAi activity exclusively in strains of the *Viannia* subgenus further suggests the use of LRV-1 as a means of achieving greater parasitic virulence by retaining the virus despite the ability to remove it entirely (Brettmann et al., 2016). The use of long-hairpin/stem-loop constructs targeting small RNAs in strains of *L. V. braziliensis* and *L. V. guyanensis,* have been shown to eliminate the viral burden through increases of specific short interfering RNAs (siRNAs) that disable the TLR3 dependent hyperinflammatory immune response (Brettmann et al., 2016; Patterson, 2017, Lye et al., 2010). A potential antiviral was discovered to be effective against the virus in 1997, where hygromycin B, an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus*, was shown to eliminate the virus in persistently infected parasites through the inhibition of protein synthesis via interference of ribosomal translocation (Ro et al., 1997).

LRV-1 Immunology

LRV-1 has been implicated in evasion of innate immune response in the human host by producing immunomodulatory effects through the TLR3 pathway, previously known to recognize dsRNA and produce an IFN-β mediated anti-viral response (Ives et al., 2011). In addition, LRV-1 recognition results in the upregulation of TNF-α, IL-6, and chemokines facilitating parasite persistence leading to organ damage (Ives et al., 2011). In TLR3 -/- mice, LRV-1 was ineffective at producing an inflammatory response (Ives et al., 2011). TLR7 has also been implicated in the immune response due to detection of LRV-1 components as demonstrated by upregulation of pro-inflammatory cytokines using *in vitro* macrophages (Ives et al., 2011). To add, Rig-like receptors and Nod-like receptors also play a role in antiviral response and inflammation through the IFN-β mediated pathway (Tripathi et al., 2007). Similarly, the virus has been implicated in subverting killing in the macrophage vacuole through upregulation of the Cu-Zn-SOD enzyme involved in protecting the cell from oxidative challenge (M.-A. Hartley et

al., 2012). LRV-1 should be evaluated as a potential immune modulator in humans given its propensity to increase parasite survival in the macrophage vacuole.

V: PULLING IT ALL TOGETHER: Immunology of American Tegumentary Leishmaniasis (ATL) and the Role of *Leishmania* RNA Virus-1 (LRV-1)

American tegumentary leishmaniasis (ATL) can be divided into two major phenotypes: severe and non-severe ATL. Severe ATL includes ML/MCL, inflammatory (e.g., exudative, erythematous, painful and/or lymphatic involvement), multifocal (e.g., ≥4 lesions in ≥2 anatomic sites), diffuse (non-ulcerating nodular lesions due to poor cell-mediated immune response), disseminated (several hundred acneiform, papular and ulcerative lesions located on 2 noncontiguous areas of the body), and atypical CL (unusual crusted, lupoid, sporotrichoid, vegetative or verrucuous cutaneous lesions) whereas non-severe ATL is characterized as localized CL (LCL) (Aronson et al., 2016; Guimaraes et al., 2016; Reithinger et al., 2007; World Health Organization, 2010). One of the defining features of ATL is the ability of CL to progress into ML/MCL in a subset of patients, which is fairly specific to Latin America.

About 1-10% of LCL infections result in ML/MCL 1-5 years after LCL has healed in South America, subject to geographic variation (Reithinger et al., 2007). In Brazil, it is believed that 0.4%, 1.4% and 2.7% of LCL lesions in the south, central and northeast regions will progress to ML/MCL (World Health Organization, 2010; Guerra et al., 2015). Countries including Bolivia and Ecuador exhibit high rates of ML/MCL, 20% and 7.7% respectively (World Health Organization, 2010). Peru and Bolivia exhibit ML/MCL cases at a rate of 7.1%, whereas Colombia and Venezuela exhibit lower frequencies, at 2.3% and 0.4% respectively (World Health Organization, 2010). Numerous hypotheses exist surrounding the progression of LCL to

ML/MCL including species, host-immune response, virulence factor expression and the controversial role of a double-stranded RNA virus known as *Leishmania* RNA Virus (LRV) identified in 20-25% of clinical isolates of the *Viannia* subgenus in South America.

LRV-1: A Predictive Biomarker of ATL?

LRV-1 has been associated with severity of disease in that patients exhibiting severe clinical phenotypes and treatment failures have been shown to harbour LRV-1 in their causative strain of *Leishmania*. In addition, there has been substantial data surrounding the role of LRV-1 in murine immune responses whereby a Th2 biased profile is produced with increases in certain pro-inflammatory cytokines, chemokines, and regulators of ROS. Given the parasite's natural ability to achieve infection by regulation of virulence factors, the question remains whether or not LRV-1 enhances or modifies the expression of these endogenous molecules for the purpose of successful infection and proliferation. To sum, if LRV-1 is bad for clinical disease manifestations and prognosis, then Th1-biased inflammatory cytokines and biomarkers should be reduced in their expression, while virulence factor expression might be enhanced. On the other hand, if LRV-1 has no influence on the pathogenesis of human ATL, then we would not expect to see alterations in biomarker or virulence factor RNA transcript expression when LRV-1-positive isolates are compared to those that are negative. LRV-1 could serve as a predictive biomarker of not only disease severity, but treatment outcome given the variable rates of CL to ML/MCL progression, relapse of initial ATL infections, and poor response to currently available therapies.

Aims/Hypotheses

LRV-1 may have a role in the pathogenicity of ATL, thus, by examining the influence of this virus on host immune and parasite responses, one might gain insight into mechanisms that might

be harnessed for improved clinical control of the disease. The aim of the project is to understand the relationship of LRV-1 to ATL by understanding: 1) prevalence of LRV-1 in American tegumentary leishmaniasis (ATL)-causing strains of *Leishmania* and its associations with clinical phenotypes of ATL; 3) the influence of LRV-1 on cytokine expression in a human macrophage model of ATL; and 4) the influence of LRV-1 on known virulence factor (VF) RNA transcript expression in pure cultures of *Leishmania* as well as the human macrophage model of ATL.

We hypothesize that LRV-1 will be over-represented among strains of *Leishmania* that cause a severe clinical phenotype, particularly ML/MCL, and that LRV-1 will be correlated to increased expression of Th2 predominant cytokines and known *Leishmania* virulence factors RNA transcripts. Conducting this body of work will enable us to better understand the potential contribution of LRV-1 to the pathogenesis of American tegumentary leishmaniasis.

Baseline prevalence of LRV-1 will be assessed to inform, at population level, any associations between the virus and clinical and demographic correlates. In addition, the possible association between clinical phenotype and LRV-1 will be further evaluated using a human macrophage model of infectivity whereby cultured strains of LRV-1 positive and negative isolates will be evaluated for changes in proinflammatory biomarker expression using a cytokine and chemokine profile previously examined in mice. Lastly, the role of endogenous VF RNA transcripts will be examined using the same model to further understand the virus-parasite dynamics prior to modulating host immune responses given the well documented literature surrounding Old World parasitic virulence factors.

By evaluating our current knowledge regarding ATL pathogenesis, we hope to illuminate data that will lead to a better understanding of how ATL interacts with populations and human hosts. Further evaluation of parasite mechanisms of survival through the analysis of *Leishmania*

virulence factor RNA transcripts, host-immune responses, and how *Leishmania* RNA Virus-1 (LRV-1) could potentially alter these mechanisms of infection will attempt to address questions pertaining to both population-level and individual health.

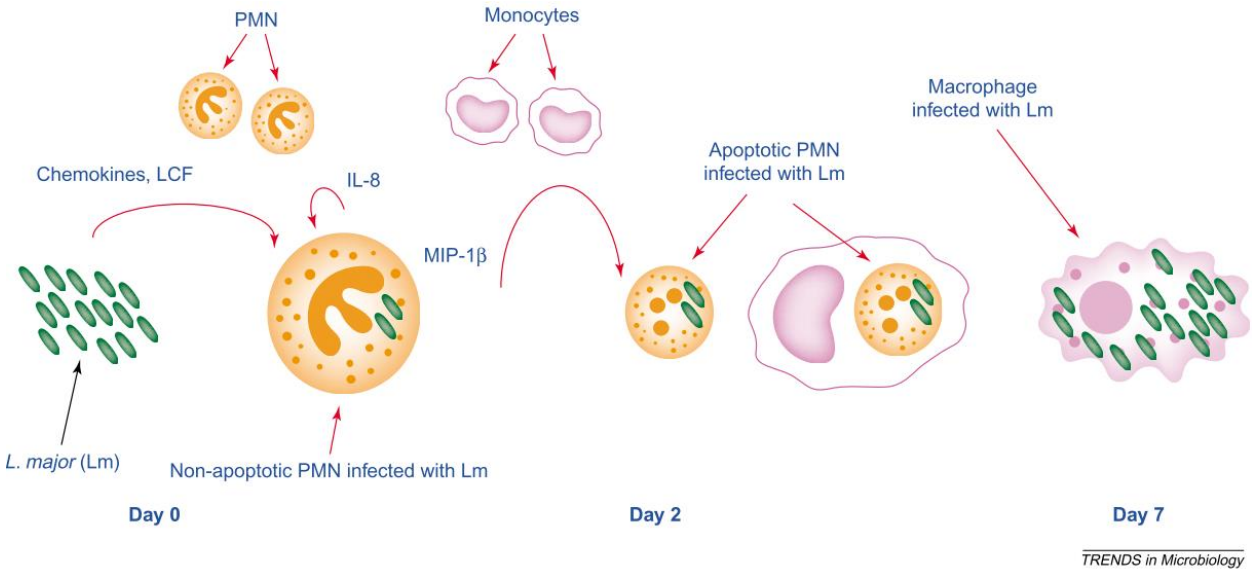
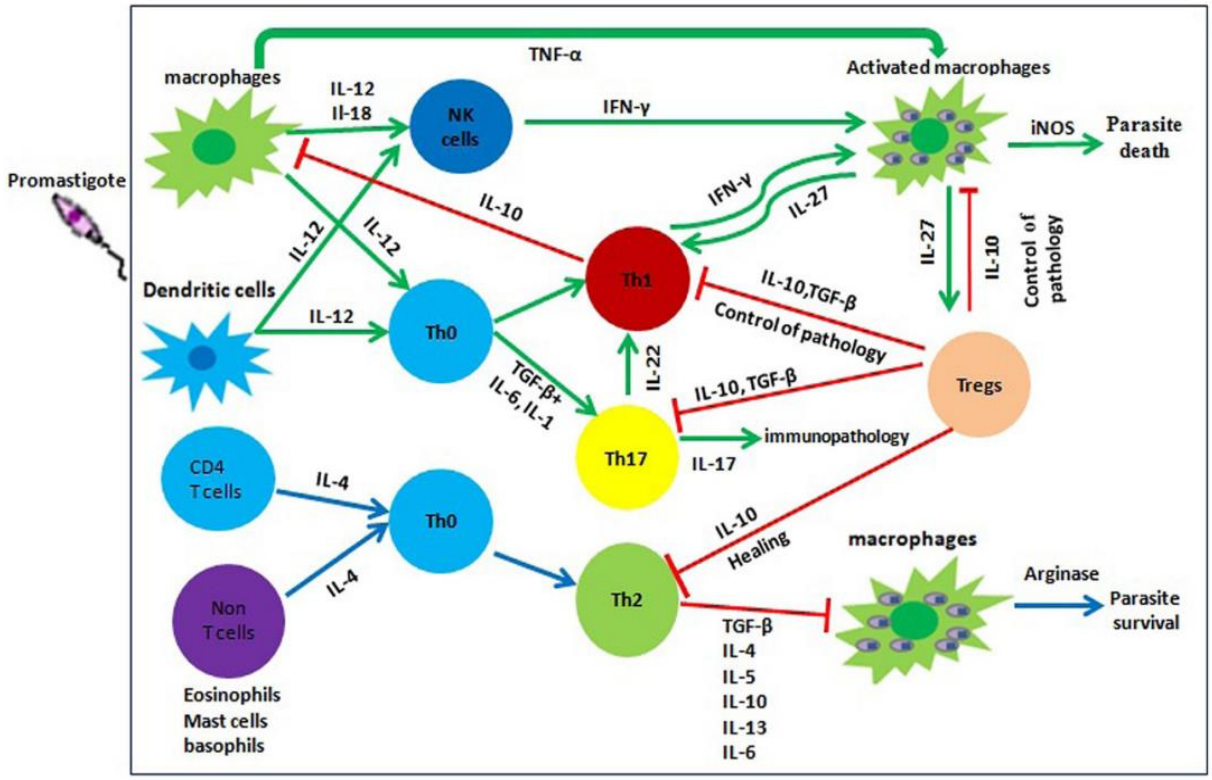


Figure 1: The Trojan horse hypothesis whereby *L. major* parasites use PMNs to multiply and silently deliver themselves to macrophages.

Adapted with permission from "Neutrophil granulocytes-Trojan horses for *Leishmania major* and other intracellular microbes" by T Laskay, G Van Zandhbergen and W Solbach, 2003, *Trends in Microbiology,* 111(5): 21-214. Copyright 2003 by Elsevier Science Ltd.

A



B

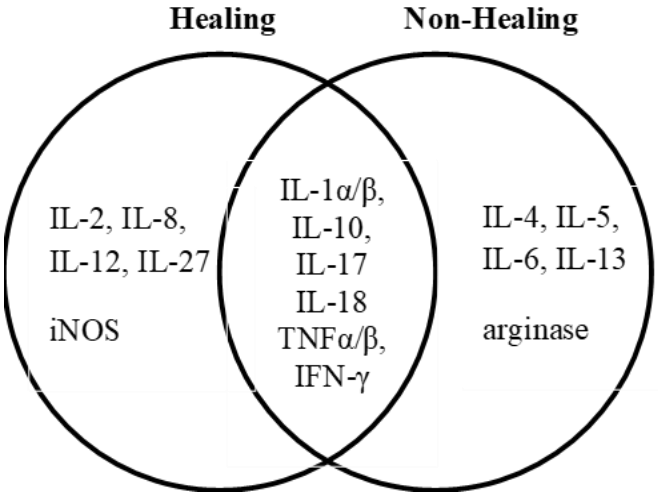


Figure 2: A) Immunological pathways against CL and MCL/ML: Th1 and Th2 mediators B) Categorization of cytokines and chemokines involved in healing or non-healing responses in ATL.

Adapted with permission from “Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review,” by N Maspi, A Abdoli and F Ghaffarifar, 2016, *Pathogens and Global Health,* 110 (6): 247-260. Copyright 2016 by Informa UK Limited, trading as Taylor & Francis Group. Classification relies on the predominant response of the immune marker. Effects contrary to the resolution of lesions are possible as dictated by the existing immune milieu.

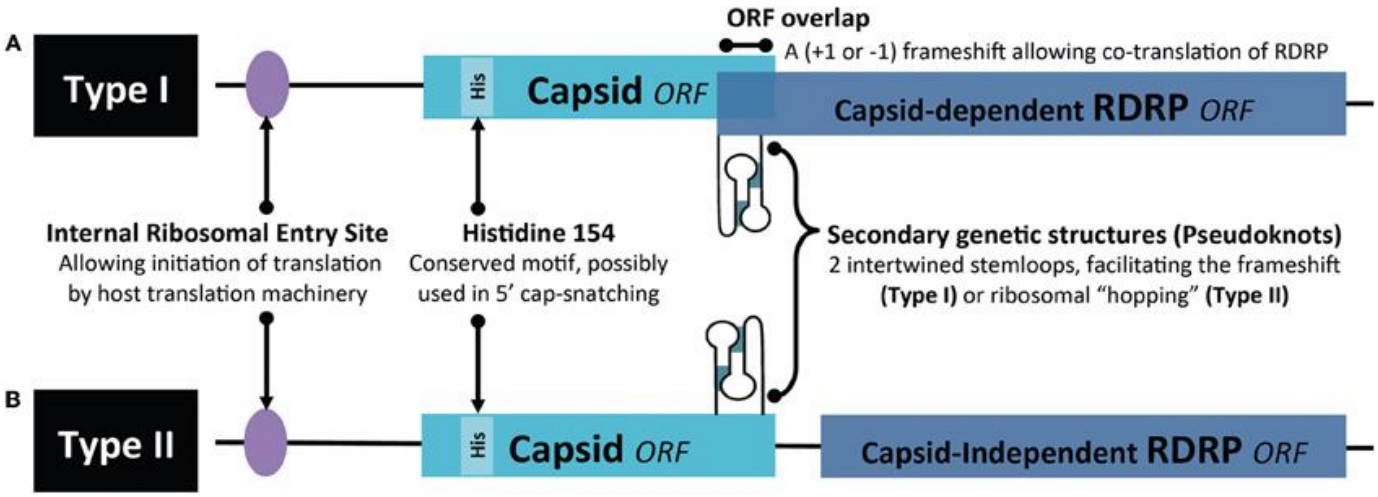


Figure 3: Characterization of A) LRV-1 and B) LRV-2 genomic structure.

Adapted with permission from "*Leishmania* RNA virus: when the host pays the toll" by MA Hartley, C Ronet, H Zangger, SM Beverley, N Fasel, 2012, *Frontiers in Cellular and Infection Microbiology-Virus and Host*, 2: 99. Copyright 2012 by Informa UK Limited, trading as Taylor & Francis Group.

Chapter 1: Prevalence and Phenotypic Associations of LRV-1 in ATL

This chapter focuses on the prevalence and phenotypic associations of LRV-1 in 5 different groupings of ATL: LRV-1 in ATL from the New World (Objective 1A), LRV-1 in ATL from Peru (Objective 1B), LRV-1 in ATL from Peru with corresponding clinical cultures (Objective 1C), LRV-1 in *L. V. braziliensis* from Peru (Objective 1D) and LRV-1 in *L. V. panamensis* from Latin America (Objective 1E) (Figure 4). Each analysis provided an overview of the spectrum of clinical phenotypes and the proportion of LRV-1 in each population of *Leishmania* isolates. Objective 1A is a descriptive piece of the entire cohort of clinical samples, heterogenous in species and geographical distribution. Objective 1B focuses on ATL from Peru, including patients from endemic settings as well as travelers. Objective 1C further looks at ATL from Peruvian patients with culturable isolates to focus on a patient population with lifelong exposure. Objective 1D further evaluates the role of LRV-1 in the species *L. V. braziliensis* causing ATL in Peruvian patients to eliminate species-specific influences. Lastly, Objective 1E looks at the novel detection of LRV-1 in a species less described in literature, *L. V. panamensis*, given the recent number of diagnoses from patients with particular travel history to Central America.

Sample Size Calculation

Based on existing literature (Ginouves et al., 2016; Valencia et al., 2014; Ogg et al., 2003; Bourreau et al., 2016; Zangger et al., 2013; Tarr et al., 1988; Adaui et al., 2016; Macedo et al., 2016; Ito et al., 2015), it is hypothesized that the prevalence of LRV-1 would be approximately 10% in strains causing the LCL non-severe phenotype, 20% in the inflammatory/multifocal CL phenotype and 40% in strains causing the ML/MCL phenotype, thus, with an alpha of 0.05 and power of 0.8, this estimate yielded a required sample size of at least 14 in each of the groups.

Therefore, an additional 50% was accounted for the unpredictable proportion of severe phenotypes. In the case where no ML/MCL phenotype is observed (see Objective 1E), the 20% inflammatory/multifocal CL phenotype and 10% LCL phenotype proportions remain, with an alpha of 0.05 and power of 0.8, requiring a sample set of 199 per group is required. Primary outcome measures include sex, age, causative species and LRV-1 positivity by clinical phenotype. LRV-1 status was analyzed by the above variables as well, and proportions calculated. Descriptive statistics (proportions, mean with SD, median, range) were calculated for all variables. Differences between categorical variables were compared using Fisher's exact test, Chi-square or Chi-square test for trend. Continuous variables were compared by Kruskal-Wallis Test or student's t-test. Significance was set at $p<0.05$. Data were analyzed using GraphPad Prism (GraphPad, CA).

Materials & Methods

Specimen Enrolment

Unique surplus discard clinical specimens of *Leishmania* spp. were identified from Public Health Ontario Laboratory (PHOL) and the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander von Humboldt", Lima, Peru between 2006-2019. Biobanked isolates were confirmed as *Leishmania* spp. by multiplex real-time PCR targeting *Leishmania* 18S rRNA, following clinical testing, which included microscopic examination of Giemsa-stained smears and/or culture by certified medical lab technologists.

Clinical Data

De-identified clinical data of source patients collected from test requisitions and case record forms were stratified into the following phenotypes: MCL/ML (simultaneous cutaneous infection and/or destruction of the mucosa), inflammatory ulcers (ulcers with associated erythema,

purulent exudate, pain with or without lymphatic involvement), or multifocal/disseminated ulcers (ulcers in ≥ 2 anatomic sites and ≥ 4 in number) as per the Infectious Diseases Society of America (IDSA) guidelines (Aronson, 2016), understanding that the pathogenesis underpinning mucosal versus severe cutaneous manifestations of *Leishmania* infection are quite different. LCL was defined as of < 4 ulcers in number (Aronson, 2016).

DNA Extraction

DNA extraction was performed using the Qiagen DNA Mini Kit (Qiagen, Germantown, MD) using 200 µL of cultured specimen with a final elution volume of 60 µL. In the case of primary clinical specimens including filter paper lesion impressions (FPLIs) (Fisher Scientific, Waltham, MA), biopsies, and cytology brushes (VWR, Radnor, PA) specimens were soaked in 200 µL of TE (ThermoFisher Scientific, Waltham, MA) prior to extraction to achieve sufficient volume and DNA concentration and eluted in 60µL nuclease-free water (ThermoFisher Scientific, Waltham, MA).

RNA Extraction

RNA was extracted from cultured promastigotes using the Cells Protocol of the QIAamp RNA Mini Kit (Qiagen, Germantown, MD) and eluted with 50µL of RNase-free Water (ThermoFisher Scientific, Waltham, MA). RNA was extracted from tissue biopsy and cytology brushes using the Fibrous Tissue Protocol from the Qiagen RNeasy Micro Kit (Qiagen, Germantown, MD) with the addition of carrier RNA (Qiagen, Germantown, MD) and eluted with 14 µL RNase-free water (ThermoFisher Scientific, Waltham, MA). RNA was extracted from FPLIs with the QIAmp RNA Blood Mini Kit (Qiagen, Germantown, MD) and eluted with 30 µL RNase-free water. An in-column DNase treatment was included using the Qiagen rDNase Set (Qiagen, Germantown, MD) as per manufacturer's protocol.

cDNA Synthesis and Purification

cDNA was performed using 10 µL of RNA in combination with the Superscript II Reverse Transcriptase and random hexamers (ThermoFisher Scientific, Waltham, MA). PCR purification was performed using the Qiagen QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and eluted with 60µL nuclease-free water (ThermoFisher Scientific, Waltham, MA).

Species Identification

Species identification was performed using the following gene targets by end-point PCR: internal transcriber space 1 (ITS1), ITS2, cysteine proteinase B (CPB), heat shock protein 70 (HSP70), mannose phosphate isomerase (MPI), zinc-dependent metalloproteinase (GP63), and confirmatory Sanger sequencing (Schonian, 2003; de Almeida, 2011; Wortmann, 2001). Restriction fragment length polymorphism (RFLP) analysis was performed on each product of end-point PCR (de Almeida, 2011; Wortmann, 2001).

Sanger Sequencing

Sanger Sequencing was performed using 1 µL of PCR product, 2 µL of Big Dye, 3 µL of Buffer, and 2 µL of 10µM of primer (ThermoFisher Scientific, Waltham, MA). The following cycling conditions were used on the Veriti ABI Thermal Cycler (Applied Biosystems, Waltham, MA): 1 min at 96°C, 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Product was cleaned using 45 µL of SAM Solution and 10 µL of beads set on a shaking incubator for 30 minutes. Products were then centrifuged for 2 minutes at 2000g prior to being loaded onto the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA). Data were standardized using the Sequencing Analyzer program and BLAST search engine was used to analyze the sequence.

LRV-1 Detection and Quantification

LRV-1 was detected by real time PCR using two primer sets, set A and set B, respectively (Zangger, 2013; Schmittgen, 2008). *Leishmania* kinetoplastid membrane protein 11 (kmp11) was used as a reference for quantification where sufficient RNA volume for quantification permitted this analysis (Tarr, 1988). A SYBR Green assay was set up using 1x SYBR Select Master Mix, 250 nM final concentration of forward and reverse primers and 5uL of cDNA in a total volume of 20 µL. The ABI 7900HT real time instrument was set to the following conditions: UDG activation at 50^{o}C for 2min, polymerase activation at 95^{o}C for 2min, followed by 45 cycles of 95^{o}C for 15sec, and 60^{o}C for 1min. A dissociation step of 95^{o}C for 15sec, 60^{o}C for 15sec, and another 95^{o}C for 15sec was added at the end to generate a melting curve to check for specificity of amplification. Each isolate was run in triplicate and contained the *L. (V.) guyanensis* ATCC® (American Type Culture Collection®) 50126™ (MHOM/BR/75/M4147) positive control to perform relative quantification using the $2^{-\Delta\Delta Ct}$ method (Ogg, 2003; Zangger, 2013; Schmittgen, 2008). If kmp11 was not detected, a pre-amplification step was performed as per Perfecta Pre-Amp Supermix guidelines. In the case that kmp11 remained undetected after pre-amplification, the 18S rRNA gene was used as a reference and a relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Bourreau, 2016; Schonian, 2003; de Almeida, 2011; Wortmann, 2011; Zangger, 2013; Schmittgen, 2008). Relative LRV-1 copy number was calculated using the methods outlined by Zangger and colleagues, and further described below (Zangger, 2013). The "gold" standard source of LRV-1 in this analysis as well as the Zangger paper is *L. V. guyanensis* (Zangger, 2013).

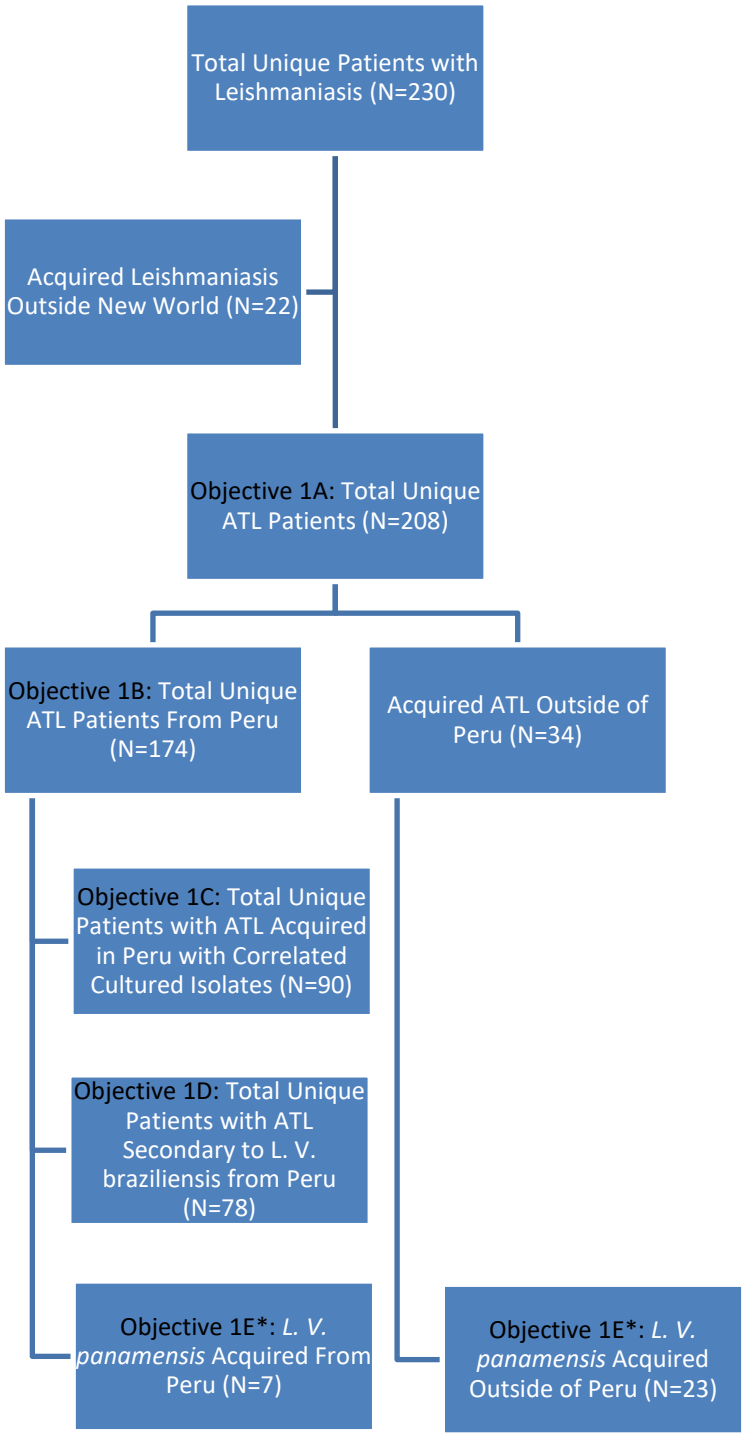
Data Analysis

Descriptive statistics (proportions, mean with SD, median, range) were calculated for all variables. Differences between categorical variables were compared using Fisher's exact test, Chi-square or Chi-Square test for trend. Fisher's exact test was used when small cell sizes (expected values less than 5) occurred, whereas chi-square test was used when the cell sizes were expected to be large. Chi-square test for trend is used to determine a linear trend between the row number and fraction of subjects in the left column, where the row numbers are arranged in a natural order (severity of disease caused by species). Continuous variables were compared by Kruskal-Wallis Test or Mann-Whitney U, comparing two or three outcomes, respectively. Significance was set at $p<0.05$. Data were analyzed using GraphPad Prism (GraphPad, CA). Relative LRV-1 copy number was calculated using the $2^{-\Delta\Delta Ct}$ method, whereby the gold standard LRV-1-containing strain, *L. (V.) guyanensis* ATCC® (American Type Culture Collection®) 50126™ (MHOM/BR/75/M4147), was used as a positive reference control for each PCR (Ogg, 2003; Zangger, 2013; Schmittgen, 2008).

Population Selection

Inclusion criteria included parasitological lesion confirmation via smear, culture or PCR and sufficient unique discard specimen for species identification and LRV-1 detection and quantification, at least. If culture was successful, specimens were considered for studies outlined in Objectives 2 and 3. Five separate analyses were performed in Objective 1, analyzing the prevalence and phenotypic associations of LRV-1 in the following cohorts: 1) ATL from the New World (N=208) [Objective 1A], 2) ATL from Peru (N=174) [Objective 1B], 3) ATL from Peru with corresponding clinical cultures (N=90) [Objective 1C], 4) *L. V. braziliensis* from Peru

(N=78) [Objective 1D] and 5) *L. V. panamensis* from Latin America (N=30) [Objective 1E] (Figure 4).



***Combined for Objective 1E**

Figure 4: Flow diagram highlighting 5 analyses derived from 208 patients with confirmed ATL.

Objective 1A: Prevalence and Phenotypic Associations of LRV-1 in Leishmania Isolates from the Full New World Cohort (N=208)

Clinical and Demographic Data

Of 208 ATL patients (Figure 4), 154 (74%) were male and 54 (26%) were female. A total of 43 (21%), 67 (32%) and 98 (47%) had the MCL/ML, inflammatory/multifocal CL and LCL phenotypes, respectively. Median age of patients was 35 years (range 0.58 years -82 years). Eighty-three (40%) patients were infected with *L. V. braziliensis*, 29 (14%) were infected with *L. V. guyanensis*, 26 (13%) were infected with *L. V. peruviana*, 9 (4%) were infected with *L. V. lainsoni*, 32 (16%) were infected with *L. V. panamensis*, 7 (3%) were infected with *Viannia* hybrids, 1 (0.5%) patient was infected with *L. mexicana* and 21 (11%) patients were infected with an unidentified species. Lastly, 55 (26%) specimens from *Leishmania* patients were LRV-1 positive compared to 136 (65%) specimens which were LRV-1 negative. Seventeen (65%) specimens could not be quantified due to lack of sufficient material.

Clinical Phenotype

Males represented 41 (95%) cases of all ML/MCL, 45 (67%) cases of inflammatory/multifocal CL and 68 (69%) cases of LCL, compared to females ($p = 0.007$). The median age of patients with ML/MCL was 41.5 years (range 7-82 years), compared to 34.5 years (range 3-80 years) and 31 years (range 0.58-76 years) for those with the inflammatory/multifocal CL and LCL phenotypes, respectively ($p = 0.015$). *L. V. braziliensis* contributed to 23 (53%) ML/MCL cases, 27 (42%) inflammatory/multifocal CL cases and 33 (34%) LCL cases. *L. V. guyanensis* contributed to 2 (5%) ML/MC cases, 7 (10%) inflammatory/multifocal CL cases and 20 (20%) LCL cases. *L. V. peruviana* contributed to 5 (12%) ML/MCL cases, 10 (15%)

inflammatory/multifocal CL cases and 11 (11%) LCL cases. *L. V. lainsoni* contributed to 2 (5%) ML/MCL cases, 4 (6%) inflammatory/multifocal CL cases and 3 (3%) LCL cases. *L. V. panamensis* contributed to no ML/MCL cases, 13 (19%) inflammatory/multifocal CL cases, and 19 (19%) LCL cases. *Viannia* hybrids contributed to no ML/MCL cases, 1 (2%) inflammatory/multifocal CL case and 6 (6%) LCL cases. *L. mexicana* contributed to only 1 (1%) LCL case. Lastly, unidentified species contributed to 11 (26%) ML/MCL cases, 5 (8%) inflammatory/multifocal CL cases, and 5 (5%) LCL cases (p=0.0007). LRV-1 positivity was identified in 15 (35%) ML/MCL cases, 17 (25%) inflammatory/multifocal CL cases and 23 (23%) LCL cases (p=0.533).

Clinical Phenotype: Sub-Analysis by Age

Given the association between age and clinical phenotype, a sub-analysis was performed. In the < 18 cohort, 3 (8%), 13 (35%) and 21 (57%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively (p=0.010). In the 18-35 group, 14 (19%), 22 (31%) and 36 (50%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively. In the 36-65 cohort, 20 (25%), 21 (26%) and 40 (49%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively. Lastly, in the >65 group, 5 (31%), 10 (62%) and 1 (6%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively.

LRV-1 Status

Males represented 42 (76%) LRV-1 positive cases compared to 100 (74%) LRV-1 negative cases (p=0.719). The median age of LRV-1 positive patients was 28 years (range 9-71 years) compared to 35 years (range 0.58 years-82 years) in LRV-1 negative patients (p=0.172). *L. V. braziliensis* contributed to 21 (38%) LRV-1 positive cases and 60 (44%) LRV-1 negative cases. *L. V.*

guyanensis contributed to 9 (16%) LRV-1 positive cases and 18 (13%) LRV-1 negative cases. *L. V. peruviana* contributed to 6 (11%) LRV-1 positive cases compared to 19 (14%) of LRV-1 negative cases. *L. V. lainsoni* contributed to 3 (7%) LRV-1 positive cases and 6 (4%) LRV-1 negative cases. *L. V. panamensis* contributed to 5 (10%) LRV-1 positive cases and 15 (11%) LRV-1 negative cases. *Viannia* hybrids contributed to 2 (4%) LRV-1 positive cases and 5 (4%) LRV-1 negative cases. *L. mexicana* contributed to only 15 (11%) LRV-1 negative cases. Lastly, unidentified species contributed to 7 (13%) LRV-1 positive cases compared to 14 (10%) LRV-1 negative cases (p=0.639). Fifteen (27%) LRV-1 positive cases were identified in patients with ML/MCL, 17 (31%) in the inflammatory/multifocal CL phenotype and 23 (42%) in the LCL phenotype. Whereas, 27 (20%) LRV-1 negative isolates were identified in patients with ML/MCL, 46 (34%) with the inflammatory/multifocal CL phenotype and 63 (46%) of the LCL phenotype (p=0.939).

LRV-1 Copy Number

Relative LRV-1 copy number (abundance) was calculated for 35/55 (64%) isolates positive for LRV-1 (Figure 5). Mean relative copy number of LRV-1 for isolates causing ML/MCL (n=11) was 14.72 ± 31.21 copies (median 1.1, range 0.03 – 103.5 copies), while for inflammatory/multifocal CL (n=10) it was 1.559 ± 4.882 copies (median $0.02x10^{-2}$, range $6.0x10^{-6}$ – 15.45 copies), and for LCL (n=14), it was 131.7 ± 477.5 (median 0.2671, range $2.57x10^{-5}$ - 1791 copies) (p=0.0067). Relative copy expression of LRV-1 in isolates causing MCL/ML was higher than that in LRV-1 positive isolates causing inflammatory CL (p=0.011). There was no difference in relative copy expression of LRV-1 in isolates causing MCL/ML versus LCL (p=0.2411). There was a difference in relative copy expression of LRV-1 in isolates causing inflammatory/multifocal CL compared to MCL/ML (p=0.0358).

Summary

The analysis of 208 ATL patients has demonstrated an association between sex, age, species and clinical phenotype, whereby patients who manifested ML/MCL were largely male, older in age (41.5 years compared to 31 years in the LCL group) and infected with *L. V. braziliensis* (Table 1). A sub-analysis of clinical phenotype by age revealed the increasing proportion of ML/MCL diagnosed with increasing age (Table 2). A breakdown of the same patient population by LRV-1 status revealed no association between sex, age, species and clinical phenotype. Relative LRV-1 copy number differed between patients manifesting ML/MCL and inflammatory CL, whereas no difference in viral burden was observed in LCL.

Table 1: Clinical and Demographic Data of 208 ATL Patients by Clinical Phenotype.

Characteristic	**Total (%)**	**Severe ATL**		**P-value**

		ML/MCL (n=43)	**Inflammatory/Multifocal CL (n=67)**	**Non-Severe ATL (LCL) (n=98)**	
Sex					
Male	154 (74%)	41 (95%)	45 (67%)	68 (69%)	0.007[a]
Female	54 (26%)	2 (5%)	22 (33%)	30 (31%)	
Median Age, years (range)	35 (0.58-82)	41.5 (7-82)	34.5 (3-80)	31 (0.58-76)	0.015[b]
Species					
L. V. braziliensis	83 (40%)	23 (53%)	27 (42%)	33 (34%)	
L. V. panamensis	32 (16%)	0 (0%)	13 (19%)	19 (19%)	
L. V. guyanensis	29 (14%)	2 (5%)	7 (10%)	20 (20%)	
L. V. peruviana	26 (12.5%)	5 (12%)	10 (15%)	11 (11%)	0.0007[c]
L. V. lainsoni	9 (4.5%)	2 (5%)	4 (6%)	3 (3%)	
Viannia Hybrids	7 (3%)	0 (0%)	1 (1.5%)	6 (6%)	
L. mexciana	1 (0.5%)	0 (0%)	0 (0%)	1 (1%)	

Unidentified Species	21 (10.5%)	11 (26%)	5 (7.5%)	5 (5%)	
LRV-1 Status					
LRV-1 Positive	55 (26%)	15 (35%)	17 (25%)	23 (23%)	0.533[a]
LRV-1 Negative	136 (65%)	27 (63%)	46 (69%)	63 (64%)	
Unknown*	17 (9%)	1 (2%)	4 (6%)	12 (13%)	

*****LRV-1 status available for 191 specimens given lack of sufficient specimen for 17

[a]Chi-square test

[b]Kruskal-Wallis test

[c]Chi-square test for trend

Table 2: Clinical Phenotype Sub-Analysis of 208 ATL patients by Age.

Age	ML/MCL (n=43)	Inflammatory CL (n=67)	Non-Severe CL (n=98)	p-value
<18 (n=37)	3 (8%)	13 (35%)	21 (57%)	0.010[a]
18-35 (n=72)	14 (19%)	22 (31%)	36 (50%)	
36-65 (n=81)	20 (25%)	21 (26%)	40 (49%)	
65+ (n=16)	5 (31%)	10 (63%)	1 (6%)	

[a]Chi-Square

246 **Table 3: Clinical and Demographic Data of 208 ATL Patients by LRV-1 Status.**

Characteristic	Total (%)	LRV-1 Status of 191 ATL Patients*		
		LRV-1 Positive (n=55)	LRV-1 Negative (n=136)	P-value
Sex				
Male	154 (74%)	42 (76%)	100 (73.5%)	0.719[a]
Female	54 (26%)	13 (24%)	36 (26.5%)	
Median Age, years (range)	35 (0.58-82)	28 (9-71)	35 (0.58-82)	0.172[b]
Species				0.639[c]
L. V. braziliensis	81 (39%)	21 (38%)	60 (44%)	
L. V. guyanensis	27 (13%)	9 (16%)	18 (13%)	
L. V. peruviana	25 (12%)	6 (11%)	19 (14%)	
L. V. panamensis	20 (10%)	5 (10%)	15 (11%)	
L. V. lainsoni	9 (4%)	4 (7%)	5 (4%)	
Viannia Hybrids	7 (3.5%)	2 (4%)	5 (3.7%)	
L. mexicana	1 (0.5%)	0 (0%)	1 (0.7%)	

Unidentified Species	21 (10%)	7 (13%)	14 (10.4%)	
Clinical Phenotype				
Severe				
ML/MCL	42 (20%)	15 (27%)	27 (20%)	0.939[d]
Inflammatory CL	63 (30%)	17 (31%)	46 (34%)	
Non-Severe (LCL)	86 (41%)	23 (42%)	63 (46%)	

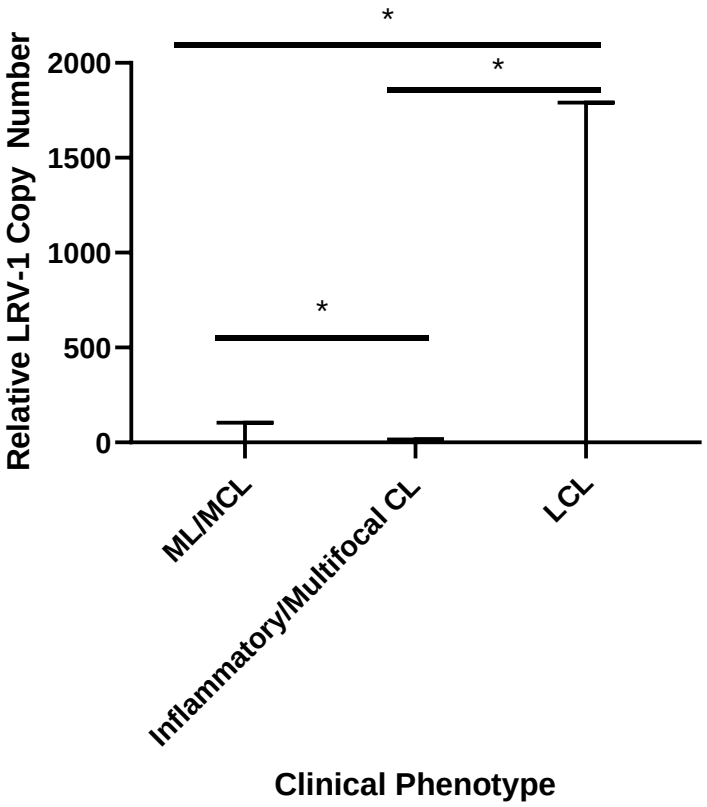
*LRV-1 status available for 191 specimens given lack of sufficient specimen for 17

[a]Fisher's Exact Test

[b]Mann Whitney

[c]Chi-Square test for trend

[d]Chi-Square



253

254 **Figure 5: Relative LRV-1 Copy Number of LRV-1 in Leishmania isolates from 208 ATL**
255 **Patients by Clinical Phenotype (median with range).**
256

Objective 1B: Prevalence and Phenotypic Associations of LRV-1 in Leishmania Isolates from Peru (N=174)

Clinical and Demographic Data

Of 174 ATL patients from Peru, 131 (75%) were male and 43 (25%) were female. A total of 40 (23%), 56 (32%) and 77 (45%) had the MCL/ML, inflammatory/multifocal CL and LCL phenotypes, respectively. Median age of patients were 32 years (range 0.58 years-82 years). Seventy-eight (45%) patients were infected with *L. V. braziliensis*, whereas 27 (16%) were infected with *L. V. guyanensis*, 25 (14%) were infected with *L. V. peruviana*, 9 (5%) were infected with *L. V. lainsoni*, 7 (4%) were infected with *L. V. panamensis*, 7 (4%) infected with *Viannia* hybrids, and 20 (12%) patients were infected with an unidentified species. Lastly, 51 (29%) specimens from *Leishmania* patients were LRV-1 positive compared to 122 (71%) specimens which were LRV-1 negative (Table 4).

Clinical Phenotype: Primary Outcomes

Males represented 38 (95%) cases of ML/MCL, 40 (72%) cases of inflammatory/multifocal CL and 53 (69%) cases of LCL, compared to females ($p = 0.005$). The median age of patients with ML/MCL was 43 years (range 7-82 years), compared to 29 years (range 3.5-66.50 years) and 29 years (range 0.58-75.42 years) for those with the inflammatory/multifocal CL and LCL phenotypes, respectively ($p = 0.004$). *L. V. braziliensis* contributed to 22 (55%) ML/MCL cases, 26 (46%) inflammatory/multifocal CL cases and 30 (39%) LCL cases. *L. V. guyanensis* contributed to 1 (2.5%) ML/MC case, 7 (12%) inflammatory/multifocal CL cases and 19 (25%) LCL cases. *L. V. peruviana* contributed to 4 (10%) ML/MCL cases, 10 (18%) inflammatory/multifocal CL cases and 11 (14%) LCL cases. *L. V. lainsoni* contributed to 2 (5%) ML/MCL cases, 4 (7%) inflammatory/multifocal CL cases and 3 (4%) LCL cases. *L. V.*

panamensis contributed to 0 (0%) ML/MCL cases, 3 (5%) inflammatory/multifocal CL cases and 4 (5%) LCL cases. *Viannia* hybrids contributed to 0 (0%) ML/MCL cases, 1 (1.8%) inflammatory/multifocal CL case and 6 (7.8%) LCL cases. Lastly, unidentified species contributed to 11 (28%) ML/MCL cases, 5 (9%) inflammatory/multifocal CL cases and 4 (5%) LCL cases (p=0.006). LRV-1 positivity was identified in 13 (33%) ML/MCL cases, 16 (28%) inflammatory/multifocal CL cases and 22 (29%) LCL cases (p=0.893).

Clinical Phenotype: Sub-Analysis by Age

In the < 18 cohort, 3 (10%), 12 (39%) and 16 (52%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively (p=0.030). In the 18-35 age group, 13 (20%), 20 (30%) and 31 (50%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively. In the 36-65 cohort, 19 (30%), 16 (25%) and 29 (45%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively. Lastly, in the >65 group, 5 (36%), 8 (57%) and 1 (7%) patients manifested ML/MCL, inflammatory/multifocal CL and LCL phenotypes, respectively

LRV-1 Status

Males represented 38 (75%) LRV-1 positive cases compared to 90 (74%) LRV-1 negative cases (p=0.4382). The median age of LRV-1 positive patients was 27 years (range 9-59 years) compared to 35 years (range 0.58-82 years) in LRV-1 negative patients (p=0.2611). *L. V. braziliensis* contributed to 21 (41%) LRV-1 positive cases and 57 (47%) LRV-1 negative cases. *L. V. guyanensis* contributed to 8 (16%) LRV-1 positive cases and 19 (16%) LRV-1 negative cases. *L. V. peruviana* contributed to 6 (12%) LRV-1 positive cases compared to 19 (16%) LRV-1 negative cases. *L. V. lainsoni* contributed to 4 (8%) LRV-1 positive cases and 5 (4%) LRV-1 negative cases. *L. V. panamensis* contributed to 3 (6%) LRV-1 positive cases and 4 (3%) LRV-1

303 negative cases. *Viannia* hybrids contributed to 2 (4%) LRV-1 positive cases and 5 (4%) LRV-1
304 negative cases. Lastly, unidentified species contributed to 7 (14%) LRV-1 positive cases
305 compared to 13 (11%) LRV-1 negative cases. Thirteen (25%) LRV-1 positive cases were
306 identified in patients with ML/MCL, 16 (31%) in the inflammatory/multifocal CL phenotype and
307 22 (43%) in the LCL phenotype. Whereas, 27 (22%) LRV-1 negative isolates were identified in
308 patients with ML/MCL, 40 (33%) with the inflammatory/multifocal CL phenotype and 55 (45%)
309 of the LCL phenotype (p=0.718).

310 *LRV-1 Copy Number*

311 Relative LRV-1 copy number (abundance) was calculated for 32/51 (63%) isolates positive for
312 LRV-1 (Figure 6). Mean relative copy number of LRV-1 for isolates causing ML/MCL (n=11)
313 was 14.72 ± 31.21 copies (median 1.1, range 0.03 – 103.5 copies), while for
314 inflammatory/multifocal CL (n=8) it was 1.933 ± 5.463 copies (median $0.08x10^{-2}$, range $6.0x10^{-6}$
315 – 15.45 copies), and for LCL (n=14), it was 131.7 ± 477.5 (median 0.2671, range $2.57x10^{-5}$ -
316 1791 copies) (p=0.1675). Relative copy expression of LRV-1 in isolates causing MCL/ML did
317 not differ from LRV-1 positive isolates causing inflammatory CL (p>0.999). There was no
318 difference in relative copy expression of LRV-1 in isolates causing MCL/ML versus LCL
319 (p=0.2987). There was a difference in relative copy expression of LRV-1 in isolates causing
320 inflammatory/multifocal CL compared to LCL (p=0.0159).

321 *Summary*

322 The analysis of 174 Peruvian ATL patients has demonstrated an association between sex, age,
323 species and clinical phenotype, whereby patients who manifested ML/MCL were largely male,
324 older in age (43 years compared to 29 years in the LCL group), infected with *L. V. braziliensis*
325 and largely LRV-1 negative (Table 4). A sub-analysis of clinical phenotype by age revealed the

increasing proportion of ML/MCL diagnosed in each age bracket (Table 5). A breakdown of the same patient population by LRV-1 status revealed no association between sex, age, species and clinical phenotype. Relative LRV-1 copy number did not differ between patients manifesting ML/MCL and inflammatory/multifocal CL or LCL, however viral burden was higher in patients manifesting LCL compared to the inflammatory/multifocal CL form.

Table 4: Clinical and Demographic Data of 174 Peruvian Patients with ATL by Clinical Phenotype.

Characteristic	Total (%)	Severe ATL		Non-Severe ATL (LCL) (n=77)	P-value
		ML/MCL (n=40)	Inflammatory/Multifocal CL (n=57)		
Sex					
Male	131 (75%)	38 (95%)	41 (72%)	53 (69%)	0.005[a]
Female	43 (25%)	2 (5%)	16 (28%)	24 (31%)	
Median Age, years (range)	32 (0.58-82)	43 (7-82)	29 (3.5-66.50)	29 (0.58-75.42)	0.004[b]
Species					
L. V. braziliensis	78 (45%)	22 (55%)	26 (46%)	30 (39%)	
L. V. guyanensis	27 (16%)	1 (2.5%)	7 (12%)	19 (25%)	0.006[c]
L. V. peruviana	25 (14%)	4 (10%)	10 (17.5%)	11 (14%)	
L. V. panamensis	7 (4%)	0 (0%)	3 (5%)	4 (5%)	
L. V. lainsoni	3 (2%)	2 (5%)	0 (0%)	1 (1%)	

Unidentified Species	22 (19%)	11 (27.5%)	5 (8.5%)	6 (8%)	
LRV-1 Status					
LRV-1 Positive	51 (29%)	13 (32.5%)	16 (28%)	22 (29%)	0.893[d]
LRV-1 Negative	123 (71%)	27 (67.5%)	41 (72%)	55 (71%)	

344 [a]Chi-Square

345 [b]Kruskal-Wallis

346 [c]Chi-Square test for trend

347 [d]Fisher's Exact

348

349

Table 5: Clinical Phenotype Sub-Analysis of 174 Peruvian Patients By Age.

Age	ML/MCL (n=40)	Inflammatory CL (n=56)	Non-Severe (n=77)	p-value
<18 (n=31)	3 (9.7%)	12 (38.7%)	16 (51.6%)	0.030[a]
18-35 (n=64)	13 (20%)	20 (30%)	31 (50%)	
36-65 (n=64)	19 (30%)	16 (25%)	29 (45%)	
65+ (n=14)	5 (36%)	8 (57%)	1 (7%)	

[a]Chi-Square

Table 6: Clinical and Demographic Data of 174 Peruvian Patients with ATL by LRV-1 Status.

Characteristic	Total (%)	LRV-1 Status of 174 Peruvian Patients with ATL		
		LRV-1 Positive (n=51)	LRV-1 Negative (n=123)	P-value
Sex				
Male	131 (75%)	38 (75%)	93 (75%)	0.4382[a]
Female	43 (25%)	13 (25%)	30 (25%)	
Median Age, years (range)	32 (0.58-82)	27 (9-59)	35 (0.58-82)	0.2611[b]
Species				
L. V. braziliensis	78 (45%)	34 (67%)	44 (36%)	0.3888[c]
L. V. guyanensis	27 (16%)	10 (20%)	17 (14%)	
L. V. peruviana	25 (14%)	9 (18%)	16 (13%)	
L. V. panamensis	7 (4%)	3 (6%)	4 (3%)	
L. V. lainsoni	3 (2%)	2 (4%)	5 (4%)	
Unidentified Species	22 (19%)	18 (35%)	4 (3%)	

Clinical Phenotype				
ML/MCL	40 (23%)	13 (25%)	27 (22%)	0.718[c]
Inflammatory CL	56 (32.4%)	16 (31%)	47 (38%)	
Non-Severe	77 (44%)	22 (43%)	55 (45%)	

363 [a]Fisher's exact

364 [b]Mann-Whitney

365 [c]Chi-square test for trend

366

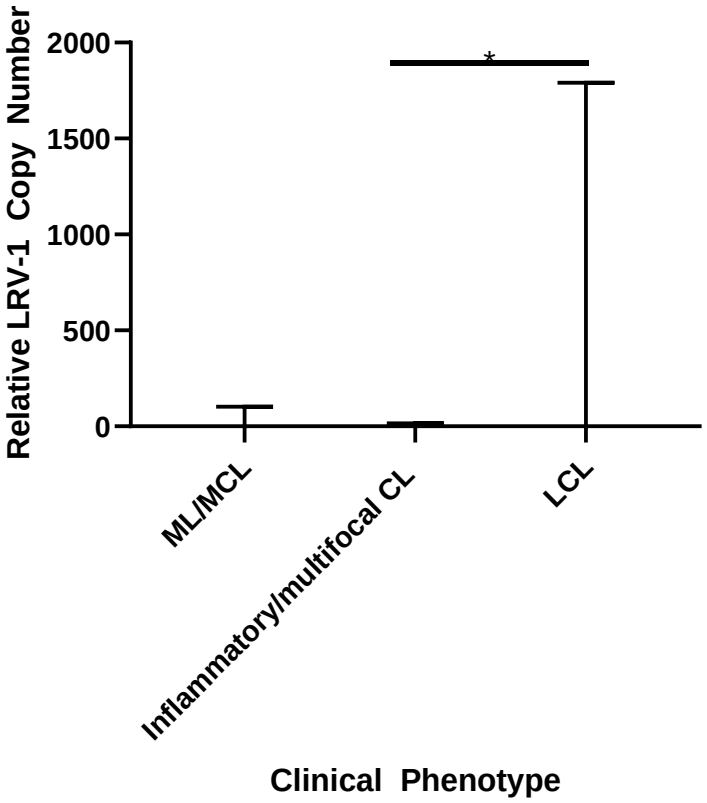


Figure 6: Relative LRV-1 Copy Number in Leishmania isolates from 173 ATL Patients by Clinical Phenotype (median with range).

Objective 1C: Prevalence and Phenotypic Associations of LRV-1 in Leishmania Isolates from Peru with Corresponding Clinical Cultures (N=90)

Clinical and Demographic Data

Of 90 ATL patients from Peru with corresponding clinical culture, 68 (76%) were male and 22 (24%) were female. A total of 15 (17%) patients manifested MCL/ML, 40 (44%) manifested inflammatory/multifocal CL and 35 (39%) manifested LCL Median age of patients were 27 years (range 0.58-76 years). Forty (45%) patients were infected with *L. V. braziliensis*, whereas 8 (9%) were infected with *L. V. guyanensis*, 8 (9%) were infected with *L. V. lainsoni*, 11 (12%) were infected with *L. V. peruviana*, 6 (7%) were infected with *L. V. panamensis*, 3 (3%) infected with *Viannia* hybrids, and 14 (16%) patients were infected with an unidentified species. Lastly, 22 (24%) specimens were LRV-1 positive compared to 66 (73%) specimens which were LRV-1 negative.

Clinical Phenotype

Males represented all 15 (100%) cases of ML/MCL, 31 (78%) cases of inflammatory/multifocal CL and 22 (64%) cases of LCL, compared to females ($p = 0.0226$). The median age of patients with ML/MCL was 35 years (range 7-68 years), compared to 25 years (range 3-70 years) and 31 years (range 0.58-76 years) for those with the inflammatory/multifocal CL and LCL phenotypes, respectively ($p = 0.4547$). *L. V. braziliensis* contributed to 9 (60%) ML/MCL cases, 19 (48%) inflammatory/multifocal CL cases and 12 (34%) of LCL cases. *L. V. guyanensis* contributed to 0 (0%) ML/MC cases, 3 (8%) inflammatory/multifocal CL cases and 5 (14%) LCL cases. *L. V. lainsoni* contributed to 1 (7%) ML/MCL cases, 4 (10%) inflammatory/multifocal CL cases and 3 (9%) LCL cases. *L. V. peruviana* contributed to 1 (7%) ML/MCL case, 5 (13%) inflammatory/multifocal CL cases and 5 (14%) LCL cases. *L. V. panamensis* contributed to 0

(0%) ML/MCL cases, 3 (8%) inflammatory/multifocal CL cases and 3 (9%) of LCL cases. *Viannia* hybrids contributed to only 3 (9%) of LCL cases. Lastly, unidentified species contributed to 4 (27%) ML/MCL cases, 6 (15%) inflammatory/multifocal CL cases and 4 (11%) LCL cases (p=0.3492). LRV-1 positivity was identified in 9 (60%) ML/MCL cases, 7 (17.5%) inflammatory/multifocal CL cases and 6 (17%) LCL cases (p=0.015).

LRV-1 Status

Males represented 20 (90%) LRV-1 positive cases compared to 45 (68%) LRV-1 negative cases (p=0.0182). The median age of LRV-1 positive patients was 31.5 years (14-70 years) compared to 27 years (1-76 years) in LRV-1 negative patients (p=0.1377). *L. V. braziliensis* contributed to 9 (41%) LRV-1 positive cases and 31 (47%) LRV-1 negative cases. *L. V. guyanensis* contributed to 1 (5%) LRV-1 positive cases and 6 (10%) LRV-1 negative cases. *L. V. lainsoni* contributed to 3 (14%) LRV-1 positive cases compared to 5 (8%) LRV-1 negative cases. *L. V. peruviana* contributed to 1 (5%) LRV-1 positive case and 10 (15%) of LRV-1 negative cases. *L. V. panamensis* contributed to 3 (14%) LRV-1 positive cases and 3 (5%) LRV-1 negative cases. *Viannia* hybrids contributed to 0 (0%) LRV-1 positive cases and 3 (5%) LRV-1 negative cases. Lastly, unidentified species contributed to 5 (23%) LRV-1 positive cases compared to 8 (12%) LRV-1 negative cases. Nine (41%) 22 LRV-1 positive cases were identified in patients with ML/MCL, 7 (32%) in the inflammatory/multifocal CL phenotype and 6 (27%) in the LCL phenotype (p=0.7901). Whereas, 6 (9%) of 66 LRV-1 negative isolates were identified in patients with ML/MCL, 33 (50%) with the inflammatory/multifocal CL phenotype and 27 (40.9%) of the LCL phenotype (p=0.525).

LRV-1 Copy Number

Relative LRV-1 copy number (abundance) was calculated for 7/22 (32%) isolates positive for LRV-1 (Figure 7). Mean relative copy number of LRV-1 for isolates causing ML/MCL (n=3) was 34.84 ± 59.43 copies (median 0.5619, range 0.4833 – 103.5 copies), while for inflammatory/multifocal CL (n=2) it was 895.8 ± 1265 copies (median 895.8, range 0.9460 – 1791 copies), and for LCL (n=4), it was 42.98 ± 69.01 (median 13.62, range $3.0x10^{-4}$ – 144.7 copies) (p=0.1675). Relative copy expression of LRV-1 in isolates causing MCL/ML did not differ from LRV-1 positive isolates causing inflammatory CL (p=0.4000). There was no difference in relative copy expression of LRV-1 in isolates causing MCL/ML versus LCL (p=0.8571). There was no difference in relative copy expression of LRV-1 in isolates causing inflammatory/multifocal CL compared to LCL (p=0.5333).

Summary

The analysis of 90 Peruvian ATL patients with corresponding clinical cultures has demonstrated an association between sex, species and clinical phenotype, whereby patients who manifested ML/MCL were largely male, infected with *L. V. braziliensis* and largely LRV-1 positive (Table 7). A breakdown of the same patient population by LRV-1 status revealed an association between sex and species whereby patient who were LRV-1 positive were largely male and infected with *L. V. braziliensis.* Relative LRV-1 copy number did not differ between all 3 clinical phenotypes.

Table 7: Clinical and Demographic data of 90 Peruvian patients with Culturable Isolates of Leishmania Causing ATL and analyzed by Clinical Phenotype.

	Total (n=90)	**ML/MCL (n=15)**	**Inflammatory CL (n=40)**	**Non-Severe (n=35)**	**P-value**
Sex					
Male	68 (76%)	**15 (100%)**	31 (78%)	22 (63%)	0.0226[a]
Female	22 (24%)	0 (0%)	9 (23%)	13 (27%)	
Age (median, years)	27 (0.58-76)	35 (7-68)	25 (3-70)	31 (0.58-76)	0.4547[b]
Species					
L. V. braziliensis	40 (44.4%)	**9 (60%)**	19 (47.5%)	12 (34%)	
L. V. peruviana	11 (12.2%)	1 (6.7%)	5 (12.5%)	5 (14%)	
L. V. guyanensis	8 (8.8%)	0 (0%)	3 (7.5%)	5 (14%)	
L. V. lainsoni	8 (8.8%)	1 (6.7%)	4 (10%)	3 (9%)	0.3492[c]
L. V. panamensis	6 (6.7%)	0 (0%)	3 (7.5%)	3 (9%)	
***Viannia* Hybrids**	3 (3.3%)	0 (0%)	0 (0%)	3 (9%)	
Unidentified Species	14 (15.6%)	4 (27.2%)	6 (15%)	4 (11%)	

LRV-1 Status*					
Positive	22 (24.4%)	**9 (60%)**	7 (17.5%)	6 (17%)	0.0153[d]
Negative	66 (73.3$)	6 (40%)	33 (82.5%)	27 (77%)	

*LRV-1 Status available for 88 specimens given lack of sufficient specimen for 2

[a]Chi-Square

[b]Kruskal-Wallis

[c]Chi-Square test for trend

[d]Fisher's Exact

443 **Table 8:** Clinical and Demographic Data of 88 Peruvian Patients with Culturable Isolates of
444 Leishmania Causing ATL and Analyzed by LRV-1 Status.

	Total (N=88)	**LRV-1 Positive (n=22)**	**LRV-1 Negative (n=66)**	**P-value**
Sex				
Male	65 (74%)	20 (90%)	45 (68%)	0.0182[a]
Female	23 (26%)	2 (10%)	21 (22%)	
Age Range (median, years)	27 (1-76)	31.5 (14-70)	27 (1-76)	0.1377[b]
Species				
L. V. braziliensis	40 (45%)	9 (41%)	31 (47%)	
L. V. peruviana	11 (12.5%)	1 (5%)	10 (15%)	
L. V. lainsoni	8 (9%)	3 (14%)	5 (8%)	
L. V. guyanensis	7 (8%)	1 (5%)	6 (10%)	0.7901[c]
L. V. panamensis	6 (7%)	3 (14%)	3 (5%)	
***Viannia* Hybrids**	3 (3%)	0 (0%)	3 (5%)	
Unidentified Species	13 (15%)	5 (23%)	8 (12%)	

Clinical Phenotype				
ML/MCL	14 (16%)	9 (41%)	6 (10%)	
Inflammatory/Multifocal CL	40 (45.5%)	7 (31.8%)	33 (50%)	0.5250[d]
LCL	33 (37.5%)	6 (27.2%)	27 (40%)	

[a]Fisher's Exact

[b]Mann-Whitney

[c]Chi-Square test for trend

[d]Chi-Square

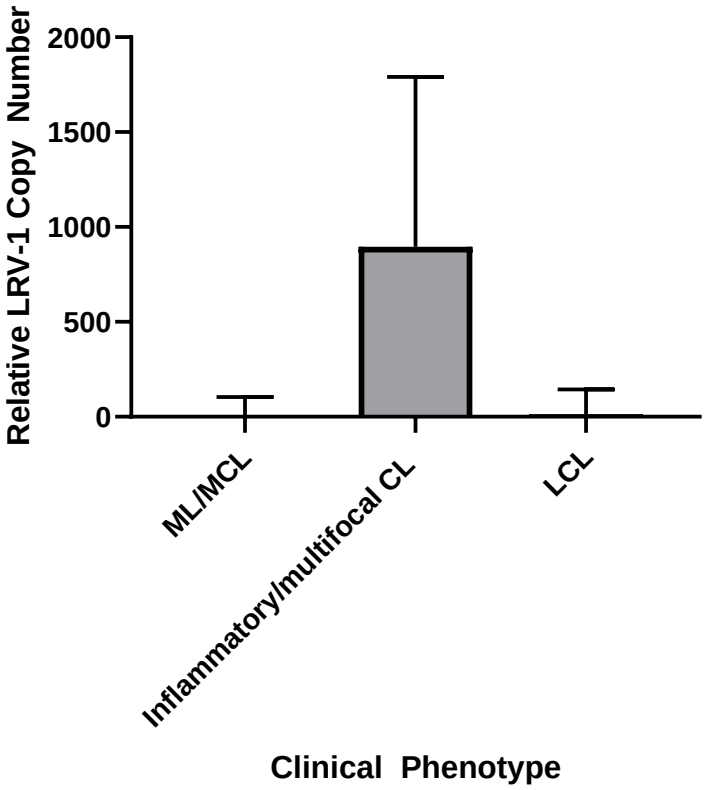


Figure 7: Relative LRV-1 Copy Number of LRV-1 in Leishmania isolates from 90 ATL Patients by Clinical Phenotype (median with range).

Objective 1D: Prevalence and Phenotypic Associations of LRV-1 in *L. V. braziliensis* from Peru (N=78)

Kariyawasam R, Lau R, Valencia BM, Llanos-Cuentas A, Boggild AK, *Leishmania* RNAVirus 1 (LRV-1) in *Leishmaia (Viannia) braziliensis* Isolates from Peru: A Description of Demographic and Clinical Correlates, 2020, 102, 2, pp, 280-285, by permission of The American Society of Tropical Medicine and Hygiene (The American Journal of Tropical Medicine and Hygiene)

Leishmania RNA Virus -1 (LRV-1) in *Leishmania (Viannia) braziliensis* Isolates from Peru: A Description of Demographic and Clinical Correlates

Ruwandi Kariyawasam BSc[1], Rachel Lau MSc[2], Braulio M. Valencia MD[3], Alejandro Llanos-Cuentas MD[3], Andrea K. Boggild MD[1,2,4,5]

Affiliations:

[1]Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada.

[2]Public Health Ontario Laboratory, Toronto, Ontario, Canada.

[3]Instituto de Medicina Tropical "Alejandro von Humboldt", Lima, Peru.

[4]Department of Medicine, University of Toronto, Toronto, Ontario, Canada.

[5]Tropical Disease Unit, Toronto General Hospital, Toronto, Ontario, Canada.

Address for Correspondence:

Dr. Andrea K. Boggild, Tropical Disease Unit, Toronto General Hospital

200 Elizabeth Street, 13EN-218

Toronto, ON M5G 2C4

Phone - 416-340-3675

Fax - 416-340-3260

Email - andrea.boggild@utoronto.ca

Keywords: mucosal leishmaniasis; cutaneous leishmaniasis**;** *Leishmania Viannia braziliensis*; Peru; *Leishmania* RNA Virus 1 (LRV-1).

Word Count: Abstract-241 words, Text-3365 words

Abstract

Leishmania RNA Virus 1-1 (LRV-1-1) is a dsRNA virus identified in isolates of *Leishmania (Viannia) braziliensis* and thought to advance localized cutaneous leishmaniasis (LCL) to mucocutaneous or mucosal leishmaniasis (MCL/ML). We examined the prevalence of LRV-1 and its correlation to phenotypes of American tegumentary leishmaniasis (ATL) caused by *L. (V.) braziliensis* from Peru to better understand its epidemiology. Clinical isolates of *L. (V.) braziliensis* were screened for LRV-1 by real-time PCR and stratified according to phenotype: LCL (< 4 ulcers in number) MCL/ML; inflammatory ulcers (erythematous, purulent, painful ulcers with or without lymphatic involvement) or multifocal ulcers (≥ 4 in ≥ 2 anatomic sites). Proportionate LRV1-positivity was compared across phenotypes. Of 78 *L. (V.) braziliensis* isolates, 26 (54.2%) had an inflammatory phenotype, 22 (28%) had the MCL/ML phenotype while 30 (38.5%) had LCL. MCL/ML was found exclusively in adult male enrollees. LRV-1 positivity by phenotype was as follows: 9/22 (41%) with MCL/ML; 5/26 (19%) with an inflammatory/multifocal CL phenotype; and 7/30 (23%) with LCL (p=0.19). LRV-1 positivity was not associated with age (p=0.55) or sex (p=0.49). Relative LRV-1 copy number was greater

in those with MCL/ML compared to those with inflammatory/multifocal CL (p=0.02). A direct association between LRV-1 status and clinical phenotype was not demonstrated, however, relative LRV-1 copy number was highest in those with MCL/ML. Future analyses to understand the relationship between viral burden and pathogenesis are required to determine if LRV-1 is truly a contributor to the MCL/ML phenotype.

Introduction

American tegumentary leishmaniasis (ATL) includes cutaneous leishmaniasis (CL), mucocutaneous (MCL), and mucosal leishmaniasis (ML), affecting 1-2 million people in the Americas (Reithinger, 2007). Localized CL (LCL) is generally a self-healing disease characterized by ulcerative, nodular, or verrucous lesions on the skin caused by several *Leishmania* spp. and endemic to many parts of the world including Peru (Reithinger, 2007; Aronson, 2016). Other clinical manifestations of CL include inflammatory CL where ulcers are associated with erythema, purulent exudate, pain and/or lymphatic involvement and more recently, atypical cutaneous leishmaniasis (ACL), which has been documented in an endemic region of Brazil (Guimares, 2016). To add, other forms include diffuse cutaneous leishmaniasis (DCL) with multiple non-ulcerative nodules (Reithinger, 2007) and disseminated leishmaniasis (DL), defined as maculopapular lesions identified in two or more anatomical sites ranging from 10-300 in number (Guimares, 2016). ML is a form of the disease affecting mucous membranes such as the nose, mouth, pharynx and larynx, more often attributed to sequela of the initial CL infection in Latin America, while MCL involves both cutaneous and mucosal lesions (Reithinger, 2007). This diverse phenotypology reflects a complex relationship between host, parasite, and vector factors[1] (extensively reviewed in [Reithinger, 2007]), with strong geographic- and species-specific preponderances to cutaneous manifestations of disease.

To add to this complexity of ATL pathogenesis, the presence of a double stranded RNA virus, *Leishmania* RNA virus 1 (LRV-1), has been identified in up to a quarter of certain strains of *Leishmania* (*Viannia*) spp., including *L. (V.) braziliensis* and *L. (V.) guyanensis*. LRV-1 found in New World *Viannia* strains are identified as LRV-1, with 14 subtypes (LRV-1-1-LRV-1-14) predominantly found in the Amazon basin (Hartley, 2012; Ginouves, 2016). Genetic diversity

between LRV-1 and parasite species exists, however the viruses from the same parasite species have shown less heterogeneity (Catanhede, 2018). It is hypothesized that the presence of LRV-1 will advance 10-15% of CL to MCL/ML stemming from an over-active immune response leading to severe immunopathological tissue infiltration and destruction (Ives, 2011; Ronet, 2011; Valencia, 2014; Ogg, 2003; Kariyawasam, 2017).

LRV-1 has been documented in 20-25% of clinical isolates of *L. (V.) guyanensis* and *L. (V.) braziliensis* found in Brazil and Peru and has been associated with first-line treatment failure (Ives, 2011; Bourreau, 2016) Studies have also indicated higher levels of LRV-1 in metastasizing versus non-metastasizing strains of *L. (V.) guyanensis*, which were correlated to increased levels of proinflammatory cytokines and chemokines including TNF-α, IL-6, CXCL10, CCL4, and CCL5 after recognition by toll-like receptor 3 (TLR3) in human and murine studies (Ives, 2011). On the other hand, in a human macrophage model, we have documented that LRV-1 in *L. (V.) braziliensis* was correlated to lower expression levels of TNF-α, IL-6, IL-1β, CXCL10, and increases in superoxide dismutase (SOD) (Kariyawasam, 2017). Given that LRV-1 may predict and correlate to more severe clinical manifestations of ATL, and given that Peru is one of the top worldwide contributors of CL, ML, and MCL, we aimed to understand its prevalence in clinical isolates of *L. (V.) braziliensis*, and the possible epidemiologic association between different clinical phenotypes of ATL from Peru.

Materials & Methods

Ethics Approval

Approval for this study was obtained from the Ethics Review Board of Public Health Ontario, the Research Ethics Board of University of Toronto, and the Institutional Review Board of Hospital Nacional Cayetano Heredia, Lima, Peru.

Specimen Enrolment

Unique surplus discard clinical specimens of *Leishmania* spp. were identified from Public Health Ontario Laboratory (PHOL) and the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander von Humboldt", Lima, Peru between 2012-2018 (Figure 8). Biobanked isolates were confirmed as *Leishmania* spp. by multiplex real-time PCR targeting *Leishmania* 18S rRNA, following clinical testing, which included microscopic examination of Giemsa-stained smears and/or culture by certified medical lab technologists.

Clinical Data

De-identified clinical data of source patients collected from test requisitions and case record forms were stratified into the following phenotypes: MCL/ML (simultaneous cutaneous infection and/or destruction of the mucosa), inflammatory ulcers (ulcers with associated erythema, purulent exudate, pain with or without lymphatic involvement), or multifocal/disseminated ulcers (ulcers in ≥ 2 anatomic sites and ≥ 4 in number) as per the Infectious Diseases Society of America guidelines (Aronson, 2016), understanding that the pathogenesis underpinning mucosal versus severe cutaneous manifestations of *Leishmania* infection are quite different. LCL was defined as of < 4 ulcers in number (Aronson, 2016).

DNA Extraction

DNA extraction was performed using the Qiagen DNA Mini Kit (Qiagen, Germantown, MD) using 200 µL of cultured specimen with a final elution volume of 60 µL. In the case of primary clinical specimens including filter paper lesion impressions (FPLIs) (Fisher Scientific, Waltham, MA), biopsies, and cytology brushes (VWR, Radnor, PA) specimens were soaked in 200 µL of TE (ThermoFisher Scientific, Waltham, MA) prior to extraction to achieve sufficient volume and

DNA concentration and eluted in 60µL nuclease-free water (ThermoFisher Scientific, Waltham, MA).

RNA Extraction

RNA was extracted from cultured promastigotes using the Cells Protocol of the QIAamp RNA Mini Kit (Qiagen, Germantown, MD) and eluted with 50µL of RNase-free Water (ThermoFisher Scientific, Waltham, MA). RNA was extracted from tissue biopsy and cytology brushes using the Fibrous Tissue Protocol from the Qiagen RNeasy Micro Kit (Qiagen, Germantown, MD) with the addition of carrier RNA (Qiagen, Germantown, MD) and eluted with 14 µL RNase-free water (ThermoFisher Scientific, Waltham, MA). RNA was extracted from FPLIs with the QIAmp RNA Blood Mini Kit (Qiagen, Germantown, MD) and eluted with 30 µL RNase-free water. An in-column DNase treatment was included using the Qiagen rDNase Set (Qiagen, Germantown, MD) as per manufacturer's protocol.

cDNA Synthesis and Purification

cDNA was performed using 10 µL of RNA in combination with the Superscript II Reverse Transcriptase and random hexamers (ThermoFisher Scientific, Waltham, MA) (Kariyawasam, 2017). PCR purification was performed using the Qiagen QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and eluted with 60µL nuclease-free water (ThermoFisher Scientific, Waltham, MA).

Species Identification

Species identification was performed using the following gene targets by end-point PCR: internal transcriber space 1 (ITS1), ITS2, cysteine proteinase B (CPB), heat shock protein 70 (HSP70), mannose phosphate isomerase (MPI), zinc-dependent metalloproteinase (GP63), and

596 confirmatory Sanger sequencing (Kariyawasam, 2017; Schonian, 2003; de Almeida, 2011;
597 Wortmann, 2001). Restriction fragment length polymorphism (RFLP) analysis was performed on
598 each product of end-point PCR (Kariyawasam, 2017; de Almeida, 2011; Wortmann, 2001).

599 ***Sanger Sequencing***

600 Sanger Sequencing was performed using 1 µL of PCR product, 2 µL of Big Dye, 3 µL of Buffer,
601 and 2 µL of 10µM of primer (ThermoFisher Scientific, Waltham, MA) (Kariyawasam, 2017).
602 The following cycling conditions were used on the Veriti ABI Thermal Cycler (Applied
603 Biosystems, Waltham, MA): 1 min at 96°C, 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min
604 at 60°C. Product was cleaned using 45 µL of SAM Solution and 10 µL of beads set on a shaking
605 incubator for 30 minutes (Kariyawasam, 2017). Products were then centrifuged for 2 minutes at
606 2000g prior to being loaded onto the Applied Biosystems 3730xl DNA Analyzer (Applied
607 Biosystems, Waltham, MA). Data were standardized using the Sequencing Analyzer program
608 and BLAST search engine was used to analyze the sequence (Kariyawasam, 2017).

609 ***LRV-1 Detection and Quantification***

610 LRV-1 was detected in isolates of *L. (V.) braziliensis* by real time PCR using two primer sets, set
611 A and set B, respectively (Figure 8) (Kariyawasam 2017; Zangger, 2013; Schmittgen, 2008).
612 *Leishmania* kinetoplastid membrane protein 11 (kmp11) was used as a reference for
613 quantification where sufficient RNA volume for quantification permitted this analysis
614 (Kariyawasam, 2017; Tarr, 1988). A SYBR Green assay was set up using 1x SYBR Select
615 Master Mix, 250 nM final concentration of forward and reverse primers and 5uL of cDNA in a
616 total volume of 20 µL (Kariyawasam, 2017). The ABI 7900HT real time instrument was set to
617 the following conditions: UDG activation at 50^{o}C for 2min, polymerase activation at 95^{o}C for
618 2min, followed by 45 cycles of 95^{o}C for 15sec, and 60^{o}C for 1min (Kariyawasam, 2017). A

dissociation step of 95ºC for 15sec, 60ºC for 15sec, and another 95ºC for 15sec was added at the end to generate a melting curve to check for specificity of amplification. Each isolate was run in triplicate and contained the *L. (V.) guyanensis* ATCC® (American Type Culture Collection®) 50126™ (MHOM/BR/75/M4147) positive control to perform relative quantification using the 2−ΔΔCt method (Ogg, 2003; Kariyawasam, 2017; Zangger, 2013; Schmittgen, 2008). If kmp11 was not detected, a pre-amplification step was performed as per Perfecta Pre-Amp Supermix guidelines. In the case that kmp11 remained undetected after pre-amplification, the 18S rRNA gene was used as a reference and a relative quantification was performed using the 2−ΔΔCt method (Kariyawasam, 2017; Bourreau, 2016; Schonian, 2003; de Almeida, 2011; Wortmann, 2011; Zangger, 2013; Schmittgen, 2008). Relative LRV-1 copy number was calculated using the methods outlined by Zangger and colleagues, and further described below (Zangger, 2013). The "gold" standard source of LRV-1 in this analysis as well as the Zangger paper is *L. V. guyanensis* (Zangger, 2013). We acknowledge that there are inter-species differences in LRV-1 viral load, however, we do not have a LRV-1 clone to calculate an absolute copy number. Moreover, by normalizing the relative abundance to the *L. V. guyanensis* MHOM/BR/75/M4147 strain, which is readily available from the ATCC, we are able to maintain consistent analysis across experiments and studies. Where copy number is recorded as N/A, this indicates an inability to calculate LRV-1 copy number due to a non-amplifiable kmp11 reference gene.

Data Analysis

Descriptive statistics (proportions, mean with SD, median, range) were calculated for all variables. Differences between categorical variables were compared using Fisher's exact test or Chi-square analysis. Continuous variables were compared by Kruskal-Wallis Test or Mann-Whitney U. Significance was set at $p<0.05$. Data were analyzed using GraphPad Prism

(GraphPad, CA). Relative LRV-1 copy number was calculated using the 2−ΔΔCt method, whereby the gold standard LRV-1-containing strain, *L. (V.) guyanensis* ATCC® (American Type Culture Collection®) 50126™ (MHOM/BR/75/M4147), was used as a positive reference control for each PCR run containing LRV-1 positive *L. (V.) braziliensis* isolates (Ogg, 2003; Kariyawasam, 2017; Zangger, 2013; Schmittgen, 2008).

Results

Clinical and Demographic Data

Of 208 specimens from patients with confirmed ATL, 78 (38%) isolates were identified as *L. (V.) braziliensis* acquired in Peru, by local Peruvians (n=76, 97%) or travelers to Peru (n=2, 3%) (Figure 8). One-hundred thirty (62%) patients were excluded due to acquisition of ATL outside of Peru, and/or infection with a non-*braziliensis* species (Figure 1). Sixty-five (83%) patients were male, while 13 (17%) were female (Tables 9 and 10). Median age was 34 years (range 2 - 76 years) (Tables 9 and 10). Thirty (38.5%) isolates were derived from patients with LCL, while 26 (33%) were from patients with inflammatory/multifocal CL, and 22 (28%) were from patients with MCL/ML (Tables 9 and 10).

Clinical Phenotype by Demographics: Secondary Outcomes

Median ages of patients were distributed across phenotypes as follows: 40.5 years (range 20 - 82 years) for those with MCL/ML; 31 years (range 13 - 68 years) for those with inflammatory/multifocal CL; and 31 years (range 2 - 76 years) for those with LCL, respectively (p=0.72) (Table 9). No children or adolescents had an MCL/ML phenotype; those in the <18 years age bracket manifested LCL (n=4, 50%) or inflammatory/multifocal CL (n=4, 50%), exclusively (Table 11). Male sex (n=65/78) was distributed across phenotypes as follows: 100% (n=22) with MCL/ML, 69% (n=18/26) with inflammatory/multifocal CL, and 83% (25/30) with

LCL (p=0.02) (Table 10). No females in the analysis had MCL/ML, while 31% and 17% of those with the inflammatory/multifocal and LCL phenotypes, respectively, were female (Table 10). To summarize the clinical phenotype by demographics data, MCL/ML was found exclusively in adult male enrollees.

LRV-1 Prevalence by Clinical Phenotype: Primary Outcome

A total of 21/78 (27%) isolates contained LRV-1 while 57/78 (73%) did not (Figure 8, Tables 9 and 10). LRV-1 was detected in 9 (41%) isolates causing MCL/ML, 5 (19%) isolates causing inflammatory/multifocal CL ulcers, and 7 (23%) isolates causing LCL, respectively (p=0.21) (Table 10). LRV-1 positivity was distributed across phenotypes as follows: 43% (9/21) of LRV-1-positive isolates were found in MCL/ML; 24% (5/21) of LRV-1-positive isolates were found in inflammatory/multifocal ulcers; and 33% (7/21) of LRV-1-positive isolates were found in LCL (p=0.19) (Table 11). However, LRV-1-positivity was detected in only 1 (10%) isolate from patients >60 years (n=10); 20 (33%) isolates from patients aged 19-59 years (n=60); and zero (0%) isolates from patients <18 years (n=8) (p=0.0591) (Table 11).

Relative LRV-1 Copy Number (abundance)

Relative LRV-1 copy number (abundance) was calculated for 17/21 (81%) isolates positive for LRV-1. Mean relative copy number of LRV-1 for isolates causing ML/MCL (n=7) was 21.6 ± 14.6 copies (median 4.7, range $9.0x10^{-2}$ – 103.5 copies), while for inflammatory/multifocal CL (n=4) it was $5.5x10^{-2} \pm 2.5x10^{-2}$ copies (median $5.8x10^{-2}$, range $2.9x10^{-3}$ – $1.0x10^{-1}$ copies), and for LCL (n=6), it was 8.3 ± 4.1 (median 7.4, range $8.5x10^{-3}$ - 27.2 copies) (p=0.11) (Figure 9). Relative copy expression of LRV-1 in isolates causing MCL/ML was higher than that in LRV-1 positive isolates causing inflammatory CL (p=0.02) (Figure 9, Table 12). There was no difference in relative copy expression of LRV-1 in isolates causing MCL/ML versus all CL

(p=0.30) and LCL (p=0.94) (Figure 9, Table 12). To summarize, LRV-1 relative copy number in MCL/ML isolates was 392.5-fold higher than in isolates causing inflammatory/multifocal CL.

Discussion

Severity of ATL has been hypothesized to be associated with the viral endosymbiont LRV-1 for decades, with the first report of LRV-1 isolated from a human with cutaneous satellite lesions and lymphatic involvement after visiting Suriname (Tarr, 1988). Since this initial report, there have been significant advancements and availability of molecular diagnostic tools to further investigate and understand the role of LRV-1 in ATL, and further accrual of data in humans (Ginouves, 2016; Valencia, 2014; Ogg, 2003; Bourreau, 2016; Cantanhede, 2015; Adaui, 2016; Macedo, 2016; Ito, 2015; Pereira, 2013). It has been shown that LRV-1 and *Leishmania* parasites have co-evolved with clustering of both the virus and the parasite in specific geographic locations. Given the species-specific and geographic correlates of observed phenotype in tegumentary leishmaniasis, LRV-1 has the potential to contribute to the diagnosis, treatment, and prognostic decision-making in the care of ATL patients (Catanhede, 2018). In this study, we examined the overall prevalence and possible correlation to clinical phenotypes of LRV-1 in clinical strains of *L. (V.) braziliensis* acquired locally and exported from Peru, a highly endemic country for CL and MCL/ML. Our analysis reflects predominantly specimens from patients both residing in and traveling to endemic areas of Peru, and thus does not constitute a full survey of representative cases restricted to endemic highland and jungle areas of Peru, where the distribution of isolates and phenotypes may differ at a population level. While we observed no direct relationship between LRV-1 positivity or negativity with 3 discrete phenotypes, we documented that patients manifesting MCL/ML had strains of *L. (V.) braziliensis* containing the highest relative copy numbers of LRV-1, a novel observation in this patient population.

We analyzed LRV-1 status in 78 isolates of *L. (V.) braziliensis* causing various clinical phenotypes of ATL from Peru and found an overall 27% prevalence, which is within the range reported previously from studies of strains in Latin America, specifically Peru (Ginouves, 2016; Cantanhede, 2016; Adaui, 2016; Macedo, 2916; Ito, 2015; Pereira, 2013; Salinas, 1996). It has been shown that LRV-1 is not preferentially associated with a specific phenotype (Adaui, 2016), although we herein determined that 41% of MCL/ML patients were LRV-1 positive, followed by LCL and inflammatory/multifocal CL at 23% and 19%, respectively. Unlike Cantanhêde and colleagues (Cantanhede, 2015), we noted no direct association of LRV-1 positivity prevalence to clinical phenotype, however, we documented an almost 400-fold higher relative LRV-1 copy number in isolates causing MCL/ML compared to isolates causing inflammatory/multifocal CL, potentially supporting a possible LRV-1 association with mucosal disease, in particular. Our findings extend what was originally documented by Ives and colleagues in a murine model of ATL where it was observed that LRV-1 quantity was several-fold higher in metastasizing strains of *L. (V.) guyanensis* (Ives, 2011). The relationship of both LRV-1 prevalence and relative viral burden to clinical manifestations and observed phenotype warrant additional work in larger cohort of patients with ATL, specifically in patients with MCL/ML.

On average, LRV-1 positive isolates in this analysis originated from patients who were 6 years younger than those whose isolates were LRV-1 negative, however, those at the extremes of age in this analysis had very low rates of LRV-1-positivity. Additionally, those with MCL/ML were an average of 8.5 years older than those with disease confined to the skin. No children or adolescents had either clinically manifest MCL/ML or LRV-1-positive *L. (V.) braziliensis* isolates causing their disease. Given that progression to ML typically occurs many years after LCL (Reithinger, 2007; Jara, 2016), that patients with MCL/ML in this analysis were older is, in

itself, unsurprising. One possible explanation for why LRV-1 may be less likely to occur in older patients who are from endemic settings is the recurrent, lifelong exposure, which could enable the parasite to harness the endogenous RNAi activity of the *Viannia* subgenus to eliminate the virus over time (Brettman, 2016). In this study, all but one isolate from patients over age 60 (n=10) were found to be LRV-1-negative, and no isolates from patients under age 18 were LRV-1-positive. Advanced age is associated with poorer T-cell response and a Th2-biased response, in particular (Salam, 2013), which in the case of ATL, is correlated to poorer immunologic control of infection and persistence of the amastigote in the phagolysosome (Hartlet, 2012). Similarly, the Th1-to-Th2 ratio has been demonstrated to be lowest in childhood and adolescence, with a peak during mid-adulthood, and slight decline thereafter (Chang, 2016). Th2 predominance over Th1 is also an important factor in the progression to ML (Moafi, 2017; Tripathi, 2007; Maspi, 2016; Hartley, 2013). Understanding the potential behavioral, socioeconomic, and biological underpinnings of the age distributions of LRV-1 noted in this analysis will be, ultimately, important to accurate interpretation of the viral role in ATL pathogenesis.

Limitations of this descriptive analysis of LRV-1 prevalence amongst *L. (V.) braziliensis* isolates originating from Peru include the comparatively small number of isolates from each age grouping (children, young adults and older adults), which may have biased our interpretation of the data. Prospective enrolment of larger cohorts that might enable more even distribution of age brackets would be worthwhile. It is also possible that significantly different proportions of LRV-1 positivity by phenotype might have emerged with a larger cohort. While our limited budget did not permit such a large scale analysis, our findings are nevertheless important as, even in this smaller cohort, they document the higher relative viral load in *L. (V.) braziliensis* isolates causing MCL/ML, and also suggest some interesting age preponderances that will be best

interrogated using a combination of epidemiologic and basic scientific approaches going forward. Relative LRV-1 quantification in this study represents a relative copy number in relation to the ATCC® *L. (V.) guyanensis* strain, where primary clinical samples are compared to a clonal line, which arguably has higher viral burden given the oligoparasitic nature of clinical samples (which contain low amastigote burden, generally). All LRV-1 positive isolates in our analysis derive from a variety of primary clinical specimens, including cytology brushes, FPLIs, and a few cultured specimens. These isolates all reflect a mixed population and are not clonal lines, thus, our findings around relative LRV-1 copy number by strain should be interpreted cautiously. All estimates of relative LRV-1 copy number are based on methods that are highly dependent on the quality of procedures used to prepare samples, and are based on a number of estimates. A truly accurate measure of LRV-1 copy number across strains will require development of improved methods. Another limitation of this analysis was our inability to resolve down to the final species level *Leishmania* isolates from 20 individuals, some of whom may have been infected with *L. (V.) braziliensis*, which, again, may have influenced our findings. A prospective study following patients who are LRV-1 positive with CL over a significant time period and evaluating the likelihood of patients developing ML could shed light on the ability of LRV-1 to contribute to mucosal diseases while demonstrating the possibility of utilizing antiviral therapy as a novel means of primarily or adjunctively treating patients.

Conclusions

We have demonstrated that relative LRV-1 viral burden was highest in *L. (V.) braziliensis* isolates causing mucosal involvement in this cohort of ATL acquired in Peru. Age emerged as an interesting bias in this cohort, where LRV-1-positive isolates originated from younger patients on average, but proportionate representation of LRV-1-positivity was not observed across age

groups, with those within the extremes of age having low rates of LRV-1-positivity in their *Leishmania* isolates. Continued exploration of LRV-1 prevalence across age groups, particularly in larger cohorts, with specific interrogation of immunological age correlates of LRV-1-positivity while controlling for behavioral, socioeconomic, and other possible biological contributors to the age biases observed herein will be essential to understanding the relevance of this demographic variable to the host-parasite-viral interplay that governs phenotype. The role of LRV-1 as a predictive biomarker of disease severity remains unclear, however the mechanistic nature, particularly regarding the immune response, will prove useful to understanding overall ATL-LRV-1 pathogenesis particularly in patients with MCL/ML.

Declarations:

Author Contributions: RK contributed to study design; data collection, analysis, and interpretation; and was primarily responsible for drafting the manuscript. RL contributed to study design; data collection, analysis, and interpretation; and to manuscript revision and critical appraisal. BMV and AL-C contributed to study design; data collection and interpretation; and to manuscript revision and critical appraisal. AKB conceived the study and contributed to study design; data collection, analysis, and interpretation; and to writing and revising subsequent iterations of the manuscript. All authors serve as guarantors of the work.

Funding: This work was supported by Public Health Ontario via the Project Initiation Fund and the University of Toronto via an Early Career Department of Medicine award.

Acknowledgements: N/A

Author Disclosure Statement: The authors have no interests to declare.

Table 9: Demographic data for 78 patients with *L. (V.) braziliensis* isolates acquired in Peru by clinical phenotype.

Characteristic	Total N (%)	MCL/ML (n=22) N (%)	Inflammatory / Multifocal CL (n=26) N (%)	LCL (n=30) N (%)	P-value
Sex					0.02
Male	65 (83)	22 (100)	18 (69)	25 (83)	
Female	13 (17)	0 (0)	8 (31)	5 (17)	
Median Age, years (range)	34 (2-82)	40.5 (20-82)	31 (10-70)	31 (2-76)	0.10
LRV-1 Status					0.21
Positive	21 (27)	9 (41)	5 (19)	7 (23)	
Negative	57 (73)	13 (59)	21 (81)	23 (77)	

804 **Table 10: Demographic data for 78 patients with *L. (V.) braziliensis* isolates acquired in**
805 **Peru by LRV-1 status.**

Characteristic	Total N (%)	LRV-1 Positive (n=21) N (%)	LRV-1 Negative (n=57) N (%)	P-value
Sex				0.50
Male	65 (83)	19 (91)	46 (81)	
Female	13 (17)	2 (9)	11 (19)	
Median Age, years (range)	34 (2-82)	29 (20 – 68)	35.5 (2 – 82)	0.55
Clinical Phenotype				0.19
MCL/ML	22 (28)	9 (43)	13 (22)	
Inflammatory/Multifocal CL	26 (33)	5 (24)	21 (37)	

LCL	30 (38.5)	7 (33%)	23 (40%)	

806

807

Table 11: LRV-1 status and clinical phenotype according to age bracket in 78 patients with *L. (V.)braziliensis* acquired in Peru.

Age Bracket	LRV-1-positivity (n=21) N (%)	Clinical Phenotype		
		MCL/ML (n=22) N (%)	Inflammatory/Multifocal (n=26) N (%)	LCL (n=30) N (%)
<18 years (n=8)	0 (0)	0 (0)	4 (50)	4 (50)
19 - 59 years (n=60)	20 (33)	18 (30)	18 (30)	24 (40)
> 60 years (n=10)	1 (10)	4 (40)	4 (40)	2 (20)

FIGURE LEGENDS

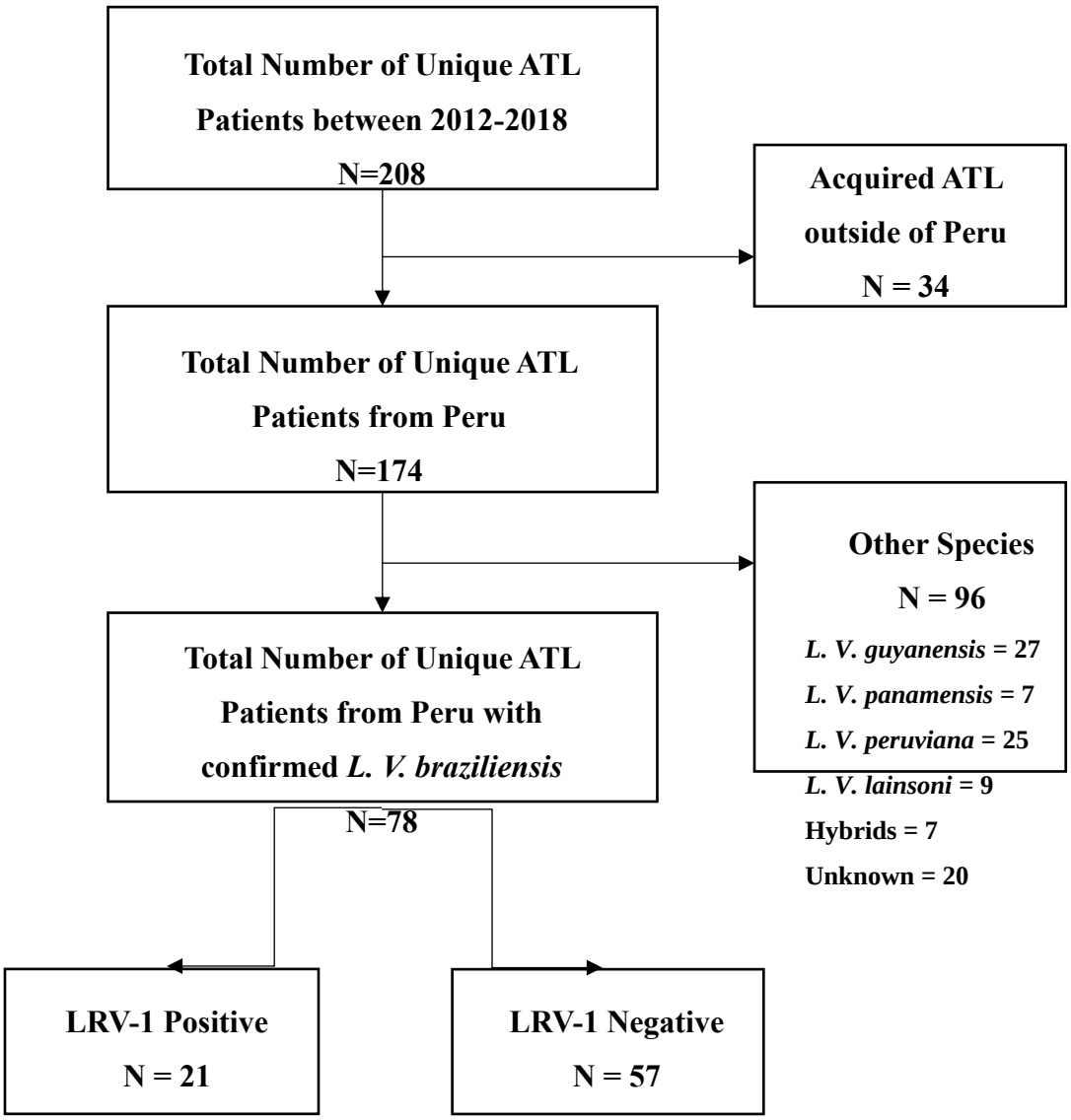
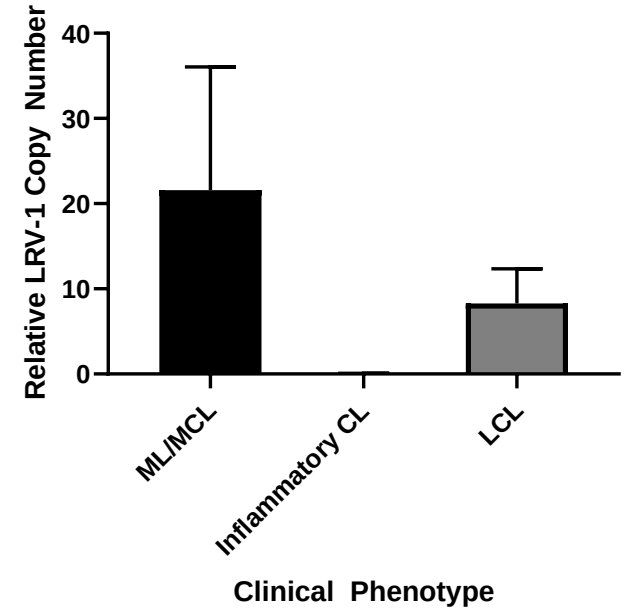


Figure 8: Workflow of sample identification and stratification of patients with confirmed *L. (V.) braziliensis.*

A



B

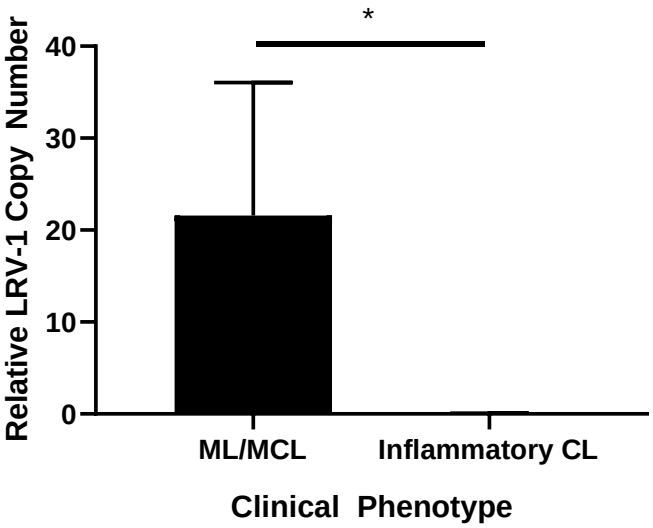


Figure 9: Mean relative LRV-1 copy number in isolates of L. (V.) braziliensis by clinical phenotype of ATL compared by Kruskal Wallis Test (A). Mean relative LRV-1 copy number in isolates of *L. (V.) braziliensis* causing MCL/ML and inflammatory/multifocal CL compared by Mann-Whitney (B).

Supplementary Information

Results

LRV-1 Prevalence by Demographics: Secondary Outcomes

Nineteen (29%) isolates from males were positive for LRV-1 versus 2 (15%) from females (p=0.50) (Table 10). Median age of patients whose isolates were LRV-1 positive and caused LCL, inflammatory/multifocal CL, and ML/MCL were: 33 years (range 23 - 57 years), 28 years (range 22 - 68 years), and 28 years (range 20-59 years), respectively (p=0.72) (Table 10). LRV-1 positivity was not associated with median age, whereby patients whose isolates were LRV-1 positive had a median age of 29 years (range 20 - 68 years) compared to LRV-1 negative patients whose median age was 35.5 years (range 2 - 82 years) (p=0.55) (Table 10).

Tables

Table 12: Demographic and clinical metrics of ATL patients with *L. (V.) braziliensis* isolates acquired in Peru

Specimen	Age	Sex	Specimen Type	LRV-1 Status	Relative LRV-1 Copy Number	Clinical Phenotype
1	28	M	FPLI	Positive	35.68	ML
2	31	M	FPLI	Negative		LCL
3	20	M	FPLI	Positive	0.35	MCL
4	28	M	Biopsy	Positive	2.9×10^{-3}	Inflammatory CL
5	50	M	Biopsy	Negative		Inflammatory CL
6	20	M	Biopsy	Negative		Inflammatory CL
7	34	M	Biopsy	Negative		Multifocal CL
8	22	M	Cytology Brush	Negative		Inflammatory CL

9	38	M	Cytology Brush	Negative		ML
10	54	M	Cytology Brush	Negative		ML
11	65	M	Cytology Brush	Negative		ML
12	20	M	Cytology Brush	Negative		LCL
13	30	M	Cytology Brush	Positive	4.7	ML
14	31	M	Cytology Brush	Negative		LCL
15	29	M	Cytology Brush	Positive	7.2	LCL
16	40	M	Cytology Brush	Negative		MCL
17	49	M	Cytology Brush	Negative		ML

18	27	M	Cytology Brush	Negative		LCL
19	31	M	Cytology Brush	Negative		LCL
20	46	M	Cytology Brush	Negative		LCL
21	53	M	Cytology Brush	Positive	N/A*	LCL
22	36	F	Cytology Brush	Positive	7.7	LCL
23	51	M	Cytology Brush	Negative		MCL
24	44	M	Cytology Brush	Negative		LCL
25	82	M	Cytology Brush	Negative		ML
26	41	M	Cytology Brush	Negative		MCL

27	33	M	Cytology Brush	Positive	7.5	LCL
28	27	M	Cytology Brush	Negative		LCL
29	22	M	Culture	Positive	0.1	Inflammatory CL
30	57	F	FPLI	Positive	$9.5x10^{-2}$	Multifocal CL
31	53	F	Culture	Negative		Multifocal CL
32	53	M	Culture	Positive	6.53	ML
33	NA	F	Culture	Negative		Inflammatory CL
34	27	M	Culture	Negative		Inflammatory CL
35	25	M	Culture	Negative		Inflammatory CL
36	35	M	Culture	Negative		MCL
37	45	M	Culture	Negative		Inflammatory CL

38	68	M	Culture	Negative		MCL
39	14	F	Culture	Negative		Inflammatory CL
40	57	M	Culture	Positive	27.2	LCL
41	35	F	Culture	Negative		Inflammatory CL
42	20	M	Culture	Positive	103.5	MCL
43	24	M	Culture	Positive	$2.8x10^{-1}$	MCL
44	2	M	Culture	Negative		LCL
45	24	M	Culture	Positive	$2.0x10^{-2}$	Inflammatory CL
46	20	M	Culture	Negative		LCL
47	13	M	Culture	Negative		Multifocal CL
48	68	F	Culture	Negative		Multifocal CL
49	53	M	Culture	Negative		Inflammatory CL

50	10	M	Culture	Negative		Inflammatory CL
51	76	F	Culture	Negative		LCL
52	12	F	Culture	Negative		LCL
53	23	M	Culture	Positive	$9.9x10^{-3}$	LCL
54	39	M	Culture	Negative		LCL
55	60	F	Culture	Negative		LCL
56	50	M	Culture	Negative		LCL
57	17	F	Culture	Negative		Multifocal CL
58	47	M	Culture	Negative		LCL
59	27	M	Culture	Positive	N/A*	ML
60	31	M	Culture	Negative		LCL
61	45	M	Culture	Negative		ML
62	26	M	Culture	Positive	$8.5x10^{-3}$	LCL
63	35	M	Culture	Positive	$9.1x10^{-2}$	ML
64	29	M	Culture	Negative		LCL

65	25	M	Culture	Negative		LCL
66	15	M	Culture	Negative		LCL
67	59	M	Culture	Positive	N/A*	ML
68	32	M	Culture	Negative		LCL
69	36	M	Culture	Negative		ML
70	12	F	Culture	Negative		LCL
71	70	M	Culture	Negative		Inflammatory CL
72	38	M	Culture	Negative		LCL
73	26	M	Culture	Negative		Inflammatory CL
74	34	M	Culture	Negative		Inflammatory CL
75	68	M	Culture	Negative		Inflammatory CL
76	68	M	Culture	Negative		ML

77	58	F	Culture	Negative		Inflammatory CL
78	68	M	Culture	Positive	N/A*	Multifocal CL

*N/A: LRV-1 relative copy number could not be calculated as the reference gene kmp11 was not amplifiable

Objective 1E: Novel Detection of Leishmania RNA Virus-1 (LRV-1) in Leishmania Viannia panamensis Clinical Isolates (N=30)

Clinical and Demographic Data

Of 208 specimens from patients with confirmed ATL, 30 (14.4%) isolates were identified as *L. V. panamensis* (Figure 4). Demographic and parasitologic factors for the 30 *L. V. panamensis* isolates from patients with ATL enrolled and analyzed are summarized in Tables 13 through 14. Eighteen (60%) patients were male, while 12 (40%) were female (Tables 13 and 14). Median age was 35 years (range 9 - 80 years) (Tables 13 and 14). Sixteen (53.3%) isolates were derived from patients with LCL, while 14 (46.7%) were from patients with inflammatory/multifocal CL, and zero (0%) patients with MCL/ML. *L. V. panamensis* was acquired in patients with the following travel history: 47% from Costa Rica (n=14), 23% from Peru (n=7), 13% from Ecuador (n=4), 3% from Belize (n=1), 3% from Brazil (n=1), 3% from Panama (n=1) and 3% from unknown (n=1), respectively (Table 13).

Clinical Phenotype

Male sex (n=18/30) was distributed across phenotypes as follows: 43% (n=6/14) with inflammatory/multifocal CL and 75% (12/16) with LCL (p=0.14) (Table 13). Twelve females were included in the analysis, of which 8 (66.7%) had the inflammatory/multifocal and 4 (33.3%) had the LCL phenotypes, respectively (Table 13). Median ages of patients were distributed across phenotypes as follows: 35 years (range 9 – 80 years) for those with inflammatory/multifocal CL and 34.5 years (range 17 - 64 years) for those with LCL, respectively (p=0.17) (Table 13). Inflammatory/multifocal CL was identified in 36% (5/14) of patients with travel history to Costa Rica, 14% (1/7) of patients with travel history to Peru, 25% (1/4) of patients with travel history to Ecuador and 100% (1/1) of patients with travel history to

Belize (p=0.42) (Table 13). Two (100%), one (100%), and one (100%) patients developed LCL after travel to an unknown country, Brazil and Panama, respectively (Table 13). One (50%) child had an inflammatory/multifocal phenotype (n=2); 9 (34.6%) individuals in the 18-65 years age bracket manifested inflammatory/multifocal CL (n=26), while those >65 (100%) exclusively manifested the inflammatory/multifocal CL phenotype (n=4) (Table 14).

LRV-1 Prevalence by Phenotype

A total of 7/30 (23%) isolates contained LRV-1 while 23/30 (77%) did not (Tables 13 and 14). Five of 14 (36%) isolates of patients with inflammatory/multifocal phenotypes were LRV-1 positive while 2/16 (13%) isolates from patients with the non-severe phenotype were LRV-1 positive (p=0.20) (Table 13).

Four (22%) isolates from males were positive for LRV-1 versus 3 (25%) from females (p=1.00) (Table 14). Median age of patients whose isolates were LRV-1 positive and caused inflammatory/multifocal CL and LCL were: 35 years (range 9-80 years) and 35 years (range 17-80 years), respectively (p=0.91) (Table 14). LRV-1 positivity was not associated with age, whereby patients whose isolates were LRV-1 positive had a median age of 35 years (range 9 - 71 years) compared to LRV-1 negative patients whose median age was 35 years (range 17 - 80 years) (p=0.91). However, LRV-1-positivity was detected in only 1 (25%) isolate from patients >65 years (n=4); 5 (21%) isolates from patients aged 18-65 years (n=24); and 1 (50%) isolate from patients <18 years (n=2) (p=0.21) (Table 15). LRV-1 isolates were detected in 43%, 43% and 14% of patients with travel history to Costa Rica (n=3), Peru (n=3) and Ecuador (n=1), respectively (p=0.554) (Table 14).

LRV-1 Copy Number

Relative LRV-1 copy number was calculated for 3/7 (43%) isolates positive for LRV-1. The mean relative copy number was identified in 3 isolates from patients with the inflammatory/multifocal phenotype was $1.08x10^{-4} \pm 1.06x10^{-3}$ (median $1.09x10^{-3}$, range $6.029x10^{-6} – 2.17x10^{-3}$ copies).

Summary

The analysis of 30 ATL patients infected with *L. V. panamensis* has demonstrated no association between sex, age, species, LRV-1 status and clinical phenotype. No ML/MCL was described in this patient population. A breakdown of the same patient population by LRV-1 status revealed no association between sex, age, species, travel history and clinical phenotype. Differences in relative LRV-1 copy number could not be accounted for given the small sample size.

Table 13: Demographic data for 30 patients with *L. (V.) panamensis* isolates by clinical phenotype.

Characteristic	Total N (%)	Inflammatory / Multifocal CL (n=14) N (%)	LCL (n=16) N (%)	P-value
Sex				0.14[a]
Male	18 (60)	6 (43)	12 (75)	
Female	12 (40)	8 (57)	4 (125)	
Median Age, years (range)	35 (9-80)	35 (9-80)	34.5 (17-64)	0.17[b]
Travel History				0.42[c]
Costa Rica	14 (47)	5 (36)	9 (65)	
Peru	7 (23)	1 (7)	6 (38)	
Ecuador	4 (13)	1 (7)	3 (19)	

Belize	1 (3)	1 (7)	0 (0)	
Brazil	1 (3)	0 (0)	1 (6)	
Panama	1 (3)	0 (0)	1 (6)	
Unknown	2 (7)	0 (0)	2 (13)	
LRV-1 Status				0.20[a]
Positive	7 (23)	5 (36)	2 (13)	
Negative	23 (77)	9 (64)	14 (87)	

[a]Fisher's Exact Test

[b]Mann-Whitney

[c]Chi-Square test for trend

Table 14: Demographic data for 30 patients with *L. (V.) panamensis* isolates by LRV-1 status

Characteristic	Total N (%)	LRV-1 Positive (n=7) N (%)	LRV-1 Negative (n=23) N (%)	P-value
Sex				1.00[a]
Male	18 (60)	4 (57)	14 (61)	
Female	12 (40)	3 (43)	9 (39)	
Median Age, years (range)	35 (9-80)	35 (9-71)	35 (17-80)	0.91[b]
Travel History				0.5554[c]
Costa Rica	14 (47)	3 (43)	11 (48)	
Peru	7 (23)	3 (43)	4 (17)	
Ecuador	4 (13)	1 (14)	3 (13)	
Belize	1 (3)	0 (0)	1 (4)	

Brazil	1 (3)	0 (0)	1 (4)	
Panama	1 (3)	0 (0)	1 (4)	
Unknown	2 (7)	0 (0)	2 (9)	
Clinical Phenotype				0.20[a]
Inflammatory/Multifocal CL	14 (47)	5 (71)	9 (39)	
LCL	16 (53)	2 (29)	14 (61)	

924 [a]Fisher's Exact Test

925 [b]Mann-Whitney

926 [c]Chi-Square test for trend

927

928 **Table 15: LRV-1 status and clinical phenotype according to age bracket in 30 patients with**
929 ***L. (V.) panamensis.***

Age Bracket	LRV-1-positivity (n=7) N (%)	Clinical Phenotype 930 931	
		Inflammatory/Multifocal (n=5) N (%)	LCL (n=2) N (%)
<18 years (n=2)	1 (50)	1 (100)	0 (0)
19 - 59 years (n=24)	5 (21)	3 (60)	2 (40)
> 60 years (n=4)	1 (25)	1 (100)	0 (0)

Chapter I General Discussion

Clinical Phenotype

ATL in each analysis is identified in a greater proportion of males than females, reflecting the societal and behavioural aspects of individuals particularly in, endemic settings (Bourreau et al., 2016; Cantanhede et al., 2015). The proportion of males manifesting ATL across each analysis ranges from 60%-83%. The median age of patients manifested with ML/MCL was 35-43 years, compared to 25-31 years for inflammatory/multifocal CL and 29-31 years for LCL. In each analysis, patients who manifesting with ML/MCL were older compared to those who manifested with inflammatory/multifocal CL or LCL, by an average of 10 years. This phenomenon occurs in the natural course of infection, whereby ML/MCL is typically identified in individuals who progress from healed LCL lesions (Reithinger et al. 2007).

The rates of *L. V. braziliensis, L. V. guyanensis, L. V. peruviana, L. V. lainsoni, L. V. panamensis, Viannia* hybrids and unidentified species identified in patients with the ML/MCL, inflammatory/multifocal and LCL phenotypes are relatively similar across each analysis. Noteworthy is the lack of *Viannia* hybrids or *L. V. panamensis* contributing to the ML.MCL phenotype across all analyses; as well as a lack of *L. V. guyanensis* contributing to ML/MCL in Objective 1C. A large proportion of ML/MCL across all objectives were identified in patients with *L. V. braziliensis* (53%-60%), compared to 3%-5% for *L. V. guyanensis*, 7%-12% for *L. V. peruviana*, 5%-7% for *L. V. lainsoni* and 26%-28% for unidentified species. Historically, ML/MCL is identified in patients infected with *L. V. braziliensis* and *L. V. guyanensis,* given the natural abundance of these species across Latin America and their propensity to cause severe disease through a number of cellular mechanisms. Moreover, rates of each species contributing to the inflammatory/multifocal CL phenotype was quite similar between analyses with the

exception of *L. V. panamensis* identified in 19% of inflammatory/multifocal CL in Objective 1A compared to 5% in Objective 1B and 8% in Objective 1C. The lack of *L. V. panamensis* in Objective 1B and 1C may be attributable to the focus on Peruvian specimens, where historically, *L. V. panamensis* is identified in Central and northern South America. Lastly, the rates of each species contributing to the LCL phenotype were not different with the exception of *L. V. guyanensis* contributing much less in the analysis of Objective 1C.

LRV-1 Status

The overall rate of LRV-1 did not differ across analyses, with a range of 23%-27%, within range of previous reported literature (Ginouves et al., 2016; Valencia et al., 2014; Cantanhede et al., 2015; Adaui et al., 2016; Macedo et al., 2016; Ito et al., 2015; Pereira et al., 2013; Salinas et al., 1996). The median age of LRV-1 positive individuals is confined to a specific age group, 27-35 years (range 0.58-82 years), typically younger than the LRV-1 negative cohort, however without significance.

The proportion of LRV-1 positive isolates identified as *L. V. braziliensis* ranges from 27% in the *L. V. braziliensis* specific analysis up to 67% in Objective 1B. *L. V. guyanensis* was identified as the causative species of LRV-1 positive isolates in 5%-20% of analyses, the former identified in Objective 1C. *L. V. panamensis* was identified in 6%-14% of LRV-1 positive isolates and brings into light the necessity to understand the influence of LRV-1 in this species. *L. V. lainsoni* was identified in 4%-14% of LRV-1 positive isolates, whereas *L. V. peruviana* was identified in 5%-18% of isolates, further warranting investigation of the role of LRV-1 in this species. *Viannia* hybrids contributed to roughly 4% of LRV-1 positive isolates, with species of unknown identify accounting for 13%-35% of these isolates. ML/MCL was identified in 25%-68% of LRV-1 positive isolates, whereas the inflammatory/multifocal CL phenotype was identified in 24%-31%

of LRV-1 positive isolates. Lastly, LCL was identified at a similar rate of 33%-42% in LRV-1 positive isolates across analyses. The high proportion of LRV-1 isolates identified in patients with ML/MCL across all objectives, but particularly Objective 1C may represent a bias due to culturability, as these specimens may represent more naturally virulent or polyparasitic strains compared to specimens received by other sources.

LRV-1 Copy Number

Relative LRV-1 copy number (abundance) varies between clinical phenotype, where the highest copy number typically came from patients who manifested ML/MCL or LCL, with lower viral burden identified in patients manifesting the inflammatory/multifocal CL phenotype. This, coupled with a higher proportion of patients with the ML/MCL phenotype detected with the virus may play a key role in the ultimate outcome of disease compared to patients with LCL.

989 **Table 16: Clinical and Demographic Data from Each Objective Analysis.**

	Objective 1A (N=208)	**Objective 1B (N=174)**	**Objective 1C (N=90)**	**Objective 1D (N=78)**	**Objective 1E (N=30)**
Sex, Male	154 (74%)	131 (75%)	68 (76%)	65 (83.3%)	18 (60%)
Median Age, Years (range)	35 (0.58-82)	32 (0.58-82)	27 (0.58-76)	34 (2-82)	35 (9-80)
ML/MCL	41.5 (7-82)	43 (7-82)	35 (7-68)	40.5 (20-82)	N/A
Inflammatory/multifocal CL	34.5 (3-80)	29 (3.5-66.50)	25 (3-70)	31 (10-70)	35 (9-80)
LCL	31 (0.58-76)	29 (0.58-75.42)	31 (0.58-76)	35.5 (20-82)	34.5 (17-64)
Species, ML/MCL	43 (20.7%)	40 (22.9%)	15 (16.7%)	22 (28.2%)	0 (0%)
L. V. braziliensis	23 (53%)	22 (55%)	9 (60%)	22 (100%)	N/A
L. V. peruviana	5 (12%)	4 (10%)	1 (6.7%)	N/A	N/A

L. V. guyanensis	2 (5%)	1 (2.5%)	0 (0%)	N/A	N/A
L. V. lainsoni	2 (5%)	2 (5%)	1 (6.7$)	N/A	N/A
L. V. panamensis	0 (0%)	0 (0%)	0 (0%)	NA	0 (0%)
Viannia Hybrids	0 (0%)	0 (0%)	0 (0%)	N/A	N/A
Unidentified Species	11 (26%)	11 (27%)	4 (27.2%)	N/A	N/A
Species, Inflammatory/Multifocal CL	67 (32.2%)	57 (32.7%)	40 (44.4%)	26 (33.3%)	14 (46.7%)
L. V. braziliensis	27 (42%)	26 (46%)	19 (47.5%)	26 (100%)	N/A
L. V. peruviana	10 (15%)	10 (17.5%)	5 (12.5%)	N/A	N/A
L. V. panamensis	13 (19%)	3 (5)	3 (7.5%)	N/A	14 (100%)
L. V. peruviana	10 (15%)	10 (17.5%)	5 (12.5%)	N/A	N/A
L. V. guyanensis	7 (10%)	7 (12%)	3 (7.5%)	N/A	N/A
L. V. lainsoni	4 (6%)	0 (0%)	4 (10%)	N/A	N/A
Viannia Hybrids	1 (1.5%)	0 (0%)	0 (0%)	N/A	N/A

Unidentified Species	5 (7.5%)	5 (8.5%)	6 (15%)	N/A	N/A
Species, LCL	98 (47.1%)	77 (44.2%)	35 (38.9%)	30 (38.5%)	16 (53.3%)
L. V. braziliensis	33 (34%)	30 (39%)	12 (34%)	30 (100%)	N/A
L. V. guyanensis	20 (20%)	19 (25%)	5 (14%)	N/A	N/A
L. V. panamensis	19 (19%)	4 (5%)	3 (9%)	N/A	16 (100%)
L. V. peruviana	11 (11%)	11 (14%)	5 (14%)	N/A	N/A
L. V. lainsoni	3 (3%)	1 (1%)	3 (95)	N/A	N/A
Viannia Hybrids	6 (6%)	0 (0%)	0 (0%)	N/A	N/A
Unidentified Species	5 (5%)	6 (8%)	3 (9%)	N/A	N/A
LRV-1, Positive	55 (26%)	51 (29%)	22 (24.4%)	21 (26.9%)	7 (23.3%)
ML/MCL	15 (35%)	13 (32.5%)	9 (60%)	9 (40.9%)	N/A
Inflammatory/multifocal CL	17 (25%)	16 (28%)	7 (17.5%)	5 (19.2%)	5 (35.7%)
LCL	23 (23%)	22 (29%)	6 (17%)	14 (29.2%)	2 (12.5%)

990

Conclusion

Overall, there is lack of evidence to support the direct association of LRV-1 and clinical phenotype at a population level, particularly LRV-1 positivity and the ML/MCL phenotype across all 5 analyses. Rather, age and species contributes to clinical manifestations of disease, whereby patients manifesting the ML/MCL group compared to LCL are on average 10 years older. In addition, *L. V. braziliensis* is a causative species found in great proportions across all forms of CL, whereas *L. V. panamensis* was identified in all forms other than ML/MCL. Determining the role of LRV-1 at a cell biological level in the context of *L. V. braziliensis* given its higher proportions in patients manifesting ML/MCL and *L. V. panamensis* given the lack of ML/MCL and high proportions in patients manifesting inflammatory/multifocal CL will prove valuable in understanding how clinical manifestation disease may vary by species and the viral endosymbiont.

Chapter 2: Influence of *Leishmania* RNA Virus-1 on Pro-Inflammatory Biomarker Expression in a Human Macrophage Model of American Tegumentary Leishmaniasis

LRV-1 in Clinical Cultures for Infectivity Studies

A total of 9 cultures including 6 clinical and 3 ATCC® were available to use for infectivity (Objective 2A and 2B) studies including a mix of *L. V. braziliensis* (n=3), *L. V. guyanensis* (n=1) and *L. V. panamensis* (n=5). Given the findings identified in Objective 1C-1D, *L. V. braziliensis* is a top contributing species to the ML/MCL phenotype observed in Peru. It is worth while understanding how LRV-1 in this species, using a human macrophage model, may contribute to the severe phenotype at a host biological level, despite the lack of direct association observed between LRV-1 status and clinical phenotype. Similarly, the host-immune response to LRV-1 in *L. V. panamensis* has yet to be described, and given a substantial number of *L. V. panamensis* contributing to the LCL and inflammatory/multifocal CL phenotypes with a lack of ML/MCL observed in Objective 1E, it is interesting to understanding the difference in dynamics that exist in this species, despite the underpowered observations in Objective 1E. The following cultures were confirmed and identified: ATCC® strains of *L. V. braziliensis* ATCC®50135™ LRV-1 negative (LVb-); *L. V. guyanensis* ATCC®50126™ LRV-1+ (LVg+); *L. V. panamensis* ATCC®50158™ LRV-1 negative (LVp0-), and 6 clinical strains including one LRV-1 positive *L. V. braziliensis* (LVbC+) and one LRV-1 negative *L. V. braziliensis* (LVbC-); four *L. V. panamensis* including two LRV-1 negative *L. V. panamanensis* (LVp1- and LVp2-) and two LRV-1 positive *L. V. panamensis* (LVp1+ and LVp2+) (see Objective 2A and 2B).

Objective 2A: Influence of Leishmania RNA Virus-1 on Pro-Inflammatory Biomarker Expression in a Human Macrophage Model of American Tegumentary Leishmaniasis

Kariyawasam R, Grewal J, Lau R, Purssell A, Valencia BM, Llanos-Cuentas A, Boggild AK, Influence of *Leishmania* RNA Virus-1 on Pro-Inflammatory Biomarker Expression in a Human Macrophage Model of American Tegumentary Leishmaniasis, 2017, 216, 7, pp, 877-886, by permission of Oxford University Press (Journal of Infectious Diseases).

Influence of *Leishmania* RNA Virus-1 on Pro-Inflammatory Biomarker Expression in a Human Macrophage Model of American Tegumentary Leishmaniasis

Ruwandi Kariyawasam[1], Jugvinder Grewal[1], Rachel Lau[2], Andrew Purssell[1], Braulio M. Valencia[3], Alejandro Llanos-Cuentas[3,4], Andrea K. Boggild[1,2,5,6]

Affiliations:

[1]Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada.

[2]Public Health Ontario Laboratories, Toronto, Ontario, Canada.

[3]Instituto de Medicina Tropical "Alejandro von Humboldt," Lima, Peru.

[4]Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru.

[5]Department of Medicine, University of Toronto, Toronto, Ontario, Canada.

[6]Tropical Disease Unit, Toronto General Hospital, Toronto, Ontario, Canada.

Address for Correspondence:

Dr. Andrea K. Boggild, Tropical Disease Unit, Toronto General Hospital

200 Elizabeth Street, 13EN-218

Toronto, ON M5G 2C4

Phone - 416-340-3675

Fax - 416-340-3260

Email - andrea.boggild@utoronto.ca

Keywords: Leishmania RNA Virus-1 (LRV-1), American tegumentary leishmaniasis (ATL), Immunophenotype; *Leishmania Viannia braziliensis*; mucosal leishmaniasis

WORD COUNT: Abstract: 197; Text: 3401.

AUTHORS' CONTRIBUTIONS: AKB conceived the study. RK, JG, RL, AP, BMV, AL-C, and AKB contributed to data collection, analysis, interpretation, and to critical appraisal of the manuscript. RK, JG, AP, and RL performed the bench experiments. AKB and RK were primarily responsible for writing the manuscript. All authors read, appraised, and approved the final manuscript.

FINANCIAL SUPPORT: This work was supported by the University of Toronto Department of Medicine, the University of Toronto Institute of Medical Science, and Public Health Ontario.

DISCLOSURES: The authors have no disclosures.

Abstract

Backgound: Species of the *Leishmania Viannia* subgenus harbour the double-stranded *Leishmania* RNA virus 1 (LRV-1), previously identified in isolates from Brazil and Peru. Higher levels of LRV-1 in metastasizing strains of *L. V. guyanensis* have been documented in both human and murine models, and correlated to disease severity.

Methods: Expression of proinflammatory biomarkers, including IL-1β, TNF-α, CXCL10, CCL5, IL-6, and SOD, in human macrophages infected with 3 ATCC and 5 clinical isolates of *L. V. braziliensis*, *L. V. guyanensis* and *L. V. panamensis* for 24- and 48- hours were measured by commercial enzyme immunoassay. Analyses were performed at 24- and 48- hours, stratified by LRV-1 status and species.

Results: LRV-1 positive *L. V. braziliensis* demonstrated significantly lower expression levels of TNF-α (p=0.01), IL-1β (p=0.0015), IL-6 (p=0.001) and CXCL10 (p=0.0004) compared to LRV-1 negative *L. V. braziliensis*. No differences were observed in strains of *L. V. panamensis* by LRV-1 status.

Conclusions: Compared to LRV-1 negative *L. V. braziliensis*, LRV-1 positive strains of *L. V. braziliensis* produced a predominant Th2-biased immune response, correlated in humans to poorer immunologic control of infection and more severe disease, including mucosal leishmaniasis. Effects of LRV-1 on the pathogenesis of ATL may be species-specific.

Introduction

Leishmaniasis is a neglected tropical disease (NTD) with over 1.5 million cases annually and 350 million people living in endemic areas such as Central and South America, the Indian Subcontinent, Middle East, and North Africa (Reithinger, 2007; CDC, 2017). Transmitted by the female *Lutzomyia* and *Phlebotomus* spp. sandflies, *Leishmania* spp. are intracellular parasites that primarily infect macrophages (Weigle, 1996). Clinical presentations vary from ulcerative lesions of cutaneous leishmaniasis (CL), destruction of the mucosal membranes of mucosal or mucocutaneous leishmaniasis (ML), or the potentially fatal visceral leishmaniasis (VL) that invades the internal organs (Reithinger, 2007; CDC, 2017; David, 2009). Both CL and ML identified in Latin America are categorized as American tegumentary leishmaniasis (ATL). *Leishmania* spp. parasites exist in two developmentally distinct stages: flagellated promastigotes in the gut of the sandfly and intracellular amastigotes upon internalization by macrophages (Pulvertaft, 1960). Within the phagolysosome of the macrophage, amastigotes are either killed by host immune responses, or persist and proliferate via immune subversion, thus propagating infection (Alexander, 1975; Chang, 1976).

Parasitological factors play an essential role in successful infection and proliferation of the parasite. One such factor may be the presence of *Leishmania* RNA virus-1 (LRV-1), found in metastasizing strains of the *Leishmania Viannia* subgenus, which appears to promote parasitic persistence in ATL (Ives, 2011). LRV-1 is a double stranded RNA virus previously identified in strains of *L. V. guyanensis* and *L. V. braziliensis* in South America (Ives, 2011; Salinas, 1996). It is believed that 10-15% of patients with CL, particularly those infected with *L. V. braziliensis*, go on to develop ML, and 20-25% of *L. V. braziliensis* or *L. V. guyanensis* isolates from Brazil and Peru harbour LRV-1 (Reithinger, 2007; Ives, 2011; Salinas, 1996). LRV-1 is hypothesized to

subvert the protective Th1 predominant response of self-healing CL (Ives, 2011; Castiglioni, 2017). A murine model of ATL wherein LRV-1 was associated with a mixed Th1/Th2 phenotype, highlighted by increases in the Th1 biomarkers TNF-α, CCCL5, and CXCL10, and Th2 cytokine IL-6, established this altered expression to occur via the TLR3 pathway (Ives, 2011). Since this initial murine model, macrophages infected with other *Viannia* strains harboring LRV-1 were also shown to induce greater expression of a number of proinflammatory cytokines and chemokines (Bourreau, 2016; Ronet, 2011; Hartley, 2012).

Human and mouse models have demonstrated that resistance to leishmanial infection and self-healing skin lesions is associated with the development of a robust Th1 response, characterized by expression of IFN-γ, IL-1, TNF-α, CXCL10, and CCL5, while susceptibility and parasite persistence is associated with a predominant Th2 response, characterized by expression of IL-4, IL-5, and IL-6 (Moafi, 2017; Tripathi, 2007; Maspi, 2016; Hartley, 2013). The detoxification of reactive oxygen species (ROS) by the CuZn-SOD enzyme further facilitates parasite persistence in the macrophage vacuole (Hartley, 2012; Maspi, 2016; Hartley, 2013; Ghosh, 2003). Thus, it is possible to characterize the ‘immunophenotype’ of macrophages by measuring the expression of these Th1 and Th2 biomarkers. To better understand the potential role of LRV-1 in the pathogenesis of ATL, an *in vitro* human macrophage model was established, and the immunophenotypes of macrophages infected by strains of LRV-1-positive and negative *L. V. braziliensis, L. V. guyanensis,* and *L. V. panamensis* were evaluated.

Methods

Ethics Approval

Approval for this study was obtained from the Ethics Review Board of Public Health Ontario (Code #2015-048.01).

Clinical Specimen Collection

Unique cultured isolates of *Leishmania* spp. were identified from the Public Health Ontario Laboratory (PHOL) between 2012-2016, and retrieved from our biobank. Biobanked clinical isolates of *Leishmania* spp. and ATCC® strains were subjected to *Leishmania* spp. confirmation and identification, following clinical testing which included microscopy examination by certified medical lab technologists.

Leishmania species identification and confirmation.

DNA was extracted using QIAamp DNA Mini Kit Blood (Qiagen). *Leishmania* genus 18S real time PCR was performed as previously described (Wortmann, 2001). Species identification included analysis of the internal transcribed spacer 1 (ITS1), ITS2, cysteine proteinase B (CPB), heat shock protein 70 (HSP70), and mannose phosphate isomerase (MPI) by PCR, restriction fragment length polymorphism (RFLP) analysis, and Sanger sequencing (Schonian, 2003; de Almeida, 2011). PCR-RFLP analysis of the ITS1 region can only differentiate *L. V. braziliensis* from the other species within the *Viannia* subgenus (*L. V. guyanensis, L. V. peruviana, L. V. panamensis, L. V. lainsoni*). Thus, PCR-RFLP and sequencing analysis of the CPB, HSP70, MPI and ITS2 regions was required to differentiate species within the *Leishmania Viannia* sub-genus complex, and to provide a confirmation of the species identified in the initial ITS1 assay. Purified PCR product was used for Sanger sequencing as per Big Dye protocol (Life Technnologies). Sequence products were purified and analyzed using the Applied Biosystems

3130xl Genetic Analyzer. Data were standardized using the Sequencing Analyzer program and the BLAST search engine was used to analyze sequences.

Clinical Phenotype of Source Patients

Unique clinical cultures that were identified from de-identified clinical data of source patients were stratified into 'severe' and 'non-severe' phenotypes, where a severe phenotype was defined as: ulcers with associated erythema, purulent exudate, pain and/or lymphatic involvement (e.g., inflammatory ulcers); or multifocal/disseminated disease (ulcers in ≥ 2 anatomic sites and ≥ 4 in number). A non-severe phenotype was defined as localized cutaneous ulcers < 4 in number.

Macrophage Differentiation and Infection

Macrophage Differentiation. ATCC® U937 CRL-1593.2™ suspension cells stored in liquid nitrogen were thawed and cultured in RPMI 1640 media (Cat# A1049101, Thermo Fisher Scientific, Carlsbad, CA,) supplemented with 10% (v/v) heat-inactivated FBS (Cat#10082-139, Thermo Fisher Scientific, Carlsbad, CA) and 1x penicillin/streptomycin (Cat#15140-122, Thermo Fisher Scientific, Carlsbad, CA) at 37°C and 5% CO_2. Cells were maintained at a concentration between $1x10^5$ and $2x10^6$ cells/mL and assessed using the trypan blue exclusion test. U937 monocytes were differentiated into macrophages by resuspending $5x10^5$ cells/ml of monocytes in RPMI-1640 supplemented with 50 ng/mL phorbol myristate acetate (PMA). One mL of monocytes were plated on removable glass cover slips onto 24-well plates and allowed to differentiate for 72 hours. Differentiated cells were identified by the presence of pseudopodia and adherence to the plate surface, while non-adherent undifferentiated monocytes were washed away with RPMI 1640 media (Sintiprungrat, 2010; Hsiao, 2011; Verhoeckx, 2004). Differentiated cells were released from the cover slip using 0.05% Trypsin-EDTA (Life Technologies) and a cell count was performed.

Leishmania strains. The following cultures were confirmed and identified: ATCC® strains of *L. V. braziliensis* ATCC®50135™ LRV-1 negative (LVb-); *L. V. guyanensis* ATCC®50126™ LRV-1+ (LVg+); *L. V. panamensis* ATCC®50158™ LRV-1 negative (LVp0-), and 5 clinical strains including one LRV-1 positive *L. V. braziliensis* (LVb+)*;* four *L. V. panamensis* including two LRV-1 negative *L. V. panamanensis* (LVp1- and LVp2-) and two LRV-1 positive *L. V. panamensis* (LVp1+ and LVp2+) [Table 17]. Promastigotes were routinely subcultured in Tobie's medium with Locke's overlay at ambient temperature every week. Prior to infection, a cell count of the promastigotes was performed.

Infection. Macrophages were infected with promastigotes at a multiplicity of infection (MOI) of 10:1 (parasite: macrophage) in triplicate. Prior to addition to the 24-well plates, promastigotes were centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended with fresh RPMI 1640 medium supplemented with 10% FBS. Subsequently, the plates were placed in an incubator set at 37°C and 5% CO_2. Supernatant was collected at 24- and 48-hours. Each sample was centrifuged at 4000 rpm for 5 minutes to pellet cells and debris, and the supernatant was collected, aliquoted, and stored at -80°C prior to commercial EIA. After supernatant removal, macrophages were washed with PBS, fixed with methanol, and Giemsa stained (5 minutes in Giemsa stain followed by 5 minutes in Giemsa buffer on a rocker set at 100 rpm). Cover slips were then removed and mounted upside down onto microscope slides using Eukitt mounting media. Slides were visualized under a microscope, to confirm MOI and the presence of amastigotes.

Analysis of cytokines and chemokines by EIA. Supernatants were thawed, brought to room temperature, and analyzed using EIA kits for: IL-1β (Cat# HSLB00C), IL-5 (Cat#D5000B), IL-4

(Cat# HS400), IL-6 (Cat# HS600B), IL-12 (Cat# HS120), CXCL10 (Cat#DIP100), CCL5 (Cat #DRN00B) and TNF-α (Cat#HSTA00D) (R&D systems, Minneapolis, MN), IFN-β (Cat# 41410, PBL Assay Science, Piscataway Township, NJ), iNOS (Cat# E-EL-H0753, Elabscience, WuHan, China), and SOD (Cat# ALX-850-033, Enzo Life Sciences, Farmingdale, NY) according to protocols provided by the manufacturer and read on a Synergy™ Plate Reader (Biotek Instruments, Winooski, VT). Concentrations were calculated according to a standard curve generated using the Gen5 Data Analysis Software (Biotek Instruments, Winooski, VT). EIAs were performed using technical triplicates on each biological triplicate.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism 6 version 6.07 software (GraphPad Software Inc, La Jolla, CA). Mean cytokine and chemokine concentrations were calculated for each strain, by species, and LRV-1 status and compared using unpaired t-tests and ANOVA. The analysis was conducted for data from both 24 and 48-hour time points.

LRV-1 Detection

RNA Extraction. RNA was extracted from cultured cells using QIAmp RNA Mini Kit (Qiagen). An in-column DNase treatment was included in all extractions as per manufacturer's protocol. Extracted RNA was split into 2 batches and stored at -20^{o}C for immediate use and -80^{o}C for longer term storage. RNA was quantified with Nanodrop spectrophotometer (Thermal Scientific).

cDNA synthesis and detection of LRV-1 by qPCR. cDNA was synthesized with 50 – 300 ng of RNA using Superscript II Reverse Transcriptase and random hexamers (Life Technologies), followed by purification with QIAquick PCR Purification Kit (Qiagen) and eluted with 50µL of nuclease-free water. Two real time PCR (qPCR) assays for detection of

LRV-1 were performed with LRV-1 set A and set B primers respectively as previously described (Zangger, 2013). *Leishmania* kinetoplastid membrane protein 11 (kmp11) was used as a quantification and extraction control (Zangger, 2013). Sybr Green real time PCR was setup with 1x Sybr Select Master Mix (Life Technologies), 250nM final concentration of forward and reverse primers, 5µL of cDNA in a total volume of 20µL (Zangger, 2013). Amplification was performed in an ABI 7900HT real time instrument with the following conditions: UDG activation at 50ºC for 2 min, polymerase activation at 95ºC for 2 min, followed by 45 cycles of 95ºC for 15 sec, and 60ºC for 1 min. A dissociation step of 95ºC for 15 sec, 60ºC for 15 sec, and again another 95ºC for 15 sec was added at the end to generate a melting curve, which was used to check for the specificity of amplification. In cases where RNA level was too low, a pre-amplification reaction to increase sensitivity of detection was performed with Perfecta Pre-Amp Supermix (Quanta Biosciences) using 100 ng of cDNA according to manufacturer's protocol with 14 cycles. The reaction was diluted 1:20 and 5µL of the pre-Amp cDNA was used in subsequent qPCR as above. ATCC®50126™ *L. V. guyanensis* strain MHOM/BR/75/M4147, known to be LRV-1 positive, was used as a positive control and RNA from a healthy human individual as negative control. LRV-1 was quantified relative to kmp11 using the $2^{-\Delta\Delta Ct}$ method (Schmittgen, 2008).

Results

Clinical and Demographic Data

Five clinical cultures were obtained from male patients with a mean age of 39.8 years (range 9-80 years) [Table 17]. Three of the 5 clinical cultures were LRV-1 positive (LVb+, LVp1+ and LVp2+) and 2 were LRV-1 negative (LVp1- and LVp2-). All clinical cultures with the exception

of LVp2- were from patients exhibiting a severe phenotype (i.e., inflammatory or multifocal as previously defined). All clinical isolates were derived from patients with a travel history to Costa Rica, with the exception of LVb+ where the travel history had been to Peru.

Biomarker Data. For Figures 10-15, letters A-C correspond to measurements at 24- hours whereas letters D-F correspond to measurements at 48- hours.

'Th2' Cytokine IL-6

IL-6 expression. At 24 and 48-hours, peak relative expression of IL-6 differed across strains regardless of LRV-1 status ($p<0.0001$) (Figure 10A and 10D). Overall, there was no difference in IL-6 expression between LRV-1 positive and negative strains at 24-($p=0.72$) and 48-($p=0.49$) hours, respectively (Figure 10B, 10E). Species stratification revealed no significant difference of LRV-1 status amongst the *L. V. panamensis* (L. V. pan) strains at both 24- ($p=0.43$) and 48- ($p=0.29$) hours (Figure 10C, 10F). IL-6 expression was significantly decreased in the LVb+ strain compared to LVb- strain at both 24- ($p<0.0001$) and 48-hours ($p=0.0012$) (Figure 10A, 10D).

'Th1' Cytokines and Chemokines: IL-1β, TNF-α, CCL5 and CXCL10

IL-1β expression. At 24- and 48-hours, peak relative expression of IL-1β differed across strains ($p=0.006$ and $p=0.0027$, respectively) (Figure 11A and 11D). There was no difference in expression by LRV-1 status of IL-1β at 24-($p=0.59$) and 48-($p=0.88$) hours, respectively (Figure 11B, 11E). Species stratification revealed no significant difference in LRV-1 status amongst the *L. V. panamensis* (L. V. pan) strains at both 24- ($p=0.23$) and 48- ($p=0.60$) hours (Figure 11C, 11F). At 48 hours, IL-1β expression was significantly decreased in the LVb+ strain compared to the LVb- strain ($p=0.0015$) (Figure 11D).

TNF-α expression. At 24- and 48-hours, peak relative expression of TNF-□ differed across strains (p=0.007 and p<0.0001, respectively) (Figure 12A and 12D). Overall, LRV-1 + strains showed no difference in relative expression of TNF-α compared to LRV-1- strains at 24-(p=0.59) and 48-(p=0.88) hours, respectively (Figure 12B, 12E). *L. V. panamensis* (L. V. pan) stratification revealed no significant difference by LRV-1 status at both 24- (p=0.43) and 48-(p=0.19) hours (Figure 12C, 12F). At 24- and 48- hours, TNF-α expression was significantly decreased in the LVb+ strain compared to the LVb- strain, respectively (p=0.0031 and p=0.01) (Figure 12A, 12D).

CCL5 expression. At 24- and 48- hours, peak relative expression of CCL5 differed across strains (p<0.0001) (Figure 13A and 13D). Overall, LRV-1 + strains revealed no difference in relative expression of CCL5 compared to LRV-1- strains at 24-(p=0.66) and 48-(p=0.71) hours, respectively (Figure 13B, 13E). Species stratification revealed no significant difference in LRV-1 status amongst the *L. V. panamensis* (L. V. pan) strains at 24 hours (p=0.76), however, a slight reduction in CCL5 expression at 48- hours was observed in the LRV-1+ strains of *L. V. pamamensis* (p=0.06) (Figure 13C, 13F). At 24- and 48- hours, CCL5 expression was not significantly reduced in the LVb+ strain compared to the LVb- strain (p=0.54 and p=0.39) (Figure 13A, 13D).

CXCL10 expression. At 24- and 48-hours, peak relative expression of CXCL10 differed across strains (p<0.0001) (Figure 14A and 14D). Overall, LRV-1 + strains revealed no difference in relative expression of CXCL10 compared to LRV-1- strains at 24-(p=0.45) and 48-(p=0.47) hours, respectively (Figure 14B, 14E). Species stratification revealed no significant difference in LRV-1 status amongst the *L. V. panamensis* (L. V. pan) strains at both 24- (p=0.21) and 48-

(p=0.35) hours (Figure 14C, 14F). At 24- and 48- hours, CXCL10 expression was significantly decreased in the clinical LVb+ strain compared to the LVb- strain, respectively (p=0.0003 and p=0.0004) (Figure 14A, 14D).

Other biomarkers

iNOS, IL-4, IL-5, Il-12 and IFN-β expression were below levels of detection and could not be quantitated.

Cu-Zn-SOD Expression

SOD expression. At 24- and 48- hours, peak relative expression of SOD differed across strains (p=0.007 and p<0.0001) (Figure 15A and 15D). Overall, LRV-1 + strains revealed no difference in relative expression of SOD compared to LRV-1- strains at 24- (p=0.97) and 48-(p=0.49) hours, respectively (Figure 15B, 15E). *L. V. panamensis* (L. V. pan) stratification revealed no significant difference in LRV-1 status at 24 hours (p=0.13), however, at 48-hours relative SOD expression was slightly higher in the LRV-1 positive *L. V. panamensis* strains (p=0.07) (Figure 15C, 15F). At 24- hours, SOD expression was significantly higher in the LVb+ strain compared to the LVb- strain (p=0.04) (Figure 15A). This difference was not observed at 48 hours (p=0.49) (Figure 15D).

Discussion

LRV-1 was first detected in the human pathogen *L. V. guyanensis* in 1988 from a visitor to Suriname with reported satellite lesions around an ulcer of CL and lymphatic involvement (Tarr, 1988). The same strain passaged through a hamster model produced a phenotype resembling ML (Tarr, 1988). Decades later, numerous reports of LRV-1 in *L. V. braziliensis* and

L. V. guyanensis strains exacerbating disease have highlighted its potential role in accelerating CL to ML. To date, LRV-1 has not been detected in isolates other than New World *Leishmania Viannia* spp. The novel documentation of LRV-1 in strains of *L. V. panamensis* in this analysis supports that LRV-1 co-infection may be more widespread in *Viannia* isolates, other than *L. V. braziliensis* and *L. V. guyanensis*, than previously recognized. Lately, the correlation of the host immune response relative to LRV-1 status has shed light on 'immunophenotypes' that exist, and which can potentially serve as a predictive biomarker of disease severity, thus allowing for therapeutic solutions harnessing both host and parasitic cellular machinery.

Using the U937 cell line, which has a long precedent of modelling human macrophages in vitro, we were able to model successful *Leishmania Viannia* spp. infection which elicits both proinflammatory cytokines and chemokines. To our knowledge, this is the first report of LRV-1 detection in strains of *L. V. panamensis*, a strain that is historically viewed as less virulent compared to *L. V. braziliensis*. Furthermore, three of four LRV-1 positive clinical isolates used in this study were derived from patients with an inflammatory/severe phenotype, characterized by ulcer erythema, purulence, and pain, along with possible lymphatic involvement, which complicates the treatment course of ATL by necessitating systemic therapy (e.g., oral miltefosine or intravenous liposomal amphotericin). It is unknown at this point whether.LRV-1 could potentially serve as an important marker of more invasive or complicated disease, which would have direct implications for treatment, however, the notion is tantalizing and thus warrants further investigation.

The expression of Th1/Th2 biomarkers and SOD did not differ by LRV-1 status amongst isolates of *L. V. panamensis*. However, a slight increase in SOD expression was observed at 48 hours alongside a slight decrease of CCL5 response in LRV-1 positive isolates which could point

to parasitic persistence given reduced killing of the parasite in the macrophage vacuole. Cu-Zn-SOD is an enzyme that protects cells from the constant oxidative challenge posed by normal oxidative metabolism (Hartley, 2012). Upon infection, macrophages produce ROS to effectively kill the parasite. It has been hypothesized that LRV-1 released from dead parasites in infected macrophages might upregulate the production of Cu-Zn-SOD by manipulation of arginase I, thereby decreasing the concentration of free radical available for parasite clearance allowing it to persist in the vacuole (Hartley, 2012).

The LRV-1 positive *L. V. braziliensis* (LVb+) isolate produced significantly lower levels of the Th1 cytokines IL-1β, CXCL10, and TNF-α as well as Th2 IL-6, compared to LRV-1 negative *L. V. braziliensis* (LVb-), and significantly higher levels of SOD resulting in an inversed mixed Th1/Th2 phenotype compared to what has been observed in other mouse and human models (Ives, 2011; Castiglioni, 2017; Bourreau, 2016; Eren, 2016; Hartley, 2016). Given that a robust Th1 response is highly correlated to clinical cure and immunological control of infection (Reithinger, 2007; Castiglioni, 2017), the Th2 biased response observed here, coupled with parasitic persistence through the upregulation of SOD, highlights a potentially severe immunophenotype which could also explain the possibility of relapse in patients months to years following clinical cure.

Previous studies have used human metastatic strains of *L. V. guyanensis* and *L. V. braziliensis* to infect mice or human PBMCs to understand the overall Th1/Th2 paradigm. The disadvantage to studying ML in mice models is the lack of nasopharyngeal pathology versus what is seen in humans, which leads to misrepresentation of inflammation-mediated tissue damage in these animals (Olivier, 2011). The use of the U937 cell line in our investigation mimics the immunological profile of what might be observed in a proximate human PBMC

model, and is more representative of human ATL than a murine model. The differences in biomarker expression observed by LRV-1 status amongst isolates of *L. V. braziliensis,* although opposite to what has been observed in highly regarded murine models, illuminate the immunological effects that could explain clinical phenotype, and even downstream potential treatment outcomes (Ives, 2011; Castiglioni, 2017; Bourreau, 2016; Eren, 2016; Hartley, 2016). Finally, a more robust immunological analysis using more clinical isolates, high-sensitivity EIA kits or a multiplex bead-based platform such as Luminex, and use of human skin tissue models could provide more insight into the mechanistic nature of LRV-1 given the limited number of clinical isolates and proinflammatory biomarkers evaluated in this study.

Conclusion

The presence of LRV-1 has shown species-specific immunomodulatory effects as demonstrated by the LRV-1 positive *L. V. braziliensis* strain resulting in a predominant Th2 response based on our panel of proinflammatory biomarkers. A more robust immunological analysis is required with additional strains to better understand the role of LRV-1 in manipulating host immune responses. The novel detection of LRV-1 in strains of *L. V. panamensis* suggests further evaluation of strains not previously known to harbour the double stranded RNA virus. Our data support that LRV-1 may be an important prognostic biomarker in ATL, which could have potential implications for treatment stratification by severity of disease. Understanding the potential role of LRV-1 in clinical relapse and progression of CL to ML warrants further prospective investigation.

Table 17: Classification, clinical, and demographic data of Leishmania spp. strains used in experiments.

***Leishmania* Strain**	**Specimen Source**	**Country of origin**	**LRV-1 Status**	**Age**	**Sex**	**Phenotype**
L. V. braziliensis (LVb-)	ATCC 50135	Brazil	LRV-1-	NA	NA	NA
L. V. braziliensis (LVb+)	Clinical biobank	Peru	LRV-1+	22	Male	Severe
L. V. guyanensis (LVg+)	ATCC 50126	Brazil	LRV-1+	NA	NA	NA
L. V. panamensis (LVp0-)	ATCC 50158	Unknown	LRV-1-	NA	NA	NA
L. V. panamensis (LVp1-)	Clinical biobank	Costa Rica	LRV-1-	80	Male	Severe
L. V. panamensis (LVp2-)	Clinical biobank	Costa Rica	LRV-1-	17	Male	Non-Severe
L. V. panamensis (LVp1+)	Clinical biobank	Ecuador	LRV-1+	9	Male	Severe

L. V. panamensis (LVp2+)	Clinical biobank	Costa Rica	LRV-1+	71	Male	Severe

FIGURE LEGENDS.

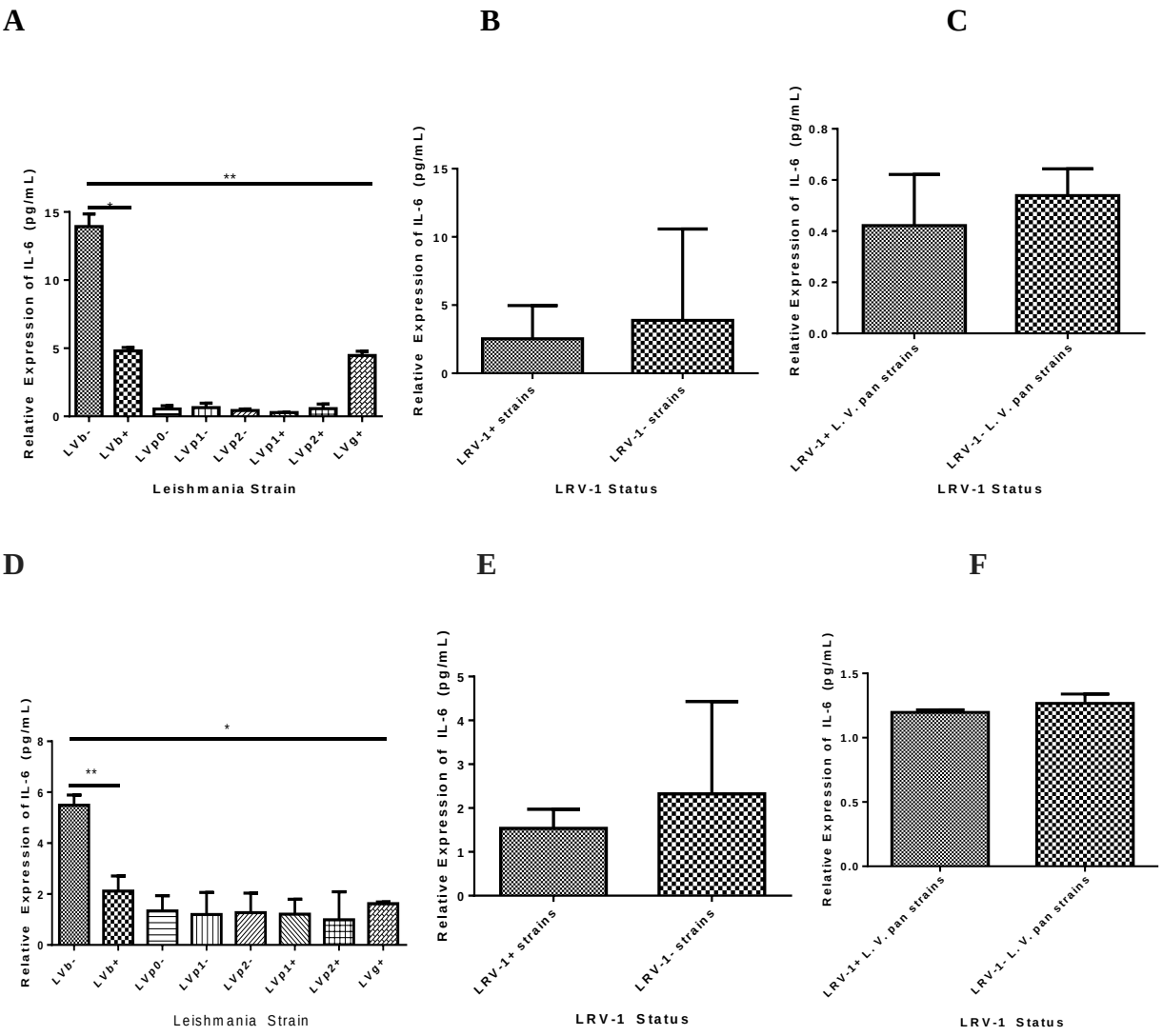


Figure 10: Relative IL-6 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by ANOVA and t-test (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

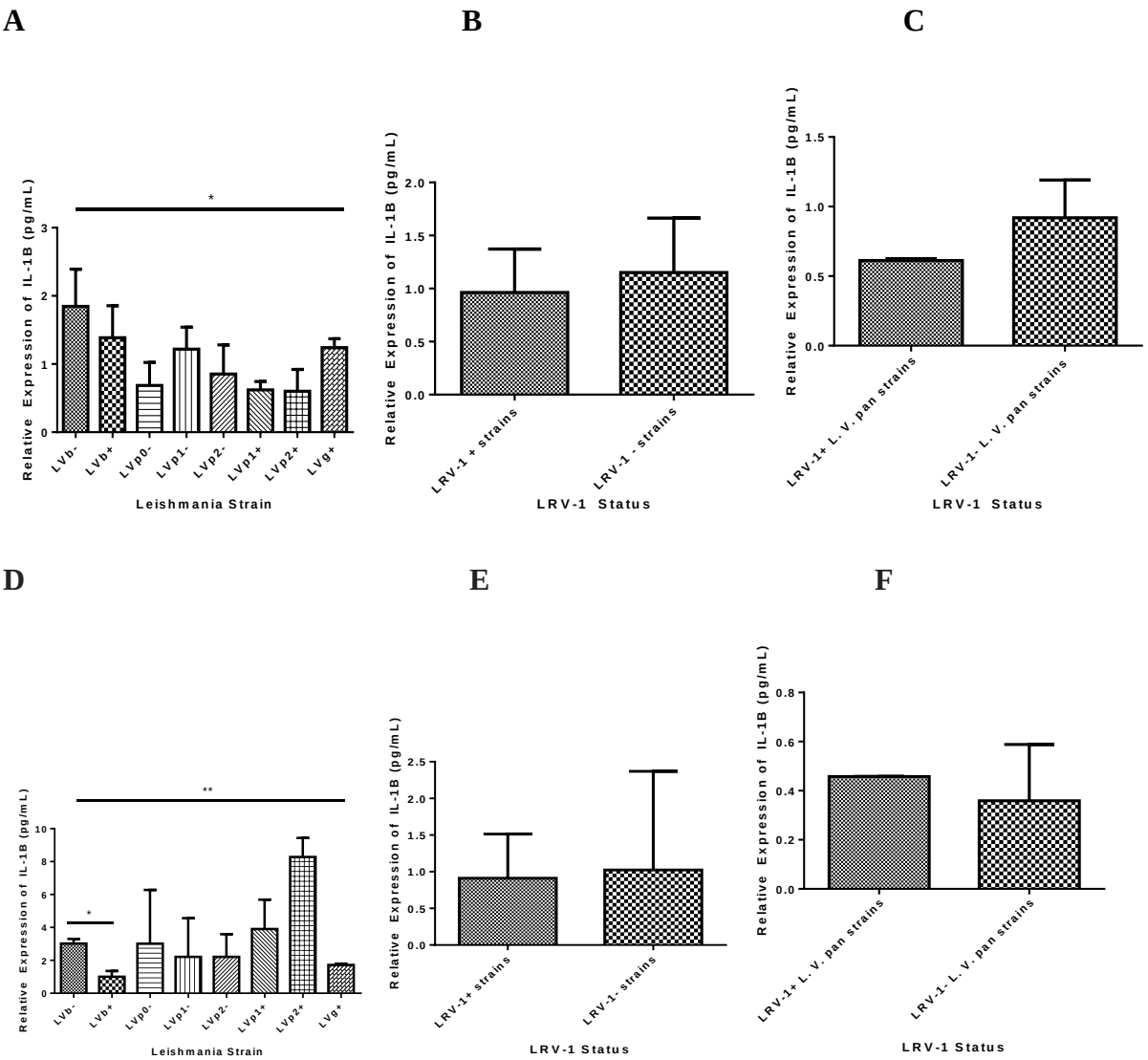


Figure 11: Relative IL-1β expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by t-test and ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

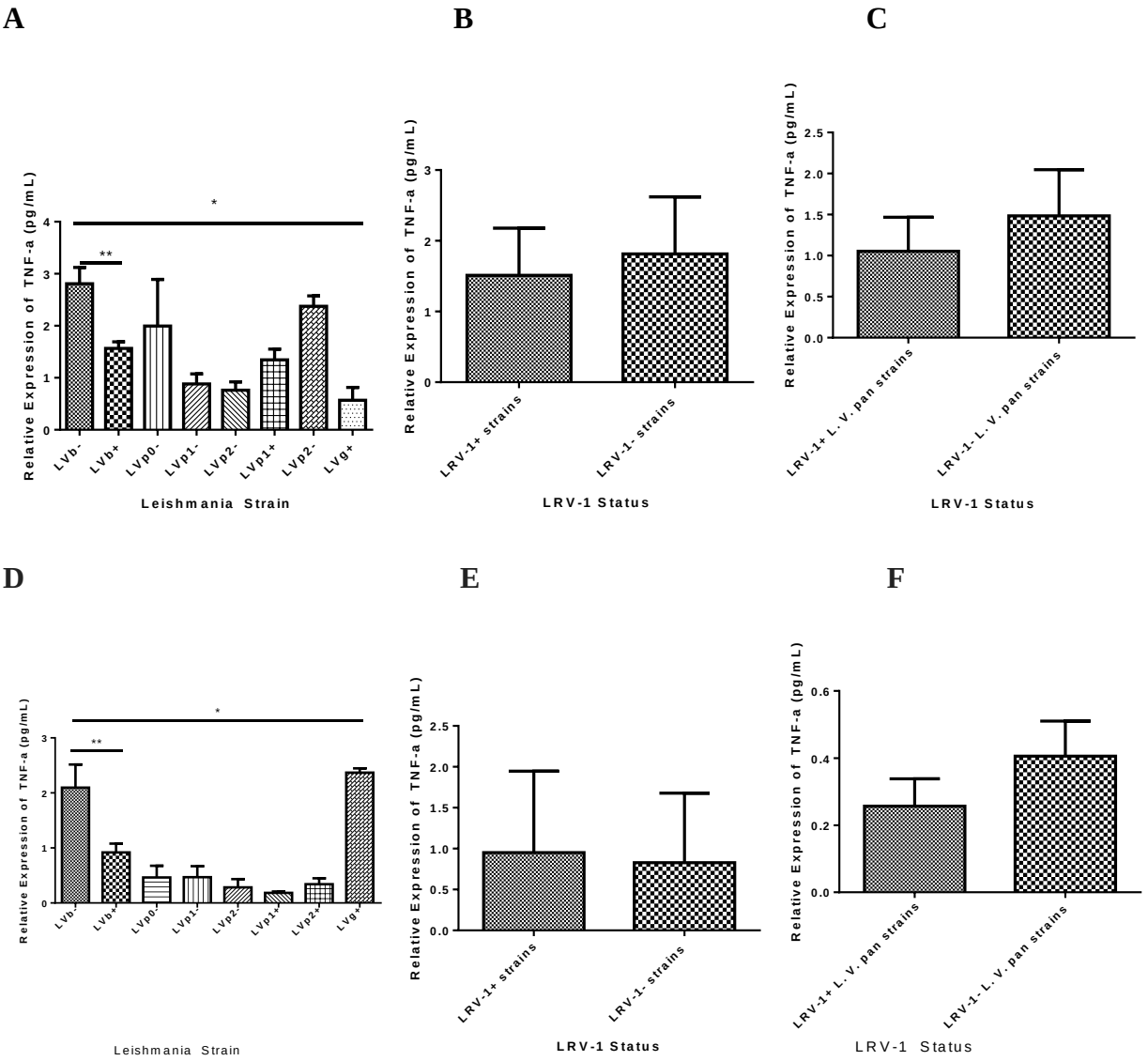


Figure 12: Relative TNF-α expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by t-test and ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

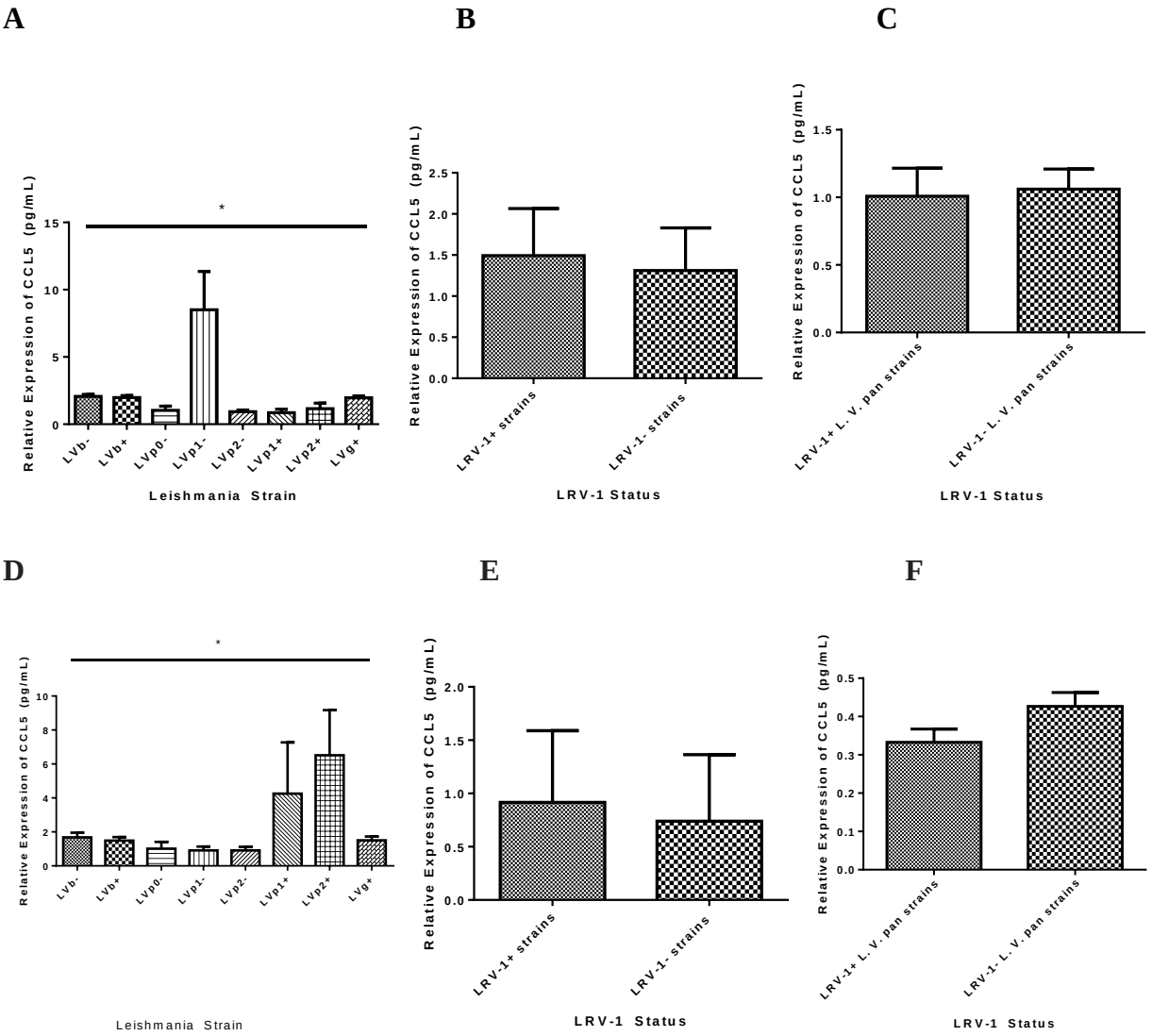


Figure 13: Relative CCL5 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by t-test and ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

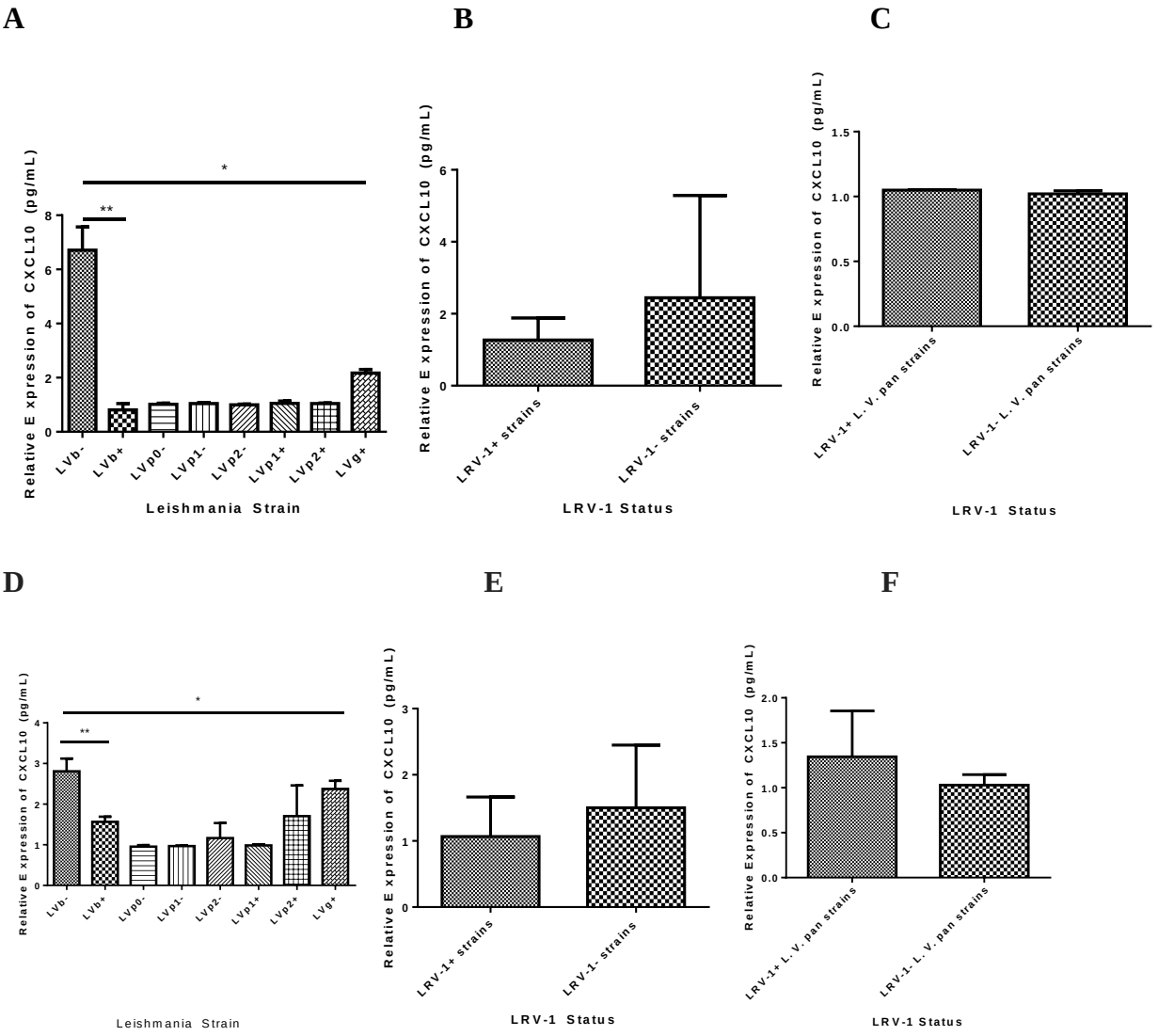


Figure 14: Relative CXCL10 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by t-test and ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

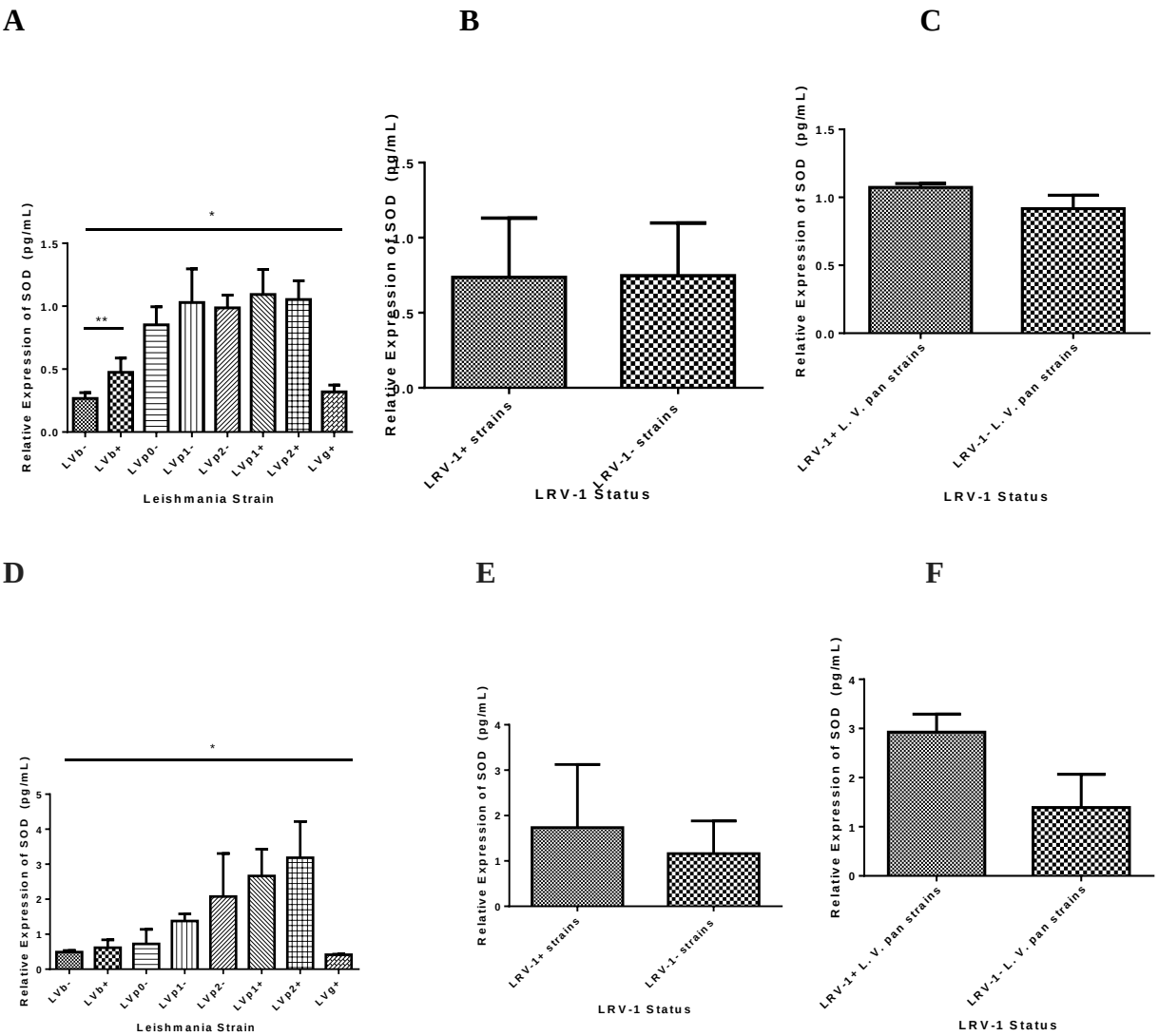


Figure 15: Relative SOD expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains and LVb+ and LVb- by t-test and ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). LRV-1 positive and negative strains of *L. V. panamensis* (L. V. pan) were compared using t-tests (C, F).

Objective 2B: Influence of Leishmania RNA Virus-1 on Pro-Inflammatory Biomarker Expression of Clinical Cultures of L. V. braziliensis in a Human Macrophage Model of American Tegumentary Leishmaniasis

Introduction

To further understand the potential role of LRV-1 in the pathogenesis of ATL, using the *in vitro* human macrophage model established, the immunophenotypes of macrophages infected by strains of clinical LRV-1-positive and negative *L. V. braziliensis* strains were evaluated, to account for the difference in strain source between the LRV-1 positive clinical isolate and the LRV-1 negative ATCC® isolate in Objective 2A.

Methods

See Objective 2A above with changes to the following:

Macrophage Differentiation. U937 monocytes were differentiated into macrophages by resuspending $4x10^4$ cells/ml of monocytes in RPMI-1640 supplemented with 50 ng/mL phorbol myristate acetate (PMA).

Leishmania strains. The following cultures were confirmed and identified: ATCC® strains of *L. V. braziliensis* ATCC®50135™ LRV-1 negative (LVb-) and *L. V. guyanensis* ATCC®50126™ LRV-1+ (LVg+) and 2 clinical strains including one LRV-1 positive *L. V. braziliensis* (LVbC+) and one LRV-1 negative *L. V. braziliensis* (LVbC-) [Table 18]. Promastigotes were routinely subcultured in Tobie's medium with Locke's overlay at ambient temperature every week. Prior to infection, a cell count of the promastigotes was performed.

Analysis of cytokines and chemokines by EIA. Supernatants were thawed, brought to room temperature, and analyzed using EIA kits for: IL-1β , IL-6 , CXCL10 , CCL5 and TNF-α

(R&D systems, Minneapolis, MN) and SOD (Enzo Life Sciences, Farmingdale, NY) according to protocols provided by the manufacturer and read on a Synergy™ Plate Reader (Biotek Instruments, Winooski, VT). Concentrations were calculated according to a standard curve generated using the Gen5 Data Analysis Software (Biotek Instruments, Winooski, VT). EIAs were performed using technical triplicates on each biological triplicate.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism 8 version 8.02 software (GraphPad Software Inc, La Jolla, CA). Mean cytokine and chemokine concentrations were calculated for each strain, by species, and LRV-1 status and compared using unpaired t-tests and ANOVA. The analysis was conducted for data from both 24 and 48-hour time points.

Results

Clinical and Demographic Data

Two clinical cultures were obtained from male patients with a mean age of 39 years (range 22-56 years) [Table 18]. One of the 2 clinical cultures were LRV-1 positive (LVb+). One of the 2 clinical cultures were from patients exhibiting a severe phenotype with travel history to Peru and Brazil, whereas the other clinical culture was from a patient exhibiting a non-severe phenotype with travel history to Brazil [Table 18].

Biomarker Data. For Figures 16-21, letters A-C correspond to measurements at 24- hours whereas letters D-F correspond to measurements at 48- hours.

IL-6 expression. At 24 and 48-hours, peak relative expression of IL-6 did not differ across strains regardless of LRV-1 status (p=0.99 and p=0.69, respectively) (Figure 16A and 16D).

Overall, there was no difference in IL-6 expression between LRV-1 positive and negative strains at 24-(p=0.86) and 48-(p=0.93) hours, respectively (Figure 16B, 16E). Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at both 24- (p=0.89) and 48- (p=0.93) hours). IL-6 expression did not differ between LVbC+ and LVbC- at both 24- (p=0.91) and 48-hours (p=0.95) (Figure 16A, 16D).

IL-1β expression. At 24- and 48-hours, peak relative expression of IL-1β did not differ across strains (p=0.52 and p=0.32, respectively) (Figure 17A and 17D). There was no difference in expression by LRV-1 status of IL-1β at 24-(p=0.22) and 48-(p=0.13) hours, respectively (Figure 17B, 17E). Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at both 24- (p=0.18) and 48- (p=0.19) hours). IL-1β expression did not differ between clinical strains LVbC+ and LVbC- at both 24- (p=0.17) and 48-hours (p=0.13) (Figure 17A, 17D).

TNF-α expression. At 24- peak relative expression of TNF-□ did not differ across strains (p=0.98) however at 48 hours there was a difference (p=0.005) (Figure 18A and 18D). Overall, LRV-1 + strains showed no difference in relative expression of TNF-α compared to LRV-1- strains at 24-(p=0.81) and 48-(p=0.81) hours, respectively (Figure 18B, 18E). Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at 24- (p=0.79), however there was a difference at 48- hours (p=0.03). TNF-α expression did not differ between the clinical strains LVbC+ and LVbC- at 24- (p=0.88), however there was a difference at 48- hours (p=0.007) (Figure 18A, 18D).

CCL5 expression. At 24- hours, peak relative expression of CCL5 did not differ cross strains (p=0.34), however at 48- hours there was a notable difference (p=0.0043) (Figure 19A and 19D).

Overall, LRV-1 + strains revealed no difference in relative expression of CCL5 compared to LRV-1- strains at 24-(p=0.10) and 48-(p=0.13) hours, respectively (Figure 19B, 19E).

Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at both 24- (p=0.37) and 48- (p=0.88) hours). CCL5 expression did not differ between LVbC+ and LVbC- clinical strains at both 24- (p=0.45) and 48-hours (p=0.26) (Figure 19A, 19D).

CXCL10 expression. At 24- hours, peak relative expression of CXCL10 did not differ across strains (p=0.83), however at 48 hours there was a notable difference (p<0.0001) (Figure 20A and 20D). Overall, LRV-1 + strains revealed no difference in relative expression of CXCL10 compared to LRV-1- strains at 24-(p=0.40) and 48-(p=0.40) hours, respectively (Figure 20B, 20E). Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at both 24- (p=0.59) and 48- (p=0.40) hours). CXCL10 expression did not differ between LVb+ clinical strain compared to LVb- clinical strain at 24 hours (p=0.91), however there was a difference at 48-hours (p<0.0001) (Figure 20A, 20D).

SOD expression. At 24- and 48- hours, peak relative expression of SOD did not differ across strains (p=0.57 and p=0.40, respectively) (Figure 21A and 21C). Overall, LRV-1 + strains revealed no difference in relative expression of SOD compared to LRV-1- strains at 24- (p=0.39) and 48-(p=0.10) hours, respectively (Figure 21B, 21E). Comparison of LRV-1 positive and negative *L. V. braziliensis* strains revealed no difference at both 24- (p=0.32) and 48- (p=0.14) hours). SOD expression did not differ between LVbC+ and LVbC- clinical strains at both 24- (p=0.23) and 48-hours (p=0.19) (Figure 21C, 21E).

Discussion

LRV-1 was first detected in the human pathogen *L. V. guyanensis* in 1988 from a visitor to Suriname with reported satellite lesions around an ulcer of CL and lymphatic involvement (Tarr, 1988). The same strain passaged through a hamster model produced a phenotype resembling ML (Tarr, 1988). Decades later, numerous reports of LRV-1 in *L. V. braziliensis* and *L. V. guyanensis* strains exacerbating disease have highlighted its potential role in accelerating CL to ML. To date, LRV-1 has not been detected in isolates other than New World *Leishmania Viannia* spp., however LRV-2 has been identified in Old World species.

Using the U937 cell line, which has a long precedent of modelling human macrophages in vitro, we were able to model successful *Leishmania Viannia* spp. infection which elicits both proinflammatory cytokines and chemokines using *L. V. braziliensis, L. V. guyanensis* and *L. V. panamensis* strains (Kariyawasam et al., 2017). Previously, LRV-1 has been shown to decrease expression of TNF-α, IL-1β, IL-6 and CXCL10 in a comparison of a clinical *L. V. braziliensis* strain to the LRV-1 ATCC® 50135 *L. V. braziliensis* strain (Kariyawasam et al., 2017). Moreover, SOD expression was significantly higher in the LRV-1 positive clinical strain at 24 hours (Kariyawasam et al., 2017). In order to account for the differences that may be attributed to reference strains, where high numbers of sub-culturing cycles and loss of important pathophysiological characteristics may have occurred over time, we sought to re-evaluate proinflammatory biomarker expression using clinical LRV-1 negative and LRV-1 positive strains of *L. V. braziliensis.*

The expression of Th1/Th2 biomarkers and SOD differed compared to Objective 2A. In this analysis, a 10-fold lower monocyte concentration was used for differentiation and infection due to time constraints. The loss of previously established associations may be attributable to the

insufficient level of macrophages eliciting immune responses as observed in Objective 2A. However, the LRV-1 positive clinical *L. V. braziliensis* (LVbC+) isolate produced significantly higher levels of the Th1 cytokines TNF-α and CXCL10 at 48 hours compared to LRV-1 negative *L. V. braziliensis* (LVbC-), similar to what has been observed in other mouse and human models (Ives, 2011; Castiglioni, 2017; Bourreau, 2016; Eren, 2016; Hartley, 2016). A predominant Th1 response is highly correlated to clinical cure and immunological control of infection (Reithinger, 2007; Castiglioni, 2017), however an exacerbated Th1 response is believed to contribute to severe phenotype as observed by increased lesions and tissue damage (Ives et al., 2011; Maspi et al., 2016). The manifestation of ML from a patient infected with LRV-1 positive *L. V. braziliensis* may be supported by this notion given the increase in TNF-α observed in comparison to the LRV-1 negative *L. V. braziliensis* strain. A number of studies have highlighted the role of increased TNF-α in severe disease resulting in localized tissue damage and secondary lesions (Carvalho et al., 2007; Vargas-Inchaustegui et al., 2010; Hartley et al., 2012; Gaze et al., 2006; Bacellar et al., 2002). Anti-TNF-α therapies, such as pentoxifylline in addition to antimony have been shown to be effective treatment of ML and may be one solution given the role of TNF-α in control of infection (Lessa et al., 2007; Amato et al., 2003). In this study, the clinical LRV-1 positive *L. V. braziliensis* isolate came from a younger patient with severe disease as compared to the LRV-1 negative *L. V. braziliensis* isolate which came from an older patient with non-severe disease. Rather than immunosenescence, differences in clinical manifestations could be attributed to a difference in TNF-α and CXCL10 expression, which has been implicated in viral infections, rather than the function of age and immunity (Steinke & Borish, 2006).

The use of the U937 cell line in our investigation mimics the immunological profile of what might be observed in a proximate human PBMC model and is more representative of human ATL than a murine model. The differences in biomarker expression observed by LRV-1 status amongst clinical isolates of *L. V. braziliensis,* illuminate more closely the immunological effects that could explain clinical phenotype, and even downstream potential treatment outcomes (Ives, 2011; Castiglioni, 2017; Bourreau, 2016; Eren, 2016; Hartley, 2016). Limitations include the use of a lower concentration of human cells infected with promastigotes which highlight perhaps the necessity of a certain threshold of human cells to be infected to elicit a robust immune response that was observed in our previous study. Finally, a more robust immunological analysis using more clinical isolates, high-sensitivity EIA kits or a multiplex bead-based platform such as Luminex and analysis of the role of anti-inflammatory cytokines such as IL-10 and TGF-β, and use of human skin tissue models could provide more insight into the mechanistic nature of LRV-1 given the limited number of clinical isolates and proinflammatory biomarkers evaluated in this study.

Conclusion

The presence of LRV-1 has shown species-specific immunomodulatory effects as demonstrated by the LRV-1 positive *L. V. braziliensis* strain resulting in an exacerbated Th1 response based on our panel of proinflammatory biomarkers. A more robust immunological analysis is required with additional strains at appropriate concentrations to better understand the role of LRV-1 in manipulating host immune responses. Our data continue to support that LRV-1 may be an important prognostic biomarker in ATL, which could have potential implications for treatment stratification by severity of disease, however warrants further prospective investigation.

Table 18: Classification, clinical, and demographic data of Leishmania spp. strains used in experiments.

***Leishmania* Strain**	**Specimen Source**	**Country of origin**	**LRV-1 Status**	**LRV-1 Copy Number**	**Age**	**Sex**	**Phenotype**
L. V. braziliensis (LVb-)	ATCC 50135	Brazil	LRV-1-	NA	NA	NA	NA
L. V. braziliensis (LVbC+)	Clinical biobank	Peru	LRV-1+	0.10	22	Male	Severe
L. V. braziliensis (LVbC-)	Clinical biobank	Brazil	LRV-1-	NA	56	Male	Non-Severe
L. V. guyanensis (LVg+)	ATCC 50126	Brazil	LRV-1+	Reference	NA	NA	NA

FIGURE LEGENDS.

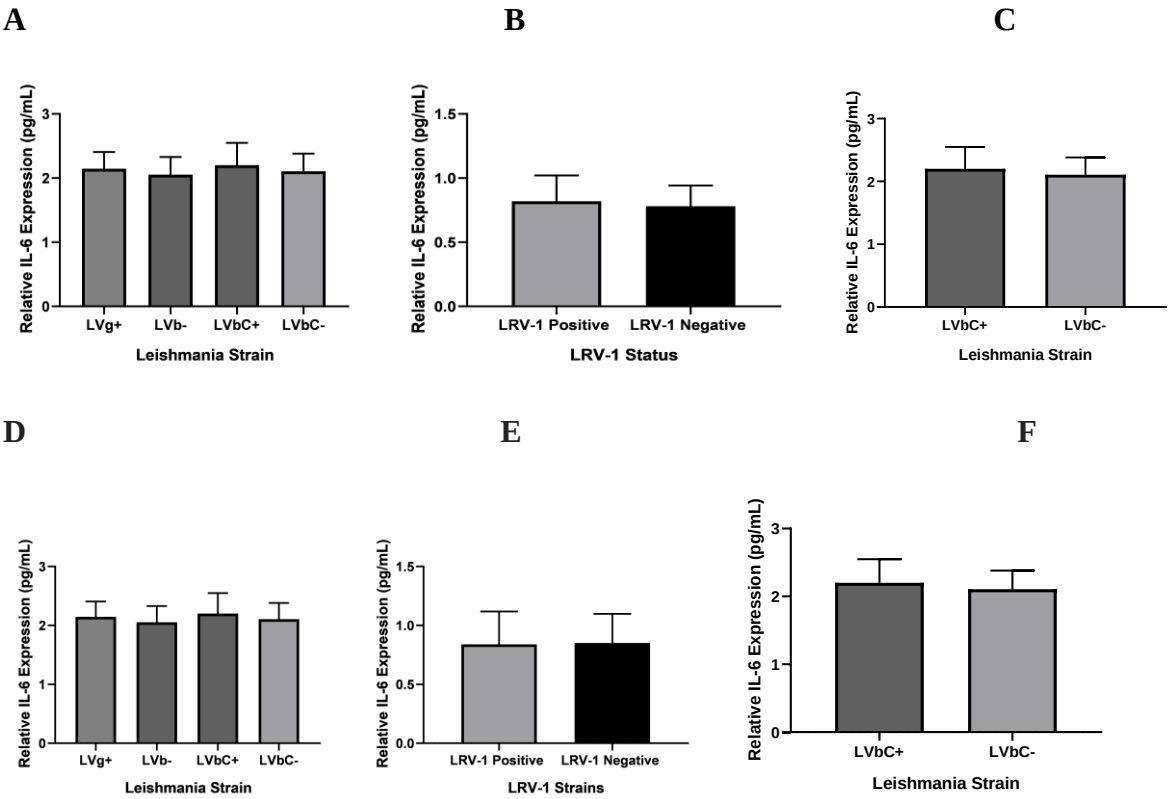
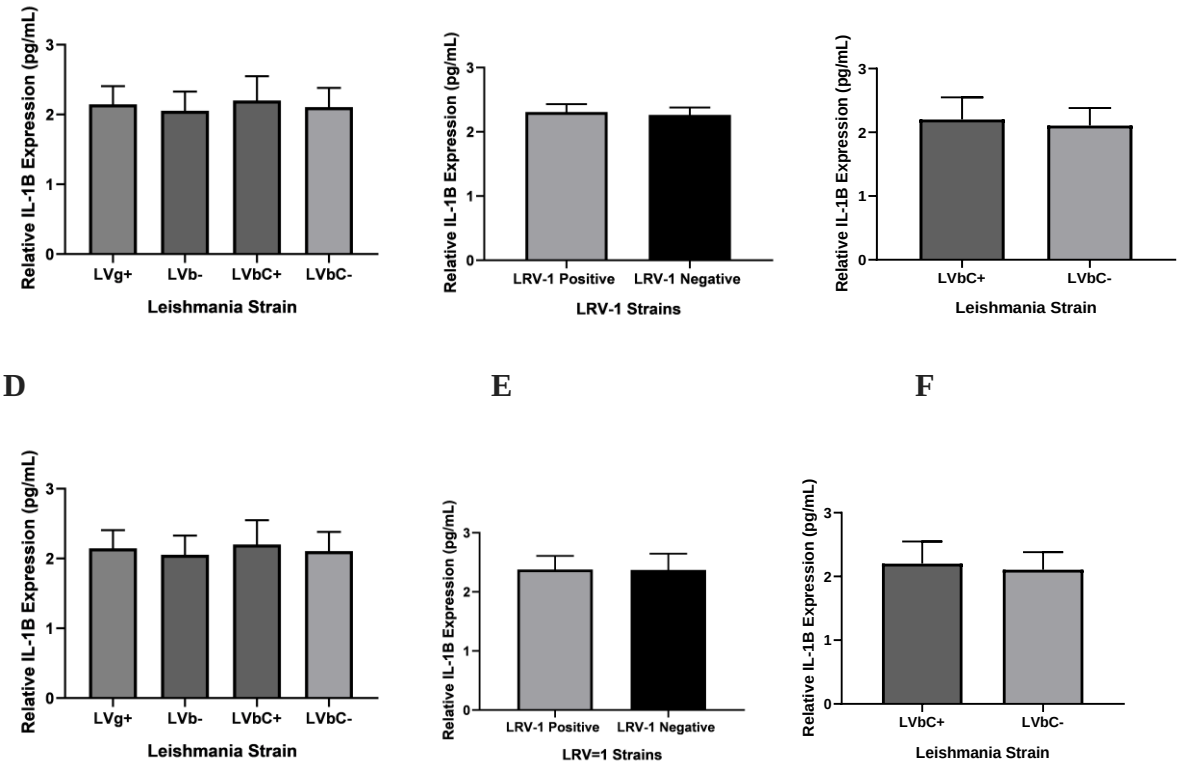


Figure 16: Relative IL-6 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).

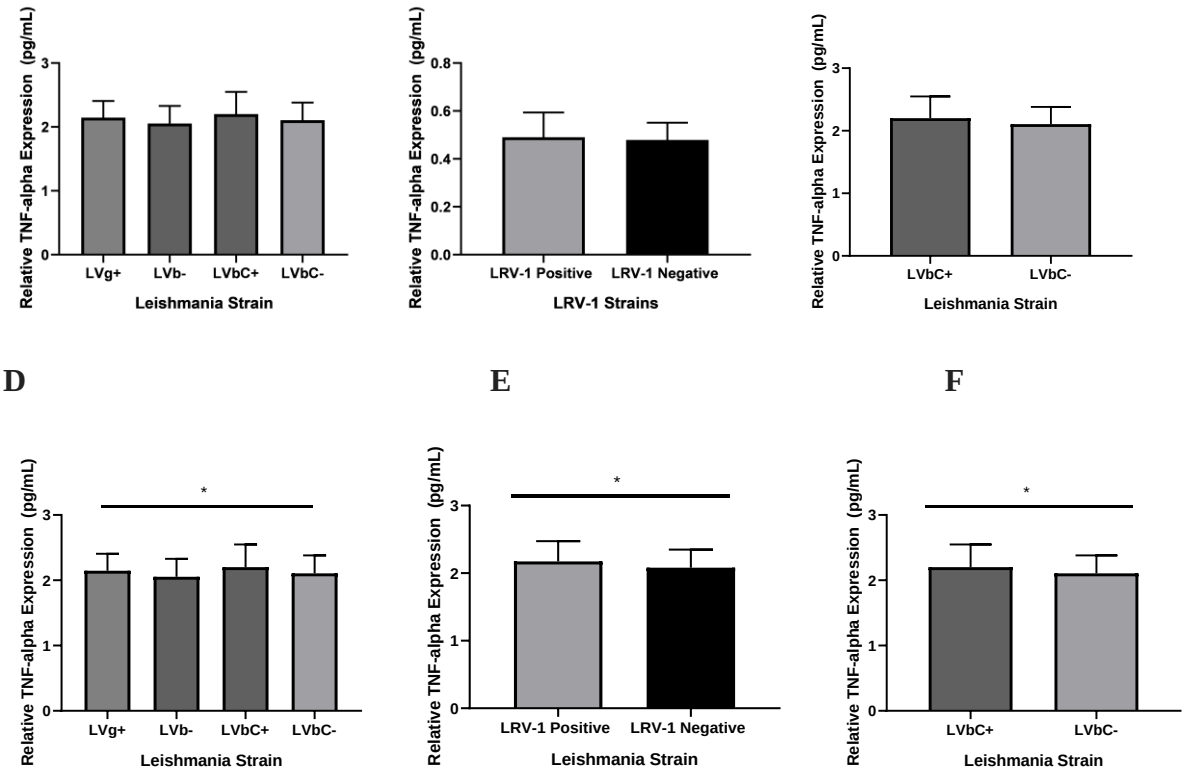


D **E** **F**

A **B** **C**

Figure 17: Relative IL-Iβ expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).

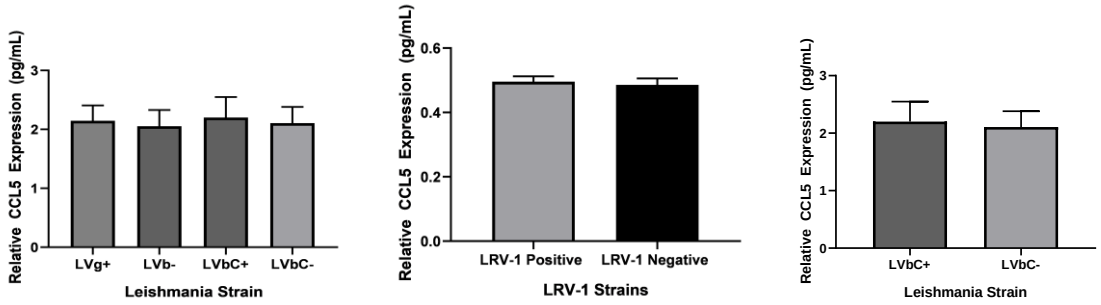


D **E** **F**

A **B** **C**

Figure 18: Relative TNF-α expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).



D **E** **F**

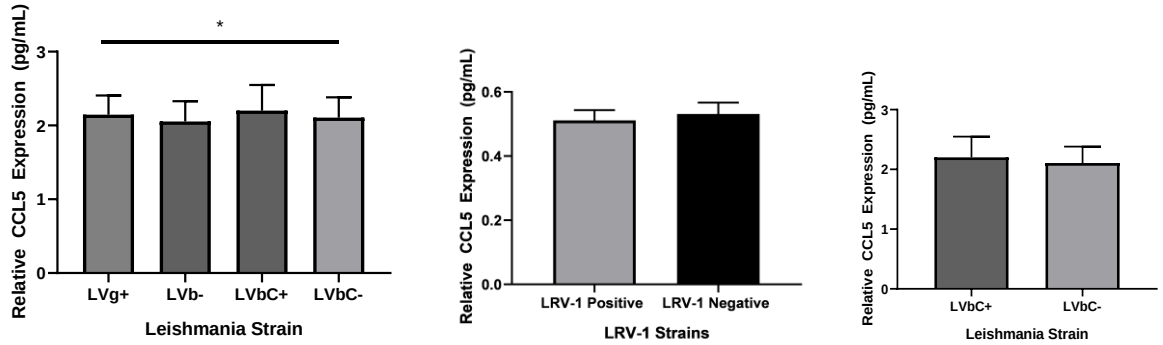
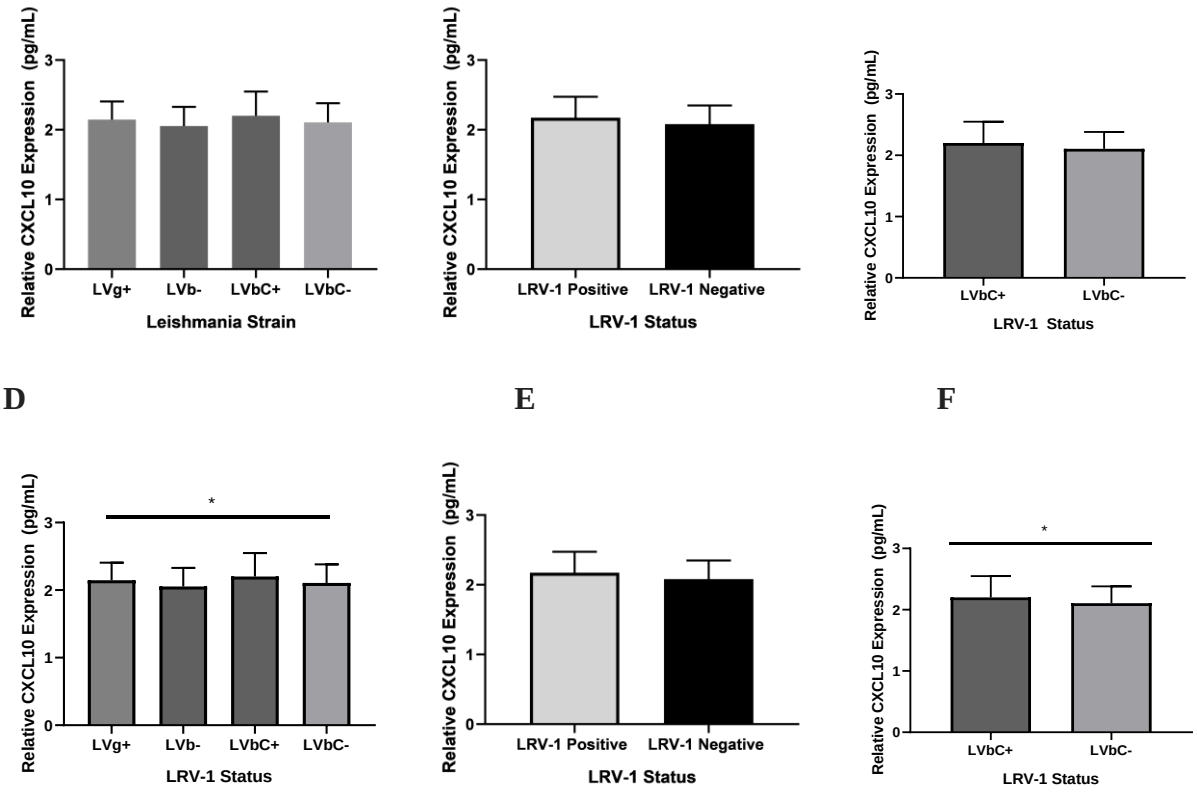


Figure 19: Relative CCL5 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).

A **B** **C**



D **E** **F**

A **B** **C**

Figure 20: Relative CXCL10 expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).

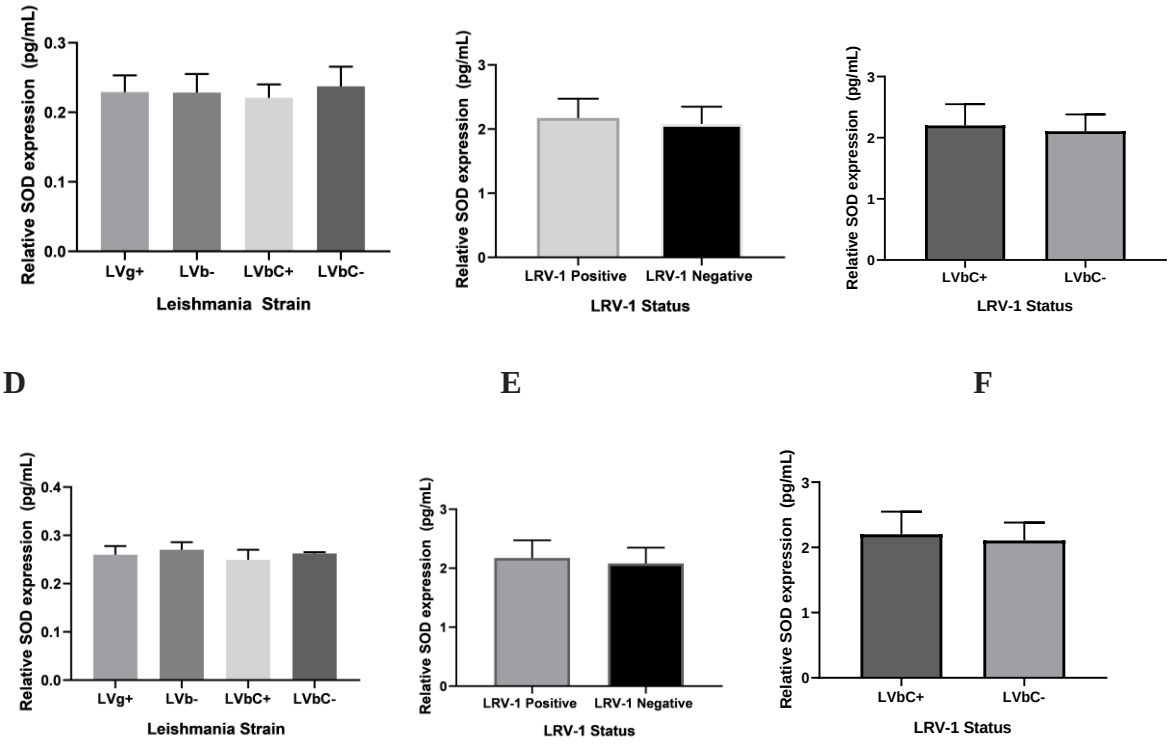


Figure 21: Relative SOD expression of infected macrophages at 24 hours (A-C) and at 48 hours (D-F).

Data were analyzed by comparing individual strains by ANOVA (A, D). Data were further analyzed by grouping strains according to overall LRV-1 status and compared using t-tests (B, E). Cytokine expression in LRV-1 positive and negative clinical strains of *L. V. braziliensis* were compared using t-tests (C, F).

Chapter III:Virulence Factor RNA Transcript Expression in the *Leishmania Viannia* Sub-genus: Influence of Species, Isolate Source and *Leishmania* RNA virus-1

LRV-1 in Clinical Cultures for VF RNA Transcript Studies

A total of 8 cultures including 5 clinical and 3 ATCC® were available to use for the VF RNA transcript study including a mix of *L. V. braziliensis* (n=2), *L. V. guyanensis* (n=1) and *L. V. panamensis* (n=5) (same cultures as Objective 2A). Analysis of VF RNA transcript observed in cultures from Objective 2B could not be performed given the low level of infectivity amounting to expression below detection limits.

Virulence Factor RNA Transcript Expression in the *Leishmania Viannia* Sub-genus: Influence of Species, Isolate Source and *Leishmania* RNA virus-1

Ruwandi Kariyawasam BSc[1], Avinash N. Mukkala[1], Rachel Lau MSc[2], Braulio M. Valencia MD[3], Alejandro Llanos-Cuentas MD[3,4], Andrea K. Boggild MD[1,2,5,6]

Affiliations:

[1]Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada.

[2]Public Health Ontario Laboratories, Toronto, Ontario, Canada.

[3]Instituto de Medicina Tropical "Alejandro von Humboldt", Lima, Peru

[4]Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru.

[5]Department of Medicine, University of Toronto, Toronto, Ontario, Canada.

[6]Tropical Disease Unit, Toronto General Hospital, Toronto, Ontario, Canada.

E-mail Addresses:

Ruwandi Kariyawasam - ruwandi.kariyawasam@mail.utoronto.ca

Avinash Mukkala - avinash.mukkala@mail.utoronto.ca

Rachel Lau - rachel.lau@oahpp.ca

Braulio M. Valencia - braulio.valencia@upch.pe

Alejandro Llanos-Cuentas - alejandro.llanos.c@upch.pe

Andrea K. Boggild - andrea.boggild@utoronto.ca

Address for Correspondence:

Dr. Andrea K. Boggild, Tropical Disease Unit, Toronto General Hospital

200 Elizabeth Street, 13EN-218

Toronto, ON M5G 2C4

Phone - 416-340-3675

Fax - 416-340-3260

Email - andrea.boggild@utoronto.ca

Word Count: Abstract: 339; Text: 3960

ABSTRACT

Background: *Leishmania* RNA virus-1 (LRV1) is a double stranded RNA virus identified in 20-25% of *Viannia* -species endemic to Latin America, and is believed to accelerate cutaneous to mucosal leishmaniasis over time. Our objective was to quantify known virulence factor (VF) RNA transcript expression according to LRV1 status, causative species and isolate source.

Methods: Eight cultured isolates of *Leishmania* were used, 4 of which were LRV1-positive (*Leishmania Viannia braziliensis* [n=1], *L. (V.) guyanensis* [n=1], *L. (V.) panamensis* [n=2]), and 4 were LRV1-negative (*L. (V.) panamensis* [n=3], *L. (V.) braziliensis* [n=1]). Promastigotes were inoculated into macrophage cultures, and harvested at 24- and 48-hrs. RNA transcript expression of *hsp23*, *hsp70*, *hsp90*, *hsp100*, *mpi*, *cpb*, and *gp63* were quantified by qPCR.

Results: RNA transcript expression of *hsp100* (p=0.012), *cpb* (p=0.016), and *mpi* (p=0.022) showed significant increases from baseline pure culture expression to 24- and 48- hours post-macrophage infection, whereas *hsp70* (p=0.004) was significantly decreased. A trend towards increased transcript expression of *hsp100* at baseline in isolates of *L. (V.) panamensis* was noted. Pooled VF RNA transcript expression by *L. (V.) panamensis* isolates was lower than that of *L.(V.) braziliensis* and *L. (V.) guyananesis* at 24- hours (p=0.03). VF RNA transcript expression did not differ by LRV1 status, or source of cultured isolate at baseline, 24- or 48- hours, however a trend towards increased VF RNA transcript expression of 2.71- and 1.93-fold change of *mpi* (p=0.11) and *hsp90* (p=0.11), respectively, in LRV1 negative isolates was noted. Similarly, a trend towards lower levels of overall VF RNA transcript expression in clinical isolates (1.15-fold change) compared to ATCC® strains at 24- hours was noted (p=0.07)

Conclusions: Our findings suggest that known VF RNA transcript expression may be affected by the process of macrophage infection. We were unable to demonstrate definitively that LRV-1 presence affected VF RNA transcript expression in the species and isolates studied. *L.(V.) guyanensis* and *L. (V.) braziliensis* demonstrated higher pooled VF RNA transcript expression than *L. (V.) panamensis*, however, further analyses of protein expression to corroborate this finding are warranted.

Keywords: American tegumentary leishmaniasis, *Leishmania Viannia braziliensis*, *Leishmania* RNA Virus-1 (LRV1), virulence factor

Background

American tegumentary leishmaniasis (ATL) is comprised of cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and mucosal leishmaniasis (ML), which are endemic to Central and South America (Fraga et al., 2012). Transmitted by sandflies, the array of clinical manifestations depend on the *Leishmania* spp. involved as well as the immunological status of the host (Reithinger et al., 2007; Olivier et al., 2012). The outcomes of infection greatly depend on host and parasitological factors whereby the protozoan parasite gains access to the host cell, and survives by either suppressing or evading the host immune response (Bifeld et al. 2015; Lamotte et al., 2017). While most CL presents as a painless ulcer, in particular, the *Viannia* subgenus has been implicated in severe disease including inflammatory CL, characterized by erythema, purulent exudate, pain and/or lymphatic involvement ("complex" as per the Infectious Diseases Society of America (IDSA) cutaneous leishmaniasis guidelines), in addition to MCL and ML (Aronson et al., 2016). Parasitological factors known to modulate the host immune response include *Leishmania* RNA virus-1 and endogenous virulence factors.

A double-stranded RNA virus, *Leishmania* RNA virus-1 (LRV1) has been identified in certain strains of the *Viannia* species predominantly found in the Amazon basin of South America (Hartley et al., 2012; Ginouves et al., 2016). Geographical expansion as a result of environmental changes and urbanization is postulated to have caused the parasite harbouring LRV1 to spread to Central America (Macedo et al., 2016; Pereira et al., 2013). LRV1 has been associated with an over-active immune response with increased expression of proinflammatory cytokines and chemokines including TNF-α, IL-6, CXCL10, CCL4, CCL5, and is believed to accelerate 10-15% of localized CL to either ML or MCL (Ives et al,. 2011; Ronet et al., 2011; Valencia et al., 2014). To add, LRV1 has been documented in 20-58% of clinical isolates of *L.*

(V.) guyanensis and *L. (V.) braziliensis* associated with first-line treatment failure and relapse (Ogg et al., 2003; Bourreau et al., 2016).

Virulence factors (VFs) are molecules that enable pathogen adaptation to adverse environmental conditions through increased expression, or via manipulation of the host immune response (Bifeld et al., 2015; Lamotte et al., 2017; Atayde et al., 2016). VFs endogenous to *Leishmania* spp. including molecular chaperones such as heat-shock proteins (HSPs), cysteine proteinases (CPB), leishmanolysins, phosphatases and proteinases, have been known to aid in the promastigote-amastigote transformation process and have certain immunomodulatory effects (Bifeld et al., 2015; Soulat et al., 2017; Sutter et al., 2017).

The current paradigm is that most genes of *Leishmania* are constitutively expressed, with fewer than 5% of mRNA transcripts varying significantly between life cycle stages (McConville et al., 2007; Duncan et al., 2004; Holzer et al., 2004; Leifso et al., 2007). Thus, regulation of gene control is thought to occur post-transcriptionally, and even post-translationally, and as such, the transcriptome has been thought of as a poor correlate of protein expression (Cohen-Freue et al., 2007; McNicoll et al., 2006; Depledge et al., 2009). Host immune pressure has also been thought not to affect parasite gene expression at the RNA level (Depledge et al., ,2009). Recent transcriptomic approaches using next-generation sequencing suggest a possible correlation between RNA abundance and ultimate protein expression, even in genes known to be constitutively expressed (Rastrojo et al., 2013). For example, HSP70 accounts for >2% of total protein in *Leishmania* promastigotes, and similarly, *hsp70* transcripts correspond to 2 of the top 3 most abundant transcripts in promastigotes of *L. major* (Rastrojo et al., 2013). Moreover, 20 of the 50 most abundant transcripts encode ribosomal proteins (Rastrojo et al., 2013). In the meta-transcriptome profiling analysis of human *L. (V.) braziliensis* infection, Christensen and

colleagues suggest that RNA transcripts identified in CL clinical lesions might be those contributing to promotion of parasite persistence, rather than just those of the most highly expressed *Leishmania* genes (Christensen et al., 2016). Such findings may suggest that in human *L. (V.) braziliensis* infection, at least, those proteins contributing to parasite subversion of the host clearance machinery are, indeed, correlated to corresponding RNA transcript levels. Furthermore, that many of the most abundant RNA transcripts encode putative proteins of as-yet undetermined function (Depledge et al., 2009; Rastrojo et al., 2013; Christensen et al., 2016), underscores that our understanding of how the parasite transcriptome might correlate to the functional proteome and ultimate virulence and pathogenesis of *Leishmania* remains an area with knowledge gaps to be filled. It has been documented that amongst CL lesions due to *L. (V.) braziliensis*, there is high uniformity of RNA transcript expression regardless of lesion size and duration (Christensen et al., 2016). Thus, we aimed to ascertain the relative abundance of known VF RNA transcripts, including *hsp23*, *hsp70*, *hsp90*, *hsp100*, *cpb*, zinc metalloproteinase GP63 (*gp63*), and mannose phosphate isomerase (*mpi*), previously evaluated in Old World strains and for which sequences were readily available through the National Center for Biotechnology Information (NCBI) database, in pure cultures and a macrophage model of infection with several species of the *Leishmania Viannia* sub-genus, a group around which few such data exist. In addition, we aimed to understand the influence, if any, of isolate source, corresponding species, and LRV1 status on VF RNA transcript expression and further comment on the virus, parasite and host dynamics in regards to infection.

Methods

Ethics Approval

Approval for this study was obtained from the Ethics Review Board of Public Health Ontario.

Clinical Data

De-identified clinical data of source patients collected from test requisitions were stratified into 'severe' and 'non-severe' phenotypes as per the IDSA guidelines (Aronson et al., 2016), where a severe phenotype was defined as: mucosal involvement; ulcers with associated erythema, purulent exudate, pain and/or lymphatic involvement (inflammatory ulcers); or multifocal/disseminated disease (ulcers in ≥ 2 anatomic sites and ≥ 4 in number) [Table 20] (Aronson et al., 2016). A non-severe phenotype was defined as localized CL (LCL) of < 4 ulcers in number (Aronson et al., 2016) [Table 20].

Cultured Leishmania spp.

Leishmania strains. Relevant characteristics of each of the *Leishmania* strains used are summarized in Table 19. Cultured isolates of *Leishmania* were obtained from the American Type Culture Collection® (ATCC®), and our *Leishmania* biobank of surplus cultured isolates at Public Health Ontario Laboratories (PHOL) as previously described (Kariyawasam et al., 2017). The following species of *Leishmania* were used: ATCC® strains of *L. (V.) braziliensis* ATCC®50135™ (MHOM/BR/75/M2903) LRV1 negative (LVb-); *L. (V.) guyanensis* ATCC®50126™ (MHOM/BR/75/M4147) LRV1+ (LVg+); *L. (V.) panamensis* ATCC®50158™ (MHOM/PA/71/LS94) LRV1 negative (LVp0-), and 5 clinical strains including one LRV1 positive *L. (V.) braziliensis* (LVb+)*;* four *L. (V.) panamensis* including two LRV1 negative *L. (V.) panamensis* (LVp1- and LVp2-) and two LRV1 positive *L. (V.) panamensis* (LVp1+ and LVp2+) [Table 19]. Promastigotes were routinely subcultured in Tobie's medium with Locke's overlay at ambient temperature every week. The following passage numbers (P#) of ATCC® and clinical isolates were used in this study: P2 (LVg+ and LVp2-), P3 (LVp2+), P5 (LVp1+), P6 (LVb-), P7 (LVb+) and P8 (LVp0- and LVp1-) [Table 19]. Prior to macrophage infection, 1.6

mL of *Leishmania* promastigotes was obtained and stored in -80°C to be used for species molecular identification, LRV1 detection and quantification.

Macrophage Infection. Macrophages were infected with promastigotes at a multiplicity of infection (MOI) of 10:1 (parasite: macrophage) in triplicate (see Supplementary Methods for macrophage differentiation). Prior to infection, a cell count of the *Leishmania* promastigotes was performed. Prior to addition to the 24-well plates, promastigotes were centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended with fresh Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (Kariyawasam et al., 2017). Subsequently, the plates were placed in an incubator set at 37°C and 5% CO_2 (Kariyawasam et al., 2017). Supernatants of infected macrophages adhering to the coverslips containing amastigotes were released using 0.05% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Life Technologies, Carlsbad, CA, USA), collected at 24- and 48- hours and were stored in -80°C until downstream RNA extraction, cDNA synthesis and VF RNA transcript expression analysis.

VF RNA Transcript Expression and LRV1 Quantification

RNA Extraction for VF Transcript Expression and Determination of LRV1 status. RNA was extracted from baseline pure culture and infected macrophages released using 0.05% Trypsin-EDTA, using QIAmp RNA Mini Kit (Qiagen, Germantown, MA, USA). An in-column DNase treatment was included in all extractions as per manufacturer's protocol.

cDNA synthesis. cDNA was synthesized with 50 – 300 ng of RNA using Superscript II Reverse Transcriptase and random hexamers (Life Technologies, Carlsbad, CA, USA), followed by purification with QIAquick PCR Purification Kit (Qiagen, Germantown, MA, USA) and eluted

with 50µL of nuclease-free water. In cases where RNA level was too low, a pre-amplification reaction to increase sensitivity of detection was performed with Perfecta Pre-Amp Supermix (Quanta Biosciences, Gaithersburg, MD, USA) using 100 ng of cDNA according to manufacturer's protocol with 14 cycles. The reaction was diluted 1:20 and 5µL of the pre-Amp cDNA was used in subsequent qPCR as above.

Detection of LRV1 by qPCR on Baseline Pure Culture. Two real time PCR (qPCR) assays for detection of LRV1 were performed with LRV1 set A and set B primers respectively as previously described (Zangger et al., 2013). *Leishmania* kinetoplastid membrane protein 11 (*kmp11*) was used as a quantification and extraction control (Zangger et al., 2013). Sybr Green real time PCR was setup with 1x Sybr Select Master Mix (Life Technologies, City, State), 250nM final concentration of forward and reverse primers, 5µL of cDNA of pure culture in a total volume of 20µL (Zangger et al., 2013). Amplification was performed in an ABI 7900HT real time instrument with the following conditions: uracil-DNA glycosylase (UDG) activation at 50^{o}C for 2 min, polymerase activation at 95^{o}C for 2 min, followed by 45 cycles of 95^{o}C for 15 sec, and 60^{o}C for 1 min. A dissociation step of 95^{o}C for 15 sec, 60^{o}C for 15 sec, and again another 95^{o}C for 15 sec was added at the end to generate a melting curve, which was used to check for the specificity of amplification. ATCC®50126™ *L. (V.) guyanensis* strain MHOM/BR/75/M4147, known to be LRV1 positive, was used as a positive control and RNA from a healthy human individual as negative control. LRV1 was confirmed by melt-curve analysis and was quantified relative to kmp11 and ATCC®50126™ *L. (V.) guyanensis* using the $2^{-\Delta\Delta Ct}$ method (Schmittgen et al., 2008).

Detection and Quantification of VF RNA Transcript Expression by qPCR. RNA transcript expression of the following virulence factors: *hsp23*, *hsp70*, *hsp90*, *hsp100*, *mpi*, *cpb*, *gp63*, and 18S, was performed on the ABI 7900HT real time instrument using primers designed using Primer Express 3.0.1 (ThermoFisher Scientific, Carlsbad, CA, USA) [Table 20]. A real-time PCR was set up in triplicate using 12.5µL 2x Taqman Universal Master Mix (ThermoFisher Scientific, Carlsbad, CA, USA), 250nM final concentration of forward and reverse primers, 10 nM probe and 5µL of cDNA from baseline pure culture or post-macrophage infection at 24- or 48- hrs, in a total volume of 20µL for each respective target [Table 20] (Zangger et al., 2013). Amplification was performed with the following conditions: UDG activation at 50^{o}C for 2 min, polymerase activation at 95^{o}C for 10 min, followed by 45 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. Virulence factor RNA transcript expression was quantified relative to 18S of each culture using the $2^{-\Delta Ct}$ method (Schmittgen et al., 2008).

Statistical Analysis

Descriptive statistics were performed on clinical data related to the surplus clinical strains housed in the *Leishmania* biobank, including age, sex, travel region, and clinical phenotype. Relative and pooled virulence factor RNA transcript expression was calculated for each strain, compared by species, LRV1 status and source of cultured isolate (ATCC® versus clinical), using Mann-Whitney U Test and Kruskal-Wallis Test at baseline pure culture, 24- and 48- hour time points post macrophage infection. For the comparison by species, we compared *L. (V.) panamensis* versus *L. V. braziliensis* and *L. V. guyanensis*, based on the premise that *L. (V.) braziliensis* and *L. (V.) guyanensis* are generally thought of as more virulent strains manifesting more severe clinical sequelae (Aronson et al., 2016; Ives et al., 2011; Ronet et al., 2011; Valencia et al., 2014). Pooled virulence factor RNA transcript expression was calculated to

determine if expression was enhanced or down regulated due to singular or multi-transcriptional genes. A log transformation was performed to graphically represent the data (Figures 22-28). All statistical analyses were conducted using GraphPad Prism 6 version 6.07 software (GraphPad Software Inc, La Jolla, CA).

Results

LRV1 Status of Isolates

Three of 5 (60%) clinical cultures were LRV1 positive (LVb+, LVp1+ and LVp2+), and 2 were LRV1 negative (LVp1- and LVp2-) [Table 19]. One of 3 ATCC® strains was LRV1 positive (LVg+), and 2 were LRV1 negative (LVb- and LVp0-) [Table 19]. Of 4 cultured clinical isolates from patients with a severe clinical phenotype, all but one were LRV1 positive [Table 19]. Relative LRV1 copy number of positive isolates ranged from $2.17x10^{-4}$ to 0.10 copies/mL [Table 19].

Virulence Factor (VF) RNA Transcript Expression

Baseline virulence factor transcript expression of all targets was detected in the 8 cultures. The following targets could not be evaluated in post-macrophage infectivity supernatants due to transcript levels below detection: *gp63* (24 hrs), *hsp23* (24- and 48- hrs), *hsp70* (48 hrs), and *hsp100* (48 hrs). Overall, VF transcript expression did not differ between baseline pure culture, 24- and 48- hours post-macrophage infectivity for *hsp90* (p=0.40) and pooled VF (p=0.78) analyses [Figure 22]. A significant increase in transcript expression from baseline pure culture to 24- and 48- hours was observed for the following VF transcripts: *cpb* (p=0.016), *mpi* (p=0.022), *gp63* (p=0.044), and *hsp100* (p=0.012) [Figure 22]. A significant decrease in transcript expression of *hsp70* by 31.4-fold was observed between baseline pure culture and 24- hours post macrophage infectivity (p=0.004) [Figure 22].

VF RNA Transcript Expression by LRV1 Status

VF transcript expression did not differ by LRV1 status for all baseline pure *Leishmania* cultures (pre-macrophage infection) for *gp63* (p=0.20), *cpb* (p=0.49), *hsp23* (p=0.34), *hsp70* (p=0.34), *hsp100* (p=0.34) and pooled (p=0.34) analyses [Figure 23]. A trend towards a 2.71- and 1.93-fold increased transcript expression of *mpi* (p=0.11) and *hsp90* (p=0.11), respectively, in LRV1 negative isolates was noted [Figure 23] VF RNA transcript expression by LRV1 status across time points is presented in Supplementary Results [Figure 24].

VF RNA Transcript Expression by Species

VF transcript expression did not differ by species (*L. (V.) panamensis* versus *L. (V.) braziliensis* and *L. (V.) guyanensis*) for all baseline pure *Leishmania* cultures (pre-macrophage infection) for the following: *gp63* (p=0.50), *cpb* (p=0.25), *mpi* (p=0.86), *hsp23* (p=0.68), *hsp70* (p=0.79), *hsp90* (p=0.50) and pooled (p>0.99) analyses [Figure 25]. Increased transcript expression of *hsp100* in isolates of *L. (V.) panamensis* was noted, however, this was not statistically significant (p=0.14) [Figure 25]. Pooled VF transcript expression by *L. (V.) panamensis* isolates was lower than that of *L.(V.) braziliensis* and *L. (V.) guyananesis* at 24- hours (p=0.03) [Supplementary Results [Figure 26].

VF RNA Transcript Expression by Source of Cultured Isolate

VF transcript expression did not differ by source of cultured isolate (ATCC® versus clinical) for all baseline pure *Leishmania* cultures (pre-macrophage infection) for the following: *gp63* (p=0.74), *cpb* (p=0.79), *mpi* (p=0.79), *hsp23* (p=0.74), *hsp70* (p=0.68), *hsp90* (p=0.79), *hsp100* (p=0.57) and pooled (p=0.86) analyses [Figure 27]. VF RNA transcript expression by source of

cultured isolate across time points is presented in Supplementary Results [Figure 27]**.** At 24-hours there was a trend towards lower levels of overall VF transcript expression in clinical isolates (1.15-fold change) compared to ATCC® strains (p=0.07) [Figure 28].

Discussion

Numerous parasitological factors enhance the ability of the *Leishmania* parasite to produce a successful infection, including infecting species, parasite load, LRV1 status, and most importantly, the expression of virulence factors (Reithinger et al., 2007; Olivier et al., 2012; Bifeld et al., 2015; Aronson et al., 2016). Many studies have separately evaluated the role of LRV1 (Hartley et al., 2012; Ginouves et al., 2016; Macedo et al., 2016; Ives et al., 2011; Ronet et al., 2011; Valencia et al., 2014) and VFs (Atayde et al., 2016; Requena et al., 2015; Hombach et al., 2014; Drini et al., 2016; Krobitsch et al., 1998; Holakuyee et al., 2012) in the pathogenesis of ATL, however, data on the combined role of both factors in ATL pathogenesis are scarce. We evaluated the contribution of LRV1 to key VF RNA transcript expression in the *Viannia* subgenus given its role as a mammalian host immunomodulator and potential influence on parasite itself, and did not demonstrate any change in relative abundance of VF RNA transcripts based on LRV1 status. However, there was a trend towards an almost 2- and 3-fold increased transcript expression of *hsp90* and *mpi*,, respectively, in LRV1 negative isolates. Overall, we noticed significant differential VF transcript expression resulting from pure cultures to the macrophage model, and also noted an overall reduction in VF transcript expression in isolates of *L. (V.) panamensis* compared to other *Vianna* strains, with a trend in decreasing expression of *hsp100* in *L. (V.) panamensis.*.Differential RNA transcript expression by source of the cultured

isolate was also not observed. This is also one of few studies to document LRV1 positive *L. V. panamensis* isolates from Central America (Parra-Munoz et al., 2018).

Molecular chaperones are key proteins involved in the maintenance of cellular homeostasis through folding of polypeptides (Raquena et al., 2015). Heat shock proteins are a subset of molecular chaperones known to increase in synthesis when presented with heat stress (Requena et al., 2015; Hombach et al., 2014). HSP23 is preferentially expressed up to 3-fold in the mammalian stage for *Leishmania* infectivity of macrophages, and is essential for stress tolerance and implicated in protection against trivalent antimonials (Hombach et al., 2014). HSP70 is the most conserved protein present in all eukaryotes and is involved in cell survival through avoidance of protein denaturation, and is often coupled with HSP90 (Hombach et al., 2014, Drini et al., 2016). HSP90, the most abundant protein in eukaryotic cytoplasm, mitochondria, and endoplasmic reticulum, is involved in the maintenance of numerous kinases and transcription factors (Hombach et al., 2014). HSP90 is also implicated in the maturation of viral proteins (Hombach et al., 2014). We found a trend towards higher expression of *hsp90* in LRV1 negative isolates compared to LRV1 positive isolates, which contravenes *hsp90*'s role in viral protein maturation. Subsequent protein work would be necessary to corroborate this finding. Lastly, HSP100 works in association with HSP70 to recognize misfolded proteins and is often an antagonist to the transformation of the amastigote back to the promastigote stage in *L. donovani* (Requena et al., 2015; Hombach et al., 2014; Drini et al., 2016; Krobitsch et al., 1998). We noted a trend towards lower expression of *hsp100* in isolates of *L. (V.) panamensis* compared to *L. (V.) braziliensis* and *L. (V.) guyanensis*, which might simply reflect that one strain of *L. (V.) panamensis* originated from a patient with non-severe CL. HSPs have also been involved in altering the immune response to *Leishmania* infection, whereby adjuvant effects of HSPs were

observed in mice infected with *L. major* and were shown to induce IL-1, IL-6, IL-12 and TNF-α expression contributing to a Th1 cytokine pattern of cellular immunity (Holakuyee et al., 2012). Overall, our data supported an increased RNA transcript expression of *hsp100* upon transformation of promastigotes to amastigotes during macrophage infection. Interestingly, we observed a significant decrease in *hsp70* transcript expression upon macrophage infection at 24 hours, with no commensurate difference in *hsp90* transcript expression, countering the observed paradigm of HSP70 and 90 coupling. It has been reported that increased synthesis of *hsp70* or *hsp90* transcripts does not correlate to increases in levels of proteins significantly, thus the reduction in *hsp70* transcripts observed in our study may not affect protein levels observed post-macrophage infection (Garami et al., 2001). Future disentanglement of how the *hsp70-hsp90* RNA transcript relationship might translate to protein expression and coupling in the in vivo situation is warranted.

Cysteine peptidases (CPs) are virulence factors present in all pathogenic kinetoplastida, and are considered potential therapeutic and vaccine candidates given their ability to modulate host-parasite interactions (Mottram et al., 2004, Casgrain et al., 2016). Three distinct genes exist: CPA, CPB, and CPC, all belonging to the same group designated Clan CA, Family C1 (Mottram et al., 2004). CPB is a key regulator of parasite stage differentiation, and is associated with a Th2 cytokine response by increasing IL-4 production, degrading NF-κβ and IL-12, thereby dampening the Th1 cytokine response (Aronson et al., 2016; Mottram et al., 2004; Pereira et al., 2012). Our data revealed an increase in expression of *cpb* transcripts from baseline to post-macrophage infection at 24- and 48- hours, and such a response, assuming correlation of RNA and protein levels, may correlate with disease severity through dampening of a Th1-directed cytokine profile and immunologic response to infection, which has been demonstrated in

previously published in vivo models (Aronson et al., 2016; Mottram et al., 2004; Pereira et al., 2012). Further evaluation of such a hypothesis using human skin models and human PBMCs is warranted.

Metalloproteases such as the zinc-dependent metalloprotease, glycoprotein 63, is a major surface antigen expressed on all *Leishmania* spp. promastigotes, and is involved in parasite adherence to macrophages and evasion of complement-mediated lysis (Olivier et al., 2012; Aronson et al., 2016; Atayde et al., 2016). GP63 activates protein tyrosine phosphatases (PTPs) to reduce nitric oxide (NO) production, thus facilitating parasite persistence in the macrophage vacuole (Silva-Almeida et al., 2012). Our data revealed an increase in *gp63* transcript expression from baseline culture to 24- and 48- hours post-macrophage infection at levels similar to *cpb* transcript expression. It has been found that CPB is required for GP63 expression, thus allowing the parasite to thrive in the macrophage (Casgrain et al., 2016). Lastly, MPI is an enzyme involved in the reversible conversion of fructose-6-phosphate and mannose-6-phosphate required for biosynthesis of various glycoconjugates (Garami et al., 2001). Lack of MPI has been associated with slowed growth in *Leishmania* spp. Our data have demonstrated a significant increase in *mpi* transcript expression from baseline to post-macrophage infection at 24- and 48-hours, as well as in LRV1 negative compared to positive isolates, which, assuming correlation of RNA and protein levels, could contribute to maintenance of the parasite's virulence to colonize host cells.

Our data have provided insight into VF RNA transcript expression in different LRV1-positive and negative *Viannia* strains causing ATL, including *L. (V.) guyanensis* and *L. (V.) panamensis* about which few such data exist. Although we did not observe significant differences in VF transcript expression attributable to source of cultured isolate or LRV1 status,

we did observe an overall diminution of VF transcript expression in *L. (V.) panamensis* compared to the historically more clinically aggressive *L. (V.) braziliensis* (Reithinger et al., 2007; Aronson et al., 2016; Hartley et al., 2012; Ginouves et al., 2016; Ives et al., 2011; Ronet et al., 2011; Valencia et al., 2014). The trend towards increased expression of *mpi* and *hsp90* in LRV1-negative isolates is interesting and requires future analyses with more isolates and a focus on protein expression to reconcile the relationship between VF expression, macrophage infection, clinical disease, and LRV1. One possibility would be that in the presence of LRV1, parasites are able to successfully infect macrophages without elaboration of specific VFs, while in its absence, cellular expenditures to produce VF that enhance macrophage infection are required. Further examination of species-specific virulence factors including leishmanolysins may illuminate aspects of infection severity, particularly as seen in *L. (V.) braziliensis* infection (Sutter et al., 2017). The host immune response may weigh heavily on the outcome of parasitic infection in addition to select virulence factors where host phosphatases such as serine threonine phosphatases (STPs) have been shown to regulate the outcome of *Leishmania* spp. infection (Soulat et al., 2017). This latter finding is consistent with the findings of Christensen and colleagues who noted uniform transcript expression across lesions due to *L. (V.) braziliensis* despite clinical variability, particularly size and lesion duration (Christensen et al., 2016).

Limitations

Limitations of this work include the small number of cultured isolates from a limited geographic range, as well as differences in the passaging of the strains in order to achieve sufficient promastigote growth phase and concentrations for successful macrophage infection. Additionally, all clinical isolates were derived from male patients, and this may affect the

generalizability of the data. Moreover, only 3 different species of *Leishmania* were found to contain LRV1, thereby limiting our ability to stratify our analyses by both LRV1 status and species. Within clinical cultures known to be LRV1 positive, the possibility that mixed LRV1 positive and negative strains exists, contributing to the lower viral load compared to *L. (V.) guyanensis* ATCC®50126™ (MHOM/BR/75/M4147). Furthermore, not all VF transcripts were detectable by our assays at all time points, which may have resulted from the limited availability of sequences for which our primers were designed and could have biased our interpretation of the data. It is possible that the expression of certain VF transcripts would be detectable at time points greater than 48- hours post-macrophage infectivity, though this premise is countered by the findings of Fernandes and colleagues, who noted maximal differential gene expression within 24-hours of macrophage infection, with little host-parasite interactions beyond that time point (Fernandes et al., 2016). We evaluated VF RNA transcript expression and did not quantify protein expression, thus, it is unknown whether or not transcript abundance would correlate to protein abundance. Finally, our macrophage model was derived from U937 cells, which may not represent the human *in vivo* or PBMC model well.

Conclusions

We have established a human macrophage model of ATL, infection of which was demonstrated to induce known VF RNA transcript expression. Differential VF transcript expression was attributable to the process of macrophage infection, despite that genes of many known VFs are thought to be constitutively expressed. Infecting species, rather than LRV1 status or source of cultured isolate, was also demonstrated to correlate to differential VF RNA transcript expression. Although trends were identified suggesting that LRV1 may inversely correlate to VF RNA

transcript expression, including *mpi* and *hsp90,* further studies focused on protein work post-macrophage infection are needed to corroborate this finding.

List of Abbreviations

American Tegumentary Leishmaniasis (ATL)

cutaneous Leishmaniasis (CL)

mucocutaneous leishmaniasis (MCL)

mucosal leishmaniasis (ML)

Infectious Diseases Society of America (IDSA)

Leishmania RNA Virus-1 (LRV1)

virulence factors (VFs)

heat shock proteins (HSPs)

cysteine proteinases (CPBs)

zinc metalloproteinase GP63 (GP63)

mannose phosphate isomerase (mpi)

National Center for Biotechnology Information (NCBI)

localized CL (LCL)

American Type Culture Collection® (ATCC®)

Public Health Ontario Laboratories (PHOL)

multiplicity of infection (MOI)

Roswell Park Memorial Institute (RPMI)

Fetal Bovine Serum (FBS)

Ethylenediaminetetraacetic acid (EDTA)

kinetoplastid membrane protein 11 (kmp11)

uracil-DNA glycosylase (UDG)

cysteine peptidases (CPs)

protein tyrosine phosphatases (PTPs)

nitric oxide (NO)

serine threonine phosphatases (STPs)

Supplementary Methods

Macrophage Differentiation. ATCC® U937 CRL-1593.2™ suspension cells stored in liquid nitrogen were thawed and cultured in RPMI 1640 media (Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated FBS (Thermo Fisher Scientific, Carlsbad, CA) and 1x penicillin/streptomycin (Thermo Fisher Scientific, Carlsbad, CA) at 37°C and 5% CO_2. Cells were maintained at a concentration between $1x10^5$ and $2x10^6$ cells/mL and

assessed using the trypan blue exclusion test. U937 monocytes were differentiated into macrophages by resuspending $5x10^5$ cells/ml of monocytes in RPMI-1640 supplemented with 50 ng/mL phorbol myristate acetate (PMA). One mL of monocytes were plated on removable glass cover slips onto 24-well plates and allowed to differentiate for 72 hours. Differentiated cells were identified by the presence of pseudopodia and adherence to the plate surface, while non-adherent undifferentiated monocytes were washed away with RPMI 1640 media (Sintiprungrat et al., 2010; Hsiao et al., 211; Verhoeckx et al., 2004; Kariyawasam et al., 2017). Differentiated cells were released from the cover slip using 0.05% Trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) and a cell count was performed.

Leishmania species molecular identification. DNA was extracted using QIAamp DNA Mini Kit Blood (Qiagen, Germantown, MA, USA). *Leishmania* genus 18S real time PCR was performed as previously described (Wortmann et al., 2001). Species identification included analysis of the internal transcribed spacer 1 (*ITS1*), *ITS2, cpb, hsp70,* and *mpi* targets by PCR, restriction fragment length polymorphism (RFLP) analysis, and Sanger sequencing (Schonian et al., 2003; de Almeida et al., 2011). PCR-RFLP analysis of the *ITS1* region can only differentiate *L. (V.) braziliensis* from the other species within the *Viannia* subgenus (*L. (V.) guyanensis, L. (V.) peruviana, L. (V.) panamensis, L. (V.) lainsoni*). Thus, PCR-RFLP and sequencing analysis of the *cpb, hsp70, mpi* and *ITS2* regions was required to differentiate species within the *Leishmania Viannia* sub-genus complex, and to provide a confirmation of the species identified in the initial *ITS1* assay. Purified PCR product was used for Sanger sequencing as per Big Dye protocol (Life Technnologies, Carlsbad, CA, USA). Sequence products were purified and analyzed using the Applied Biosystems 3130xl Genetic Analyzer. Data were standardized using

774 the Sequencing Analyzer program and the Basic Local Alignment Search Tool (BLAST) search
775 engine was used to analyze sequences.

776 Detection and Quantification of VF RNA Transcript Expression by qPCR.

777 Primers were validated against Taqman™ Control Genomic DNA (male) (Thermofisher
778 Scieintific, Carlsbad, CA, USA) to ensure no cross-reactivity. In addition, primers were
779 validated against the following ATCC® strains to ensure detection: *L. (V.) braziliensis* ATCC®
780 50135™ (MHOM/BR/75/M2903), *L. (V.) guyanensis* ATCC®50126™ (MHOM/BR/75/M4147),
781 *L. (V.) panamensis* ATCC®50158™ (MHOM/PA/71/LS94), *L. amazonensis* ATCC®50159™
782 (IFLA/BR/67/PH8), *L. chagasi* Cunha and Chagas ATCC®50133™ (MHOM/BR/74/PP75), *L.*
783 *donovani* (Laveran and Mesnil) Ross ATCC®50212™ (MHOM/IN/80/DD8), *L. infantum*
784 Nicolle ATCC®50134™ (MHOM/TN/80/IPT-1), *L. major* ATCC®50122™
785 (MHOM/IL/67/JERICHO II), *L. mexicana* (Biagi) Garnham ATCC®50157™
786 (MHOM/BZ/82/BEL21) and *L. tropica* (Wright) Luhe ATCC®50129™ (MHOM/SU/74/K27).

787 *Supplementary Results*

788 *VF RNA Transcript Expression by LRV1 Status*

789 At 24- hours of macrophage infectivity, VF transcript expression of LRV1 positive and LRV1
790 negative isolates were not significantly different for *cpb* (p=0.33), *mpi* (p>0.99) and *hsp70*
791 (p=0.67) [Figure 24]. The following targets could not be evaluated at 24-hours due to transcript
792 levels below detection: *gp63*, *hsp23* and *hsp100*. Similarly, no significant differences were
793 observed at 48- hours post-macrophage infection for pooled VF transcript analysis (p=0.63) , *cpb*
794 (p=0.49) and *mpi* (p>0.99) [Figure 24]. The following targets could not be evaluated at 48- hours
795 due to transcript levels below detection: *gp63*, *hsp23*, *hsp70* and *hsp100*.

VF RNA Transcript Expression by Species

Apparent increased transcript expression of *hsp100* in isolates of *L. (V.) panamensis* was noted, however this was not statistically significant (p=0.14) [Figure 25]. Pooled VF and *cpb* transcript expression was analyzed at 24- and 48- hours after macrophage infection for *L. (V.) panamensis* isolates verses other (*L. (V.) guyanensis* and *L. (V.) braziliensis*) [Figure 26]. Pooled VF transcript expression of *L. (V.) panamensis* isolates at 24- hours was significantly lower ($1.31x10^6$ fold) in comparison to the other isolates (p=0.04) [Figure 26]. No difference in *cpb* transcript expression at 24- hours post-macrophage infection was observed (p=0.33). Similarly, there was no difference in pooled VF and *cpb* transcript expression at 48- hours post-macrophage infection (p>0.99) [Figure 26].

VF RNA Transcript Expression by Source of Cultured Isolate

Pooled VF transcript expression of ATCC and clinical strains could only be evaluated at 24-hours where a trend towards lower levels of overall VF transcript expression in clinical isolates (1.15-fold change) was observed (p=0.07) [Figure 28]. At 48- hours post-macrophage infection, VF transcript expression was not significantly different between ATCC and clinical isolates for the following: pooled VF transcript (p=0.57), *cpb* (p=0.25), and *mpi* (p=0.67) [Figure 28].

List of Abbreviations

phorbol myristate acetate (PMA)

internal transcribed spacer 1 (ITS1)

restriction fragment length polymorphism (RFLP)

Basic Local Alignment Search Tool (BLAST)

Declarations

Ethics approval and consent to participate: Approval for this study was obtained from the Ethics Review Board of Public Health Ontario.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors have no competing interests.

Funding: This work was supported by the University of Toronto Department of Medicine, the University of Toronto Institute of Medical Science, and Public Health Ontario.

Authors' contributions: AKB conceived the study. RK, ANM, RL, BMV, AL-C, and AKB contributed to data collection, analysis, interpretation, and to critical appraisal of the manuscript. RK, ANM, and RL performed the bench experiments. AKB and RK were primarily responsible for writing the manuscript. All authors read, appraised, and approved the final manuscript.

Acknowledgments: not applicable.

Table 19: Classification of Leishmania spp. strains used in experiments.

***Leishmania* Strain**	**Source of Isolate**	**Country of acquisition**	**LRV1 Status**	**Relative LRV1 Copy Number**	**Age**	**Sex**	**Clinical Phenotype**
L. (V.) braziliensis (LVb-)	ATCC (MHOM/BR/75/ M2903)	Brazil	LRV1-	NA	Unk	Unk	Unk
L. (V.) braziliensis (LVb+)	Clinical	Peru	LRV1+	0.1	22	Male	Severe
L. (V.) guyanensis (LVg+)	ATCC (MHOM/BR/75/ M4147)	Brazil	LRV1+	Reference (1)	Unk	Unk	Unk
L. (V.) panamensis (LVp0-)	ATCC (MHOM/PA/71/ LS94)	Unknown	LRV1-	NA	Unk	Unk	Unk
L. (V.) panamensis (LVp1-)	Clinical	Costa Rica	LRV1-	NA	80	Male	Severe

L. (V.) panamensis (LVp2-)	Clinical	Costa Rica	LRV1-	NA	17	Male	Non-Severe
L. (V.) panamensis (LVp1+)	Clinical	Ecuador	LRV1+	$2.17x10^{-4}$	9	Male	Severe
L. (V.) panamensis (LVp2+)	Clinical	Costa Rica	LRV1+	$1.02x10^{-4}$	71	Male	Severe

Table 20: Primer and probe sequences used to detect virulence factor RNA transcripts by real-time PCR.

Target	Sequence
18S	
Forward	5’- AAGTGCTTTCCCATCGCAACT-3’
Reverse	5’- GACGCACTAAACCCCTCCAA-3’
Probe	FAM-CGGTTCGGTGTGTGGCGCC-NFQ
GP63	
Forward	5’- GGCTTCTACCAGGCGGACTT-3’
Reverse	5’-TGATGY†Y†BTBCR*CCATGCACTT-3’
Probe	FAM-AGGCCGAGGTGATG-MBG
CPB	
Forward	5’- GCTCGTCGGGTACAACAAGAC-3’
Reverse	5’- AGTCCTCACCCCACGAGTTCT-3’
Probe	FAM-TTCCGTACTGGGTGATC-BHQ1
MPI	
Forward	5’-GCTGCGAGGCCGGATAA-3’
Reverse	5’-GGAGTCAAGGCGCAR*ATGAG-3’
Probe	FAM-TACAAGGACCCGAACCACAR*GCCTGA-BHQ1
HSP23	

Forward	5’-GAR*CGS‡TGCTTCGAGCTT-3’
Reverse	5’-GAAGS‡TGGCCTTGATTTTGC-3’
Probe	FAM-CTGTTCGAGCTTC-BNFQ
HSP70	
Forward	5’- GTGGAW¶ATCATCGCGAACGA-3’
Reverse	5’- GAGTCCGTGAACGCAACGTA-3’
Probe	FAM-AGGGY†AACCGCACGACACCGT-BHQ1
HSP90	
Forward	5’- CAAGAAGCGCAACAACATCAA-3’
Reverse	5’- TCGCAGTTGTCCATGATGAAC-3’
Probe	FAM-TGTACGTGCGCCGCG-BHQ1
HSP100	
Forward	5’-CCGACTTCCAR*GACGACAAC-3’*
Reverse	5’-GCCTGCTTGCAGAGATCR*A-3’
Probe	FAM-ACGAGTCACTGAACAAG-BHQ1

*R=A,G

†Y=C,T

‡S=C,G

¶W=A,T

FIGURE LEGENDS

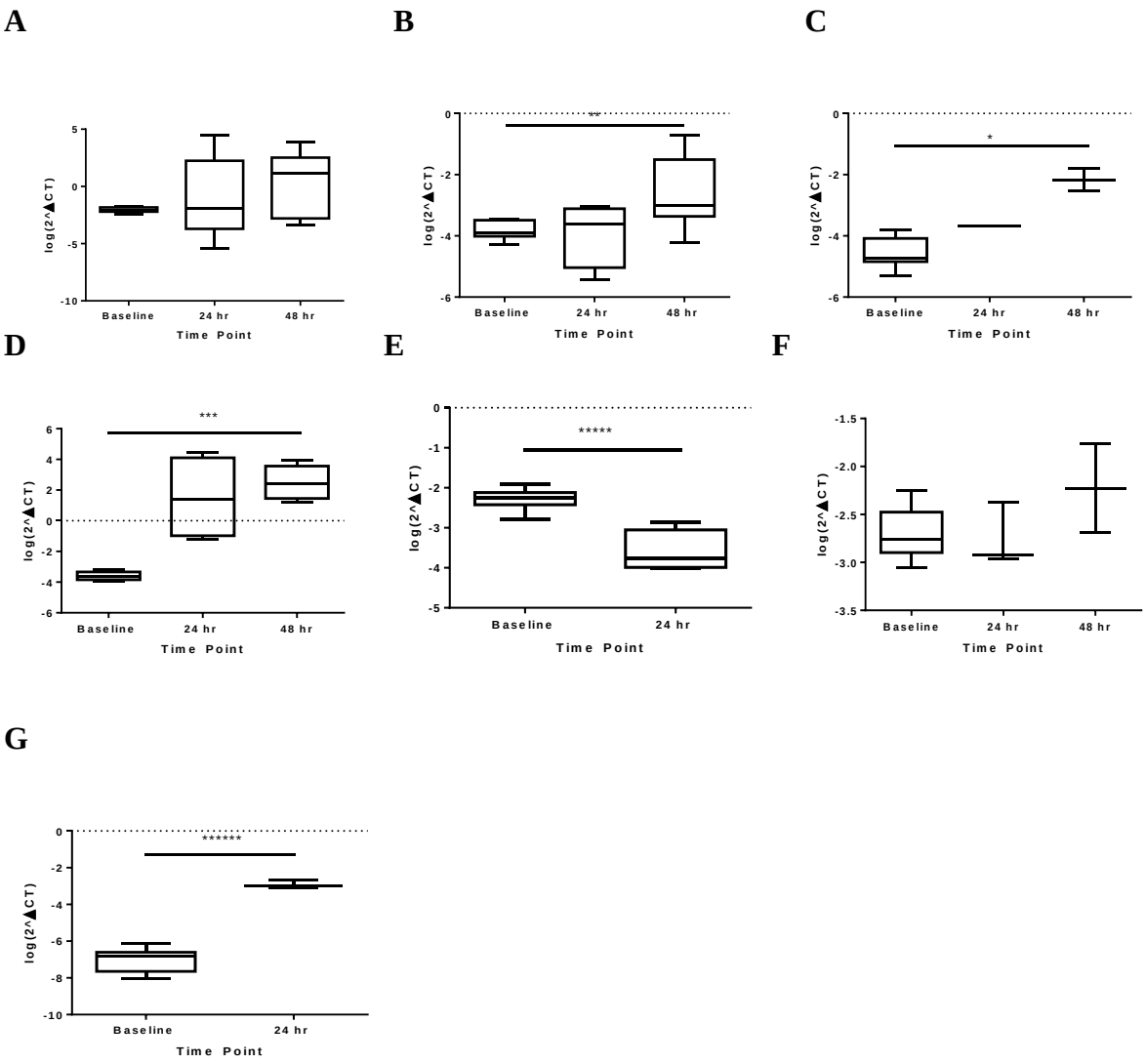


Figure 22: Log transformed virulence factor RNA transcript expression in baseline cultures and supernatants post-macrophage infectivity at 24- and 48- hours compared by Kruskal-Wallis or Mann-Whitney for the following targets:

pooled expression (A), *cpb* (B), *gp63* (C), *mpi* (D), *hsp70* (E), *hsp90* (F) and *hsp100* (G).

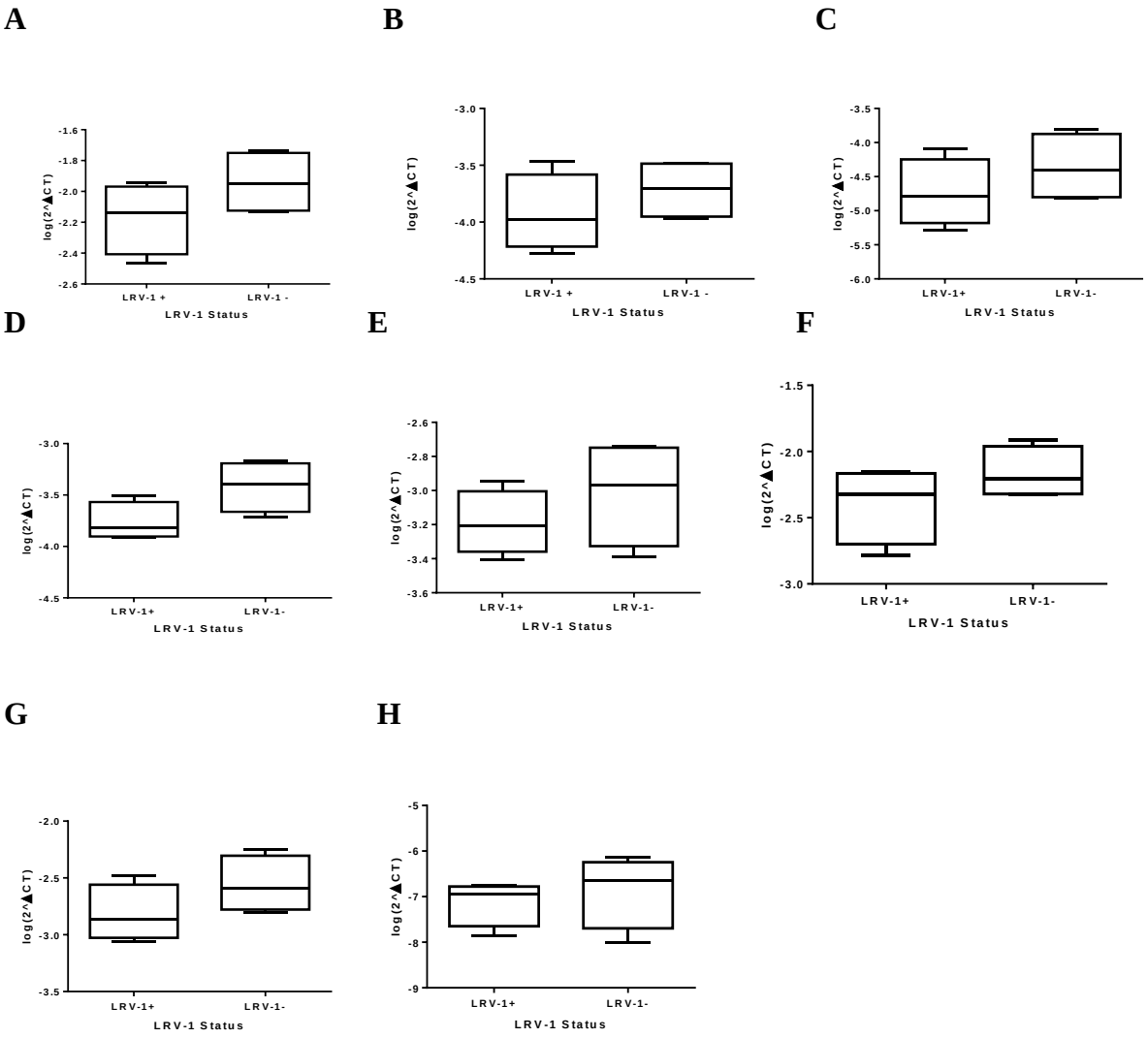


Figure 23: Log transformed virulence factor RNA transcript expression in baseline cultures analyzed by grouping strains according to LRV-1 status compared by Mann-Whitney for the following targets:

pooled expression (A), *cpb* (B), *gp63* (C), *mpi* (D), *hsp23* (E), *hsp70* (F), *hsp90* (G) and *hsp100* (H).

A **B**

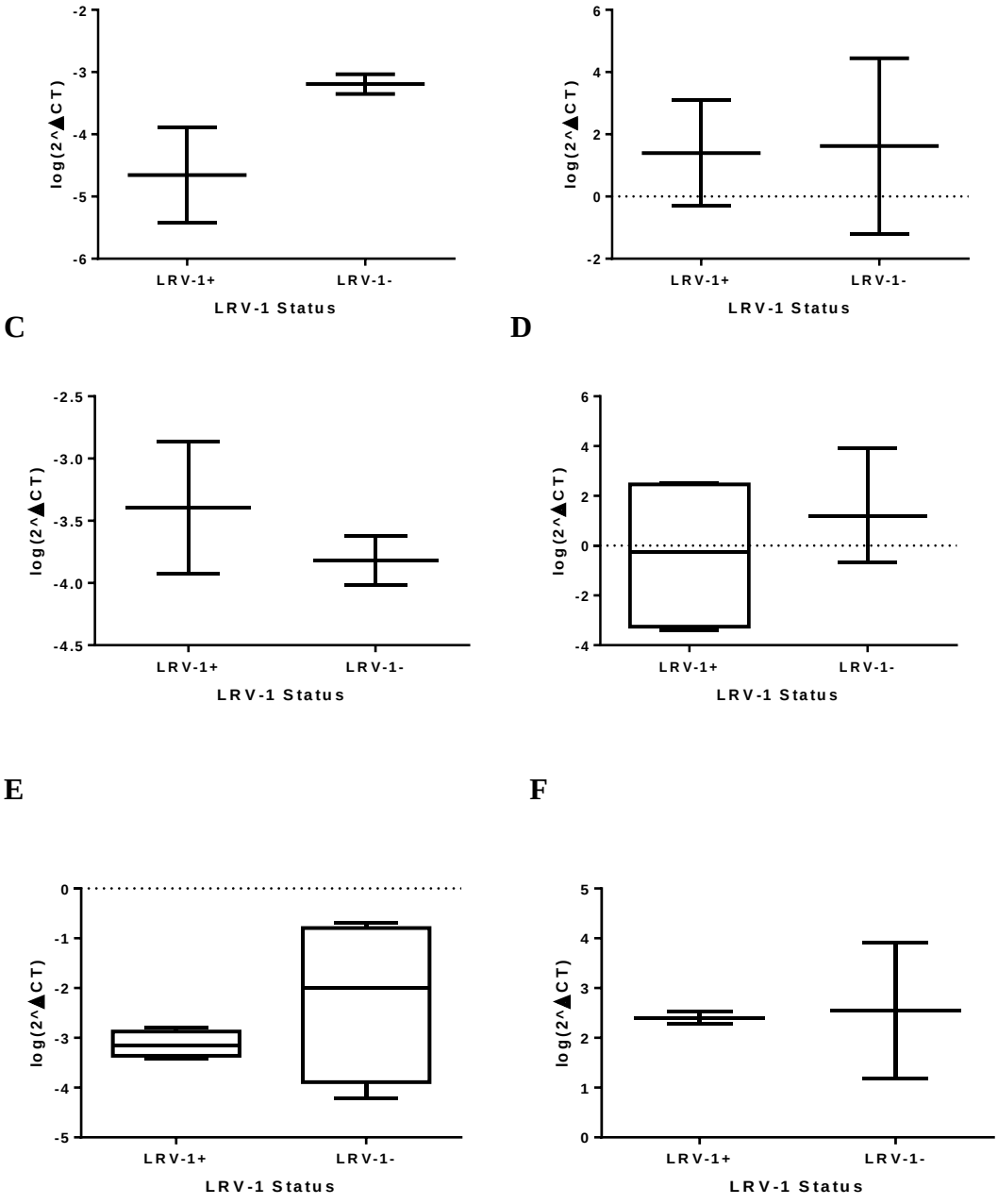


Figure 24: Log transformed virulence factor RNA transcript expression in supernatants post-macrophage infectivity at 24- and 48- hours compared by LRV-1 status using t-test for the following targets: *cpb*-24 hrs (A), *mpi*-24 hrs (B*), hsp70*-24 hrs (C), pooled-48 hrs (D), *cpb*-48 hrs (E) and *mpi*-48 hrs (F).

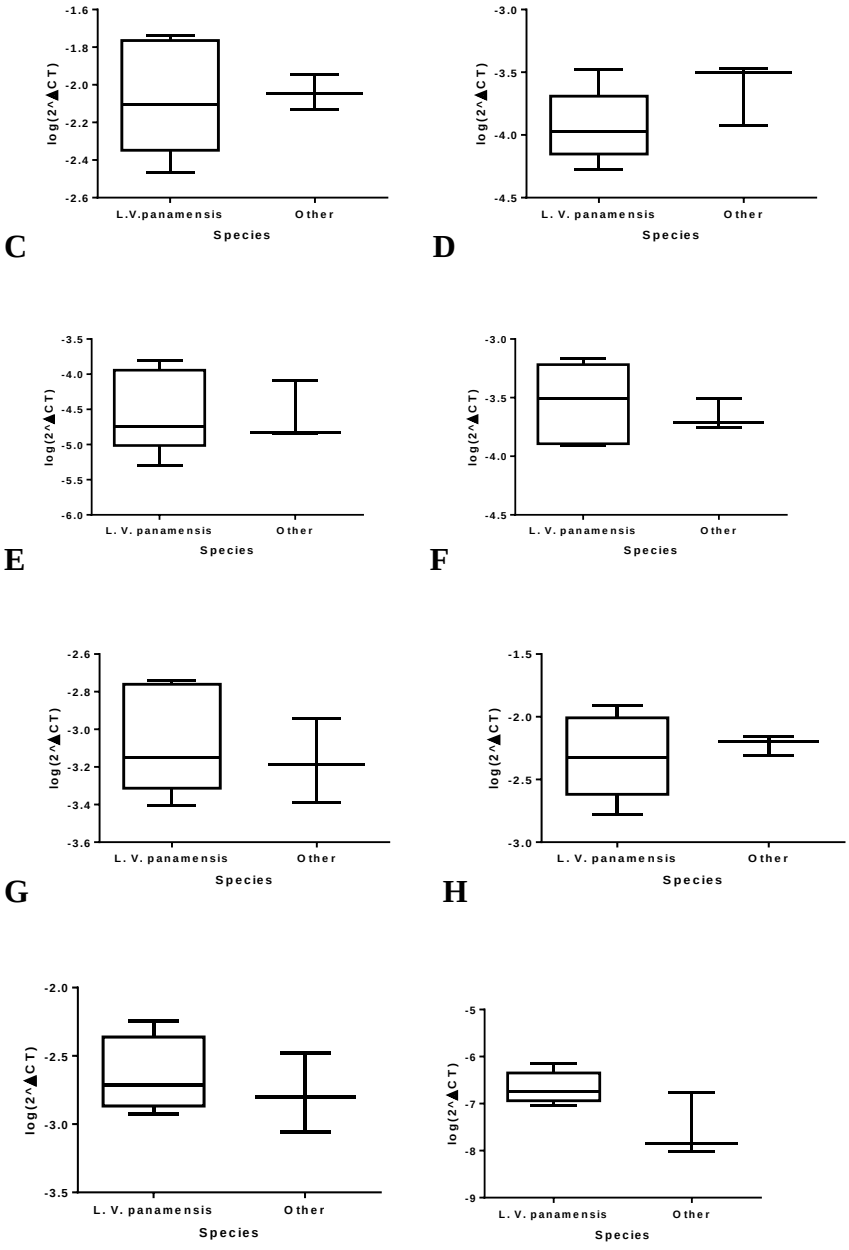


Figure 25: Log transformed virulence factor RNA transcript expression in baseline cultures analyzed by grouping strains according to species (L. V. panamensis versus other) compared by Mann-Whitney for the following targets: pooled expression (A), *cpb* (B), *gp63* (C), *mpi* (D), *hsp23* (E), *hsp70* (F), *hsp90* (G) and *hsp100* (H).

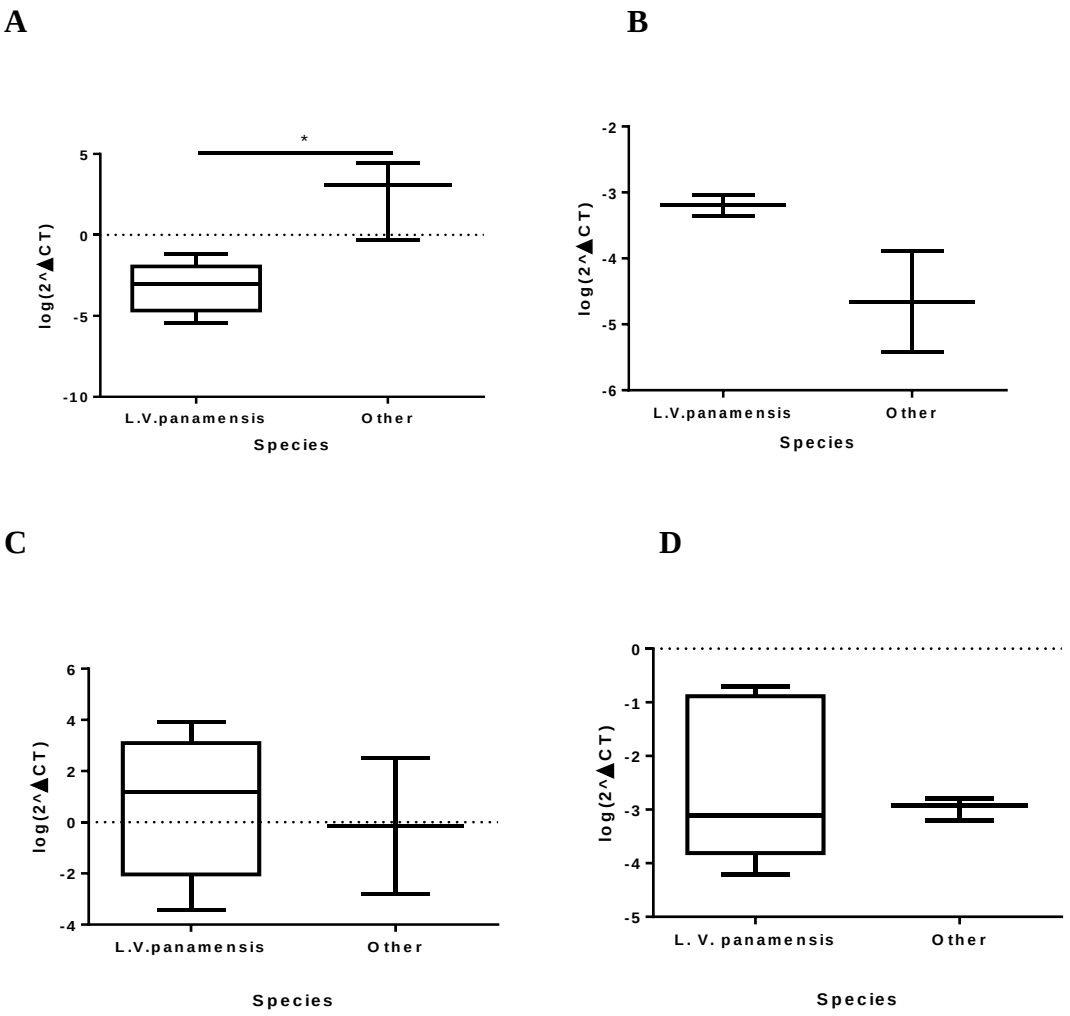


Figure 26: Figure 26: Log transformed virulence factor RNA transcript expression in supernatants post-macrophage infectivity at 24- and 48- hours compared by species using t-test for the following targets: pooled VF-24 hrs (A), *cpb*-24 hrs (B), pooled VF-48 hrs (C) and *cpb*-48 hrs (D).

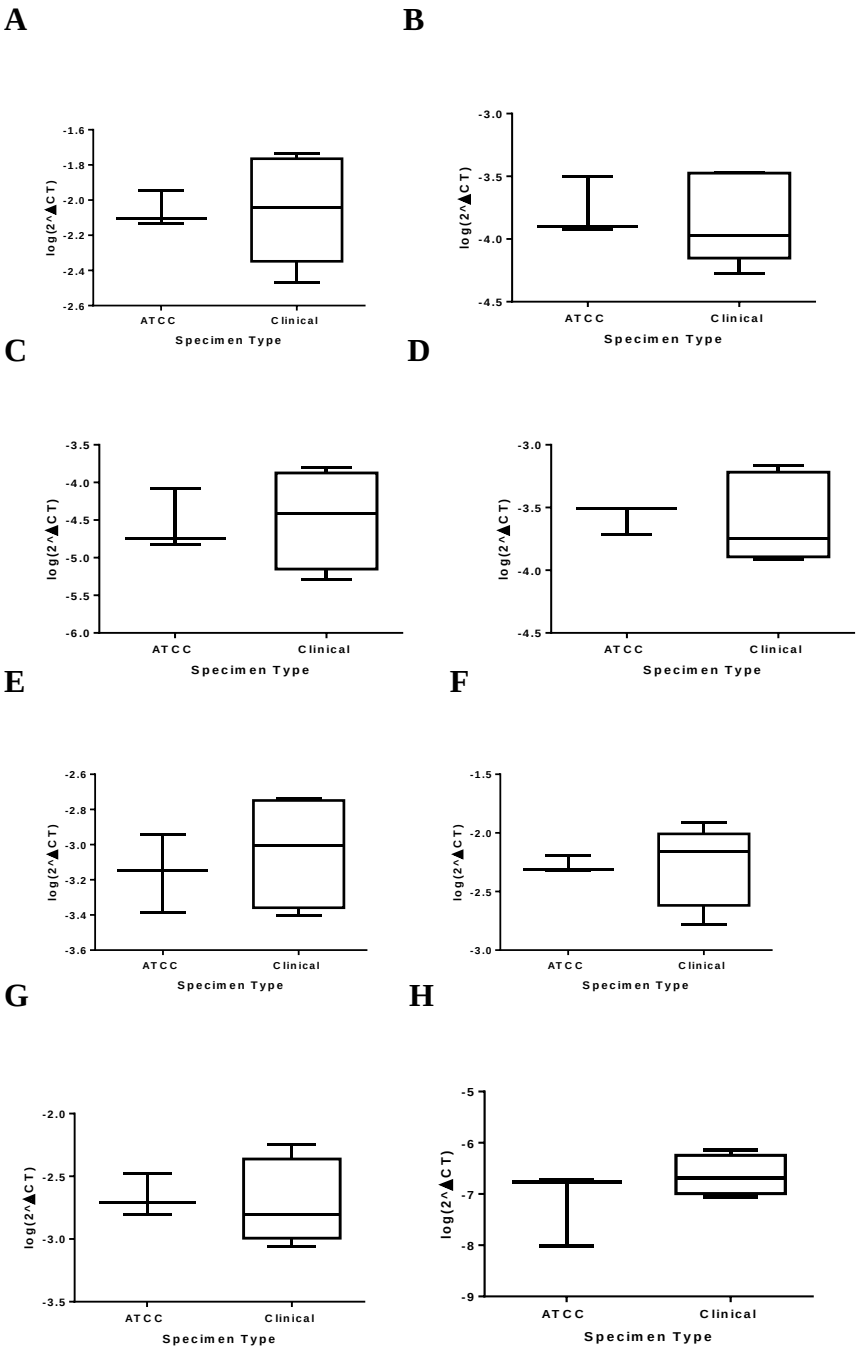


Figure 27: Log transformed virulence factor RNA transcript expression in baseline cultures analyzed by grouping strains according to source of cultured isolate (ATCC® versus clinical) compared by Mann-Whitney for the following targets: pooled expression (A), *cpb* (B), *gp63* (C), *mpi* (D), *hsp23* (E), *hsp70* (F), *hsp90* (G) and *hsp100* (H).

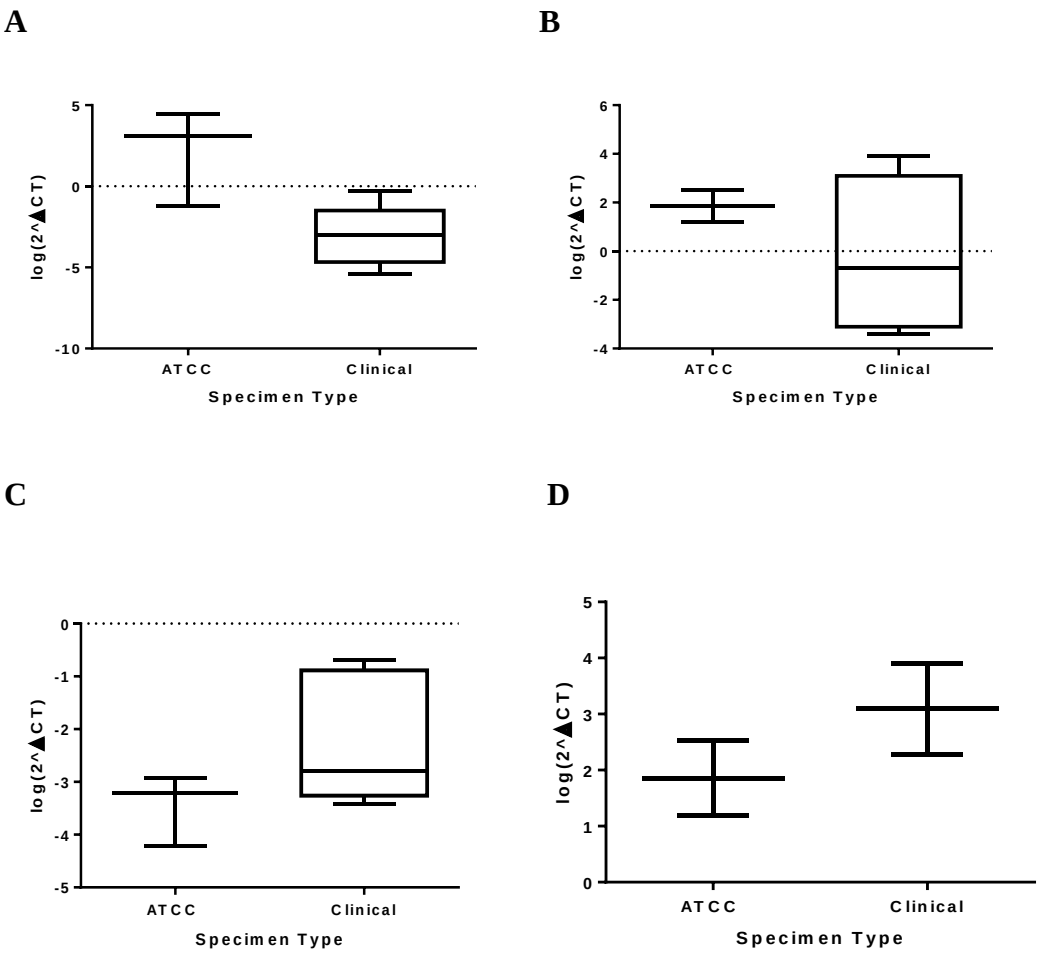


Figure 28: Log transformed virulence factor RNA transcript expression in supernatants post-macrophage infectivity at 24- and 48- hours compared by source of cultured isolate using t-test for the following targets: pooled VF-24 hrs (A), pooled VF-48 hrs (B), *cpb*-48 hrs (C) and *mpi*-48 hrs (D).

General Discussion

Through a combination of epidemiological, cell biological, and molecular methods, we have highlighted the complexities of LRV-1 in ATL pathogenesis, and our work supports the concept of a role for LRV-1 in the modulation of New World *Leishmania* strain pathogenicity that is potentially species-specific, and potential role as a diagnostic, prognostic and therapeutic marker. ATL is a complex disease that is over-simplified by binary classifications of cutaneous and mucosal involvement. An array of symptomatic CL infections exist where presentations other than LCL can be construed as "severe phenotypes", including inflammatory, multifocal, diffuse, or disseminated ulcers, in addition to the naturally severe ML and MCL infections. It is believed these rare forms of CL are a reflection of the underlying host immune status, whereby patients are often in an immunocompromised state. Globally, CL presents itself in all areas of *Leishmania* endemic countries, but the MCL and ML phenotypes are generally restricted to species acquired in Latin America. In Latin America, approximately 10-15% of healed LCL progresses to ML/MCL 1-5 years after healing, subject to geographic variation (Reithinger et al., 2007; Jara et al., 2016). Given that 20-25% of clinical isolates of the *Viannia* subgenus harbour LRV-1, it is of interest to understand the role of LRV-1 in these patients to truly determine the utility of evaluating LRV-1 as a diagnostic, prognostic and therapeutic marker of severe disease.

The role of infecting species weighs heavily on the clinical outcome of disease, however, the underlying host immune status and response to infection further contributes to the course of infection. Both infecting species and host immunological response display marked geographic variation as it relates to clinical course and outcome, both of which are naturally influenced by other biological, environmental, and socioeconomic factors, not the least of which being access to medical care. The ability of the parasite to achieve successful infection is reflective of its inherent virulence, whether it be by endogenous mechanisms or, potentially, exogenous factors

such as coinfection with the endosymbiont LRV-1. The work conducted during this thesis attempted to illustrate a fulsome picture of LRV-1 in ATL pathogenesis by addressing questions at both sample population and cell biological levels. A brief summary of each chapter will be provided along with placing it in the current realm of literature with a focus on the *L. V. braziliensis* and *L. V. panamensis* species. A discussion surrounding the current landscape of diagnostics, prognostics and therapeutics will illuminate gaps that currently exists and how LRV-1 may serve as a marker intersecting at all three levels. Lastly, the next steps for each chapter will be addressed in Future Directions, culminating into concluding remarks for this project.

Objective 1: Prevalence and Phenotypic Associations of LRV-1 in ATL

Understanding the prevalence of LRV-1 in our patient population comprised the first step in creating a picture of LRV-1's interaction with New World strains of *Leishmania* causing ATL, and provided a window into what environmental or geographic factors may influence the host and parasite response to LRV-1. It is clear that specific public health measures targeting disease prevention could be made at a higher levels to address population health, rather than individual-level health, were there political will and resources. Given that leishmaniasis is a neglected disease of rural poverty, many gains could be made in disease control through relatively simple but costly and large-scale public health interventions targeting improved case detection, vector control, and reservoir control. As such, by understanding LRV-1 at a population level, specific targeting measures can be put in place that may have downstream effects for individual health and overall impact on vector and parasite populations.

Five separate analyses were performed evaluating LRV-1 in different populations including the entire New World cohort (N=208) (Objective 1A), ATL acquired in Peru (N=174) (Objective 1B), ATL acquired in Peru with corresponding clinical cultured isolates (N=90) (Objective 1C),

ATL from isolates of *L. V. braziliensis* from Peru (N=74) (Objective 1D) and ATL in isolates of *L. V. panamensis* from Latin America (N=30) (Objective 1E). Each subsequent analysis with the exception of Objective 1E, eliminates potential confounders, including heterogenous sampling, species and geography. In achieving this objective, it became clear that the rate of LRV-1 (23.3%-29%) in each analysis did not differ and was within range of previously reported literature (Ginouves et al., 2016; Valencia BM., 2014; Ogg et al., 2003; Bourreau et al., 2016; Cantanhede et al., 2015; Adaui et al., 2016; Macedo et al., 2016; Ito et al., 2015; Pereira et al., 2013). Phenotypic manifestation of severe disease varied by species, where patients infected with *L. V. brazliensis* were represented in our defined clinical groups including LCL, inflammatory/multifocal CL and made up a large proportion of ML/MCL; whereas patients infected with *L. V. panamensis* presented with either LCL and inflammatory/multifocal CL. Historically, ML/MCL has been identified in patients infected with *L. V. braziliensis* and *L. V. guyanensis*,with few case reports citing *L. V. panamensis* as a causative species (Reithinger et al., 2007; World Health Organization, 2010; Gonzalez et al., 2017; Achtman et al., 2016; Morales et al., 2014). Our work has supported the current knowledge of species and clinical phenotype, and in particular, shed light on the clinical spectrum of illness of *L. V. panamensis* observed in patients acquiring ATL from Central and northern South America. Furthermore, this work supports the longstanding theory of age and severity of disease, where age was found to be associated with clinical phenotype in the larger analyses (Cincura et al., 2017; Carvalho et al., 2015; Salam et al., 2013). Patients manifesting ML/MCL were on average 10 years older than those manifesting inflammatory/multifocal CL or LCL, which was lost in subsequent analyses including the Peruvian cultures, *L. V. braziliensis* and *L. V. panamensis* specific analyses, attributable to a shrinking sample size. This finding is incongruent with the large body of work surrounding the role of LRV-1 in disease severity in animal models. However, in humans, it has

been well documented that older patients may manifest severe infectious diseases due to poorer T-cell responses, and the effect of co-morbidities must also be considered as factors that can enhance disease severity (Salam et al., 2013). Although the presentation of ML and MCL in older patients is justified based on the long incubation period of mucosal disease, potentially decades, those in the older age bracket in our studies also had more severe manifestations of cutaneous disease. However, underlying biological and host immunological factors may exist, whereby patients in endemic settings are likely to be reinfected multiple times resulting in low level infection of macrophages producing robust responses observed in patients of older age (Bosque et al., 2000; Cooper et al., 1994). Furthermore, age was not associated with LRV-1 status despite being associated with severe clinical phenotype. The lack of association between the detection of LRV-1 in clinical isolates and age is not surprising, as biologically speaking there is no reason for one age group to be diagnosed with more virus than the other. However, the severe disease observed into the older patients may highlight the natural course of ATL given that ML/MCL is historically observed in patients with a prior history of CL well over 5 years before (Teles et al., 2019; Guerra et al., 2015). In addition, there is a biological basis for the occurrence of ML/MCL in patients from endemic areas given the recurrent infections that have been shown to increase permissiveness of macrophages and long durations of low levels of infection, regardless of the virus (Bosque et al., 2000; Cooper et al., 1994).

Overall, LRV-1 copy number was no different in patients with ML or MCL compared to LCL, however a noteable difference was observed when compared to inflammatory/multifocal CL, whereby the latter would have much lower levels. The lack of viral burden observed in patients manifesting the inflammatory/multifocal forms of CL may be attributed to the baseline host immune status in this patient population, identified as individuals in an immunocompromised state. As such, less viral load would be required to modulate the host

immune response in patients with inflammatory/multifocal CL, compared to those with CL or ML/MCL. One must also consider the role of the active RNAi pathway in the parasite and its ability to control the amount of LRV-1 translated, suggesting that the viral burden may not be a truly proportional to disease severity. The quantification of viral load may not be an accurate representation of the actual load at the time of infection, as a number of these specimens were derived from clinical cultures, where the parasite has routinely been subcultured for a timeline of weeks to months, depending on the growth of the parasite.

Our findings mirrored that of previously reported epidemiological literature (Bourreau et al., 2016; Ito et al., 2013; Teles et al., 2019), whereby males were over-represented in all groups clinical and LRV-1 groups compared to females, which may be influenced by biological predilections as well as behavioural and social factors, such as work and family dynamics. Overall, our epidemiological data suggests phenotypic associations with age, rather than LRV-1 and dependent on species, whereby patients infected with *L. V. panamensis* developed either inflammatory/multifocal CL or LCL in our sub-analysis, whereas patients infected with *L. V. braziliensis* manifested one of the three major clinical manifestations evaluated in this study. LRV-1 may be an important diagnostic marker as observed by its varying rates in different species and clinical phenotype groups.

Objective 2: Influence of LRV-1 on Proinflammatory Biomarker Expression of L. V. braziliensis and L. V. panamensis

Given the lack of ML/MCL clinical manifestations observed in patients infected *L. V. panamensis*, and a large proportion of ML/MCL identified in LRV-1 positive isolates of *L. V. braziliensis* despite no direct association of LRV-1 and clinical phenotype in either species, we sought out to analyze the profile of proinflammatory biomarkers known to contribute to ATL pathogenesis. Using a human macrophage model from the U937 cell line derived from a 37-year-

old male patient with histiocytic lymphoma previously used in *Leishmania* spp. studies, we were able to elicit proinflammatory biomarker expression using culturable isolates of *Leishmania* from specimens incorporated into our initial epidemiological analysis of LRV-1. Comparison of TNF-α, IL-1β, IL-6, CCL5, CXCL10, and SOD expression at 24- and 48-hours were analyzed using clinical and ATCC® cultured isolates of *L. V. braziliensis* and *L. V. panamensis.*

Proinflamamtory Biomarker Expression of L. V. braziliensis

Overall, the LRV-1 positive *L. V. braziliensis* isolate, producing clinically severe disease, stimulated lower levels of the Th1 biomarkers IL-1β, TNF-α, and CXCL10, as well as Th2 IL-6 at both 24- and 48- hour time points, whereas SOD was upregulated at 24 hours when compared to the ATCC® LRV-1 negative *L. V. braziliensis* isolate. A subsequent set of experiments was performed comparing directly a clinical LRV-1 positive isolate to clinical LRV-1 negative isolate. In this analysis, a 10-fold lower macrophage cell count was used and prior associations between the clinical LRV-1 positive isolate and the ATCC® LRV-1 negative isolate were lost. Overall, the presence of LRV-1 was found to stimulate higher levels of TNF-α and lower levels of CXCL10 at 48 hours. The differences observed in these two experiments, particularly the inverse association of TNF-α may be attributed to two things: the change in isolate source to clinical strains and an infection of promastigotes at a rate of 10:1 for a monocyte count of $1x10^5$ rather than $1x10^6$, which represents a lower level of infection. Overexpression of TNF-α has been documented and attributed to a hyperactive Th1 response in MCL responsible for the destructive tissue damage (Gaze et al., 2006; Bacellar et al., 2002; Carvalho et al., 2007), however in studies not necessarily evaluating LRV-1. Overall, an effective immune response requires infection of macrophages in order to elicit proinflammatory biomarkers to circulate and control infection. Parasite load and the concentration of infected macrophages may represent important factors to consider, where lower levels of infection may not be efficient to mount a

robust immune response, however continuous infection in patients reinfected in endemic settings may elicit a more robust T cell reaction (Bosque et al., 2000; Cooper et al., 1994).

Proinflammatory Biomarker Expression of L. V. panamensis

Using 4 clinical isolates of *L. V. panamensis*, 2 positive for LRV-1 and 2 negative for LRV-1, it was found that there was no difference in expression of Th1 biomarkers IL-1β, TNF-α, and CXCL10, as well as Th2 IL-6 at both 24- and 48- hour time points, including SOD. This finding may explain the lack of ML/MCL phenotype observed in Objective 1, where the presence of LRV-1 does not alter immune responses based on the evaluated biomarkers. Rather, the baseline host immune status may have an influence as to the manifestation of clinical disease in patients infected with this species, as *L. V. panamensis* is historically viewed as a less virulent species (Reithinger et al., 2007; Aronson et al., 2016; Hartley et al., 2012; Ginouves et al., 2016).

Overall, our biological data suggests LRV-1's function is dependent on species and host immune status, whereby patients infected with *L. V. panamensis* developed either inflammatory/multifocal CL or LCL, not relate to the presence of LRV-1. However, in our evaluation of LRV-1 in clinical isolates, overexpression of TNF-α in the LRV-1 positive isolate may be responsible for the tissue destruction and damage observed in patients with ML/MCL infected by *L. V. braziliensis.* Overall, LRV-1 may have an important role as a prognosticator and therapeutic marker, given it's potential source as an immunomodulator thereby making it a factor to consider in the drug development pipeline and treatment plan.

Objective 3: Influence of LRV-1 on VF RNA Transcript Expression

In addition to host-parasite interaction, it is important to examine other factors including the role of endogenous molecules known as virulence factors, which assist the parasite in establishing successful infection. The expression of proinflammatory biomarkers analyzed herein

was corroborated by our analysis of VF RNA transcripts in pure *Leishmania* cultures and our macrophage model. As previously reported, VF RNA transcript expression is enhanced by the act of infection, as exhibited by the increases of CPB, MPI, GP63, and HSP100 RNA transcripts from baseline pure culture expression to 24- and 48- hour post-macrophage infection, which are important in promastigote-to-amastigote differentiation, invasion, and modulation of the host immune response. A significant body of literature exists around the effects of HSPs eliciting a Th1 biased response by increasing IL-1, IL-6, TNF-α, and IL-12 (Holakuyee et al., 2012; Drini et al., 2016; Hombach et al., 2014; Bifeld et al., 2015). The overall increase of HSP100 in our isolates following macrophage infection, coupled with the increased TNF-α observed in the examination of clinical specimens of LRV-1 positive *L. V. braziliensis* supports a biological underpinning to the severe phenotype exhibited by the LRV-1 positive strain of *L. V. braziliensis* in our model, and as observed in a number of studies examining the ML/MCL phenotype. Similarly, the role of CPB coupled with GP63 in creating a Th2 response could be demonstrated through increasing IL-4, degrading NF-κβ and IL-12, thereby dampening the curative Th1 response. Although IL-4 and IL-12 could not be examined due to levels of expression below the limits of the assays, future understanding of the relationships between VF RNA transcript and biomarker expression would benefit from examining the more fulsome cytokine and chemokine profile.

VF RNA transcript expression did not differ by species, when comparing *L. V. panamensis* to *L. V. braziliensis* and *L. V. guyanensis* in all observed VFs, however a notable decrease in pooled expression by *L. V. panamensis* was identified. Historically, *L. V. panamensis* has been viewed as less clinical aggressive compared to *L. V. braziliensis*, as observed by the lack of difference between VF RNA transcript expression (Reithinger et al., 2007; Aronson et al., 2016; Hartley et al., 2012; Ginouves et al., 2016). Overall, LRV-1 has shown no direct effect on observed VF

RNA transcripts, regardless of species, however there is a paucity in data surround post-translational effects. Thus, continued efforts musts be made to fully understand how and why the virus co-exists with the parasite, and whether this may be an important factor to consider as a diagnostic or therapeutic marker.

Could LRV-1 be a potential a diagnostic, prognostic and/or therapeutic marker?

There are a number of diagnostic, prognostic and therapeutic gaps that currently exist in ATL. Laboratory diagnostics in ATL have made huge strides, coinciding with the advancements in technology that have allowed for robust methods of detection and speciation, including WGS. More often than not, these diagnostic tools are limited to areas of resourced settings with strong laboratory infrastructure or reference centers, however analysis may be limited due to lack of technical expertise and/or cost. Speciation represents a huge problem with many species falling within a 1 nucleotide difference in selected genes, as well as ill-produced reference sequences, further complicating the matter. Moreover, a knowledge gap exits surrounding the prognosis of patients, whereby management of patients with CL is difficult given the inability to predict relapse or development of ML/MCL, thus duration of follow-up and intensity of treatment often falls short. Drug development pipelines have also been at a standstill for over half a century, with limited drugs and treatment regimens highly toxic in nature. To further exacerbate this situation, there are few trials to evaluate drug efficacy, as complexities arise given the spectrum of clinical illness with CL, species, geography and route of administration; making treatment decisions very difficult, especially in low-resource settings.

To date, patients with a clinical diagnosis of CL require confirmation of *Leishmania* spp. infection, to rule out competing and/or concurrent infections including fungal and or bacterial etiologies of disease (Aronson et al., 2016). As per the IDSA guidelines, treatment regimens are

individualized based on a multitude of factors including but not limited to: infecting *Leishmania* spp., risk of ML/MCL (as per geography if species identification can't be performed), age, childbearing competency, obesity, comorbidities including hepatic, pancreatic, renal or cardiac conditions, sociobehavioural impact of lesions on day to day activities and self-esteem, cost, travel logistics and route of administration (Aronson et al., 2016). Follow-up includes nasal and oropharyngeal examination for up to 1 year, 2 years if there is an increased risk of ML/MCL upon healing of lesion or completion of treatment. As of the publication of the IDSA guidelines in 2016, "The risk factors for the development of ML are poorly understand, as are the factors that affect the progression and anatomic expression of ML over time. Investigational testing for the presence of *Leishmania* RNA virus (a purported virulence factor), is not readily available, nor, to date, has it been found useful for identifying persons who may have or be at risk of ML (Aronson et al., 2016)." Fast forward 4 years later, LRV-1 continues to represent a potential marker that intersects at the current diagnostic, prognostic and therapeutic algorithms, with the possibility of advancing the current approaches in place, given the recent literature surrounding this virus, as well as the work conducted throughout this thesis.

LRV-1 may be an important diagnostic marker in the context of ATL, given it's species-specific modulation of host immune responses, that has the potential to dictate the course of infection and outcome in patients. Throughout this thesis, it was observed that ML/MCL was the most severe phenotype in the *L. V. braziliensis* analysis, while inflammatory CL was the most severe in the *L. V. panamensis* analysis. Furthermore, a large proportion of LRV-1 positive isolates were identified in patients with these severe forms. Although these data reflect studies of smaller samples and a heterogenous populations (travelers and non-travelers), there is potential to improve upon these study designs in the future and determine whether to include LRV-1 in the diagnostic algorithm. While species identification is difficult and complex, LRV-1 confirmation

may be more tangible and achievable, particularly in underresourced settings. Rather than species identification dictating the course and nature of treatment, perhaps a simple confirmation of *Leishmania Viannia* genus and the presence or absence of LRV-1 may suffice to stratify patients into appropriate groups.

Given 10-15% of patients with healed LCL progress to ML/MCL in Latin America, LRV-1 represents a potential prognosticator that may add value to this patient population. In the context of ML/MCL in *L. V. braziliensis* infection, LRV-1 may be of importance when considering the likelihood of developing ML/MCL from LCL. In the context of inflammatory CL in *L. V. panamensis*, the weight of LRV-1 on patient outcome may not be as significant, however with future studies undertaking the extent of LRV-1 in this species, it may be an important factor. Given the current follow-up timelines of 1-2 years, and knowing ML/MCL is often seen decades later, if LRV-1 is continued to be identified in immunocompetent patients who eventually develop severe disease, LRV-1 may be an important prognostic marker to dictate duration and intensity of follow-up, to include a more aggressive nasal and oropharyngeal examination and longer duration of follow-up. As demonstrated by immunological and cell biological data, there are differences attributable to LRV-1 which supports it's role as a potential prognosticator in ATL, in addition to the existing literature primarily around host immune modulation (Atayde et al., 2019; de Carvalho et al., 2019; Ives et al., 2011; Eren et al., 2016; Kariyawasam et al, 2017).

Lastly, LRV-1 can be considered a novel therapeutic target to treat patients infected with the parasite-containing virus and may be a means of shifting course of infection from what may be severe (ML/MCL or inflammatory CL), to limited non-severe disease (LCL). Given the limited regimens and overall uncertainties in efficacy surrounding species, clinical phenotype; and a long list of counterindications, changes to treatment plans can be made and perhaps represent a

new method of disease eradication in this population. With a number of studies evaluating potential antivirals such as hygromycin B and harnessing endogenous pathways such as RNAi to inhibit viral production through short interfering RNAs (siRNAs) and compounds including 2'C-methyladenosine triphosphate, there is huge potential to design specific antivirals to reduce the development of ML/MCL and inflammatory CL in a subset of ATL patients with the virus (Robinson et al., 2018; Kuhlmann et al., 2017; Brettman et al., 2016; Ro et al., 1997).

Summary

With the advent of more sensitive and robust molecular technologies such as whole-genome sequencing, novel viruses are constantly being discovered. "Every day, more than 800 million viruses are deposited per square meter above the planetary boundary layer – that's 25 viruses for each person in Canada," making viruses the most abundant microbe on the plant. The exact role of all these viruses have yet to be identified, however in a number of human, animal and plant organisms, the role of the co-existence of certain viruses is questionable. To date, LRV-1 has been described as an 'endosymbiont,' by definition, 'any organism that lives within the body or cells of another organism most often, though not always, in a mutualistic relationship' (Casem, 2016). Although viruses are not considered living organisms, the analogy of the relationship remains the same. This relationship is thought to benefit the parasite in achieving successful infection of the human host, however this would believe to occur in up to 25% of isolates of the *Viannia* subgenus. Furthermore, the lack of influence of LRV-1 on analyzed VF RNA transcripts further supports the overall low level benefit achieved by maintaining this virus within the parasite. Without the parasite, LRV-1 can not propagate successfully, raising the question of whether the virus exists in the parasite in a mutual or commensal relationship. Our overall analysis of LRV-1 in various populations of ATL identified LRV-1 in 23%-29% of clinical isolates of the *Viannia* subgenus, largely identified in the *L. V.*

braziliensis species. We observed no direct relationship between LRV-1 status and clinical phenotype, however at a biological level, LRV-1 may contribute to differences of proinflammatory biomarker expression. Despite RNAi machinery only identified the *Viannia* subgenus being able to control LRV-1 expression, the virus is only identified in 20-25% of clinical isolates to date. Upon analyzing a select number of VF RNA transcripts, we observed no differences in expression attributable to LRV-1 status, however this does not rule out expression post-transcriptionally or post-translationally. A number of viruses have been identified in other eukaryotic organisms including *Saccharomyces* spp. and *Trichomonas* spp. (Gupta et al., 2007). Given that LRV-1 may not persist when introduced into uninfected strains, the overall fitness of the parasite is brought into question. The virus may benefit a subset of those parasites whose overall fitness is not comparable to hypervirulent strains of the same species, and remains in these parasites in order to benefit the parasite during host infection. Regardless, the exact benefit of the virus in the parasite requires much work and should be considered when developing targeted strategies against parasitic infections overall.

Current treatment for ATL is dictated by clinical presentation and if available, infecting species, and consists of a range of topical, oral, and intravenous pharmacologic options, many of which have substantial toxicities. Clearly, the presence of LRV-1 is not always associated with severe disease, given that some strains are correlated to more severe infection without it. The assumption that certain strains of *Leishmania* are more virulent may be valid, however, our analysis of targeted VF RNA transcripts demonstrated no difference in expression between species known to cause ATL. We can conclude, however, that the host immune response varies depending on infecting strain and in regards to the *L. V. braziliensis* strain in our work, specifically, the presence of LRV-1.

Given the large proportions of treatment failures in patients with ATL (Reithinger et al., 2007; Adaui et al., 2016; Brettman et al., 2016), some of whom will have been infected with LRV-1-positive and potentially more virulent strains of *Leishmania*, both the host immune response and parasite factors remain critical to achieving clinical cure. Targeted therapies based on immunoprofiling of patients as well as viral detection could be an area for future development, however, at this time, such an approach in resource limited settings would be challenging. Future validation of such a concept would be informative to allocation of patients to specific treatment options, aiming for the least toxic medication with greatest likelihood of cure. Moreover, given our finding of LRV-1 in strains of *L. V. panamensis* , which has not been previously reported, it is worth expanding our surveillance efforts for this potential co-factor in disease pathogenesis.

Future Directions

A number of studies have attempted to evaluate the prevalence of LRV-1 in various patient populations, however are confounded by a number of factors or contain very small sample sizes. Ideally, a prospective study of healed uncomplicated LCL patients from an endemic population, such as in the highlands of Peru were ML/MCL is documented in 95% of the country, infected with either *L. V. braziliensis,* would be useful in truly understanding the role of LRV-1. A thorough follow-up including nasal and oropharyngeal examination for up to 5-10 years would be ideal to determine the rate of ML/MCL development and whether LRV-1 may be a contributing factor. As such, with LRV-1 identified at 26.9% a population level and 23.3% in LCL patients and 41% of patients with MCL/ML from Peru infected with *L. V. braziliensis,* with an alpha of 0.05 and power of 80%, a sample size of 216 is required. Regarding *L. V. panamensis,* LRV-1 was identified at an overall rate at 23%, however only 13% of LCL were positive compared to 36% of inflammatory/multifocal CL patients were positive, thus a sample size of 108 is required to further understand the difference in LRV-1 in this species. In addition

the collection of PBMCs at the start and end of the study would be useful for infectivity and VF RNA transcript studies as explained below. Continued collection of PBMCs during follow-up can address questions regarding re-infection and low level persistence of both parasite and virus that may ultimately influence patient outcomes.

Animal models of the mechanistic nature of LRV-1 fail to truly represent that which happens in the human host. Certain mouse models, including C57BL/6, CBA, and C3H/He strains are naturally resistant to *Leishmania* infection, whereas BALB/c mice remain susceptible, and this polarity can heavily influence interpretation of immunologic data (Maspi et al., 2016) Moreover, studies using human macrophages isolated from clinical patients presents a problem where the supportive immune response is lost after translation to the *in vitro* situation. The best way to understand the pathogenesis of ATL, as well as the potential contribution of LRV-1, should rely on a model reflective of human beings, particularly in endemic areas of the world where malnutrition and co-morbidities may affect the way the immune system interacts with the parasite. Future analysis of biomarker expression from PBMCs of patients to create 'immunophenotypes' stratified by patients' baseline immune status, will be necessary to better understand the potential role of LRV-1 in ATL disease. The use of 3-dimensional human skin models such as the EpidermFT could prove useful in understanding how the parasite and LRV-1 might interact at different levels of the skin to contribute to disease pathogenesis. Such work could also further enhance clinical diagnostics. In addition, models enabling more prolonged time point assessments should be evaluated given that clinical presentations are often weeks to months after the initial sandfly bite, and may provide more meaningful insight into the true pathogenesis of disease. As observed in our studies, experimental conditions will have impact on overall results, thus maintaining a steady macrophage concentration to infect with along with eliminating variability from isolate source is key when performing such experiments.

Furthermore, understanding the role of other innate immune pathways, particularly around the role of Type I IFNs and interferon-stimulated genes (ISGs) in antiviral action by blocking viral replication will provide useful.

Analysis of VF RNA transcripts by infecting PBMCs and using RNA sequencing and microarrays could further confirm the whether these transcripts are being translated at unvarying rates and are being upregulated or modulated by specific factors, like LRV-1, in addition to being present in high numbers. This could also provide insight into other genes not previously evaluated but which could potentially serve as therapeutic targets, which is an especially germane line of inquiry given the substantial toxicities associated with recommended first-line antimonials, and alternates including amphotericin.

Conclusions

ATL is a complicated parasitic infection with many factors contributing to the course of disease and treatment outcome. Our data have further suggested a role of LRV-1 as a potential contributor to disease pathogenesis, and certainly, biomarker expression in a human macrophage model of *L. V. braziliensis* infection. Further evaluation of additional immune biomarkers is warranted to fully understand the host-pathogen dynamics in modulation of immune responses. Lastly, the combined analysis of virulence factor RNA transcript has shed light on the overall ability of the parasite to achieve successful macrophage infection independent of LRV-1 status. Our *L. V. braziliensis* and *L. V. guyanensis* strains exhibit increased expression of targeted VF RNA transcripts, rather than *L. V. panamensis,* and this finding require further investigation using other technologies to expand our understanding of VFs in ATL pathogenesis. Our findings highlight the possibility that LRV-1 may contribute to severe disease by altering immune responses in the context of *L. V. braziliensis,* regardless of age, however that severity of disease

increases with age attributable to re-activation, re-infection or immunosuppression following senescence. Further work to disentangle these inter-related concepts in the pathogenesis of ATL is required to place LRV-1 as a diagnostic, prognostic and therapeutic marker.

References

Adaui, V., Lye, L. F., Akopyants, N. S., Zimic, M., Llanos-Cuentas, A., Garcia, L., … Beverley, S. M. (2016). Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human leishmaniasis caused by leishmania braziliensis in Peru and Bolivia. *Journal of Infectious Diseases*, *213*(1), 112–121. https://doi.org/10.1093/infdis/jiv354.

Amato VS, Andrade Jr. HF, Neto VA, Duarte MIS. (2003). Persistence of Tumor Necrosis Factor-α In Situ After Lesion Healing in Mucosal Leishmaniasis. *Am J Trop Med Hyg 68(5): 527-528.*

Ameen M (2010). Cutaneous and mucocutaneous leishmaniasis: emerging therapies and progress in disease management. *Expert Opin. Pharmacother 11(4).*

Aronson, N., Herwaldt, B. L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., … Magill, A. (2017). Diagnosis and treatment of leishmaniasis: Clinical practice guidelines by the infectious diseases society of America (IDSA) and the American Society of tropical medicine and hygiene (ASTMH). *American Journal of Tropical Medicine and Hygiene*, *96*(1), 24–45. https://doi.org/10.4269/ajtmh.16-84256.

Armstrong TC, Keenan MC, Widmer G, Patterson JL. (1993). Successful transient introduction of *Leishmania* RNA virus into a virally infected and uninfected strain of *Leishmania. Proc Natl Acad Sci 90: 1736-1740.*

Atayde VD, Filho ASL, Chaparro V, Zimmerman A, Martel C, Jaramillo M, Olivier M (2019). Exploitation of the *Leishmania* exosomal pathway by *Leishmania* RNA Virus. *Nature Microbiology, 4: 714-723.*

Bacellar O, Faria D, Nascimento M, Cardoso TM, Gollob KJ, Dutra WO, Scott P, Carvalho EM (2009). Interleukin 17 Production among Patients with American Cutaneous Leishmaniasis. *JID 200; 75-78.*

Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, Gollob KJ, Carvlaho EM (2002). Up-Regulation of Th1-Type Responses in Mucosal Leishmaniasis Patients. *Infection and Immunity 70(12) 6734-6740.*

Bailey MS, Lockwood DNJ (2007). Cutaneous leishmaniasis. *Clin Dermatol 25: 203-11.*

Balasegaram, M., Ritmeijer, K., Lima, M. A., Burza, S., Ortiz Genovese, G., Milani, B., … Chappuis, F. (2012). Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opinion on Emerging Drugs*, *17*(4), 493–510. https://doi.org/10.1517/14728214.2012.748036

Bañuls, A. L., Hide, M., & Prugnolle, F. (2007). Leishmania and the Leishmaniases: A Parasite Genetic Update and Advances in Taxonomy, Epidemiology and Pathogenicity in Humans. *Advances in Parasitology*, *64*. https://doi.org/10.1016/S0065-308X(06)64001-3.

Barros N, Vasquez N, Woll F, Sanchez C, Valencia B, Llanos-Cuentas A, White Jr. AC, Montes M. (2018). Regulatory T-Cell Dynamics in Cutaneous and Mucocutaneous Leishmaniasis due to *Leishmania braziliensis. Am J Trop Med Hyg 98(3): 753-758.*

Ben Salah A, Zakroui H, Zaatour A, Ftaiti A, Zaafouri B, Garraoui A, Olliaro PL, Dellagi K, Ben Ismail R (1995). A randomized, placebo-controlled trial in Tunisi treating cutaneous leishmaniasis with paromomycin ointment. *Am J Trop Med Hyg 53(2): 16206.*

Bezerra de Menezes, J. P., Guedes, C. E. S., Petersen, A. L. de O. A., Fraga, D. B. M., & Veras, P. S. T. (2015). Advances in development of new treatment for Leishmaniasis. *BioMed Research International*, *2015*, 1–12. https://doi.org/10.1155/2015/815023

Bifeld, E., & Clos, J. (2015). The genetics of Leishmania virulence. *Medical Microbiology and Immunology*, *204*(6), 619–634. https://doi.org/10.1007/s00430-015-0422-1.

Bifeld E, Chrobak M, Zander D, Schleicher U, Schonian G, Clos J (2015). Geographical sequence variation in the *Leishmania major* virulence factor P46. *Infection, Genetics and Evolution 30: 195-205.*

Blackwell JM (1999). Tumor Necrosis Factor alpha and Mucocutaneous Leishmaniasis. *Parasitology Today 15(2): 73-75.*

Boaventura VS, Santos CS, Cardoso CR, Andrade J, Dos Santos WLC, Clarencio J, Silva JS, Borges VM, Barral-Netto M, Brodskyn CI, Barral A (2010). Human mucosal leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. *Eur J Immunol, 40: 2830-2836.*

Boggild, A. K., Miranda-Verastegui, C., Espinosa, D., Arevalo, J., Adaui, V., Tulliano, G., … Low, D. E. (2007). Evaluation of a microculture method for isolation of Leishmania parasites from cutaneous lesions of patients in Peru. *Journal of Clinical Microbiology*, *45*(11), 3680–3684. https://doi.org/10.1128/JCM.01286-07

Boggild, A. K., Ramos, A. P., Espinosa, D., Valencia, B. M., Veland, N., Miranda-Verastegui, C., … Llanos-Cuentas, A. (2010). Clinical and demographic stratification of test performance: A pooled analysis of five laboratory diagnostic methods for American cutaneous leishmaniasis. *American Journal of Tropical Medicine and Hygiene*, *83*(2), 345–350. https://doi.org/10.4269/ajtmh.2010.09-0414

Boggild, A. K., Ramos, A. P., Valencia, B. M., Veland, N., Calderon, F., Arevalo, J., … Llanos-Cuentas, A. (2011). Diagnostic performance of filter paper lesion impression PCR for secondarily infected ulcers and nonulcerative lesions caused by cutaneous leishmaniasis. *Journal of Clinical Microbiology*, *49*(3), 1097–1100. https://doi.org/10.1128/JCM.02457-10

Boggild, A. K., Valencia, B. M., Espinosa, D., Veland, N., Ramos, A. P., Arevalo, J., … Low, D. E. (2010). Detection and Species Identification of *Leishmania* DNA from Filter Paper Lesion Impressions for Patients with American Cutaneous Leishmaniasis. *Clinical Infectious Diseases*, *50*(1), e1–e6. https://doi.org/10.1086/648730

Boggild, A. K., Valencia, B. M., Veland, N., Ramos, A. P., Calderon, F., Arevalo, J., … Llanos-

Cuentas, A. (2011). Non-invasive cytology brush pcr diagnostic testing in mucosal leishmaniasis: Superior performance to conventional biopsy with histopathology. *PLoS ONE, 6*(10), 2–7. https://doi.org/10.1371/journal.pone.0026395

Borish, L. C., & Steinke, J. W. (2003). 2 . Cytokines and chemokines, *2*(Table I), 460–475. https://doi.org/10.1067/mai.2003.108.

Borges AF, Gomes RS, Ribeiro-Dias F (2017). Leishmania (Viannia) guyanensis in tegumentary leishmaniasis. *Pathogens and Disease, 76, fty025.*

Bosque F, Saravia NG, Valderrama L, Milon G (2000). Distinct Innate and Acquired Immune Responses to *Leishmania* in Putative Susceptible and Resistant Human Populations Endemically Exposed to *L. (Viannia) panamensis* Infection. *Scand J Immunol 51, 533-541.*

Bourreau, E., Ginouves, M., Prévot, G., Hartley, M. A., Gangneux, J. P., Robert-Gangneux, F., … Ronet, C. (2016). Presence of leishmania RNA virus 1 in leishmania guyanensis increases the risk of first-line treatment failure and symptomatic relapse. *Journal of Infectious Diseases*, *213*(1), 105–111. https://doi.org/10.1093/infdis/jiv355.

Bourreau E, Ronet C, Couppie P, Sainte-Marie D, Tacchini-Cottier F, Launois P (2007). IL-10 producing CD8+ T cells in human infection with *Leishmania guyanensis. Microbes and Infection 9, 1034-1041.*

Brettmann, E. A., Shaik, J. S., Zangger, H., Lye, L.-F., Kuhlmann, F. M., Akopyants, N. S., … Beverley, S. M. (2016). Tilting the balance between RNA interference and replication eradicates *Leishmania* RNA virus 1 and mitigates the inflammatory response. *Proceedings of the National Academy of Sciences*, *113*(43), 11998–12005. https://doi.org/10.1073/pnas.1615085113.

Cadd TL, Patterson JL (1994). Synthesis of Viruslike Particles by Expression of the Putative Capsid Protein of *Leishmania* RNA Virus in a Recombinant Baculovirus Expression System. *Journal of Virology, 68(1): 358-365.*

Cantanhede, L. M., da Silva J??nior, C. F., Ito, M. M., Felipin, K. P., Nicolete, R., Salcedo, J. M. V., … Ferreira, R. de G. M. (2015). Further Evidence of an Association between the Presence of Leishmania RNA Virus 1 and the Mucosal Manifestations in Tegumentary Leishmaniasis Patients. *PLoS Neglected Tropical Diseases*, *9*(9), 1–11. https://doi.org/10.1371/journal.pntd.0004079.

Carlsen ED, Jie Z, Liang Y, Henard CA, Hay C, Sun J, Guedes HM, Soong L (2015). Interactions between Neutrophils and Leishmania braziliensis Amastigotes Faciliate Cell Activation and Parasite Clearance. *J Innate Immun, 7(4): 354-63.*

Carneiro FP, Magalhaes AV, Couto MAL, Bocca AL, Muniz-Junqueira MI, Sampaio RNR (2009). Foxp3 expression in lesions of the different clinical forms of American tegumentary leishmaniasis. *Parasite Immunology 31; 646-651.*

Carvalho SH, Frezard F, Pereira NP, Moura AS, Ramos LMQC, Carvalho GB, Rocha MOC (2019). American tegumentary leishaniasis in Brazil: a critical review of the current

therapeutic approach with systemic meglumine antimoniate and short-term possibilities for an alternative treatment. *Tropical Medicine and International Health 24(4): 380-391.*

Carvalho AM, Amorin CF, Barbosa JLS, Lago AS, Carvalho EM (2015). Age Modifies The Immunologic Response and Clinical Presentation of American Tegumentary Leishmaniasis. *Am J Trop Med Hyg 92(6): 1173-1177.*

Casem ML. Cell Systems. Case studies in cell biology-1st Edition, 2016.

Cassagne C, Pratlong F, Jedidi F, Benikhlef R, Aoun K, Normand AC, Faraut F, Bastein P, Piarroux R (2013). Identification of *Leishmania* at the species level with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Microbiol Infect 20; 551-557.*

Carvalho LP, Passos S, Bacellar O, Lessa M, Almeida RP, Magalhaes A, Dutra WP, Gollob KJ, MAchado P, Ribeiro de Jesus A (2007). Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model for pathogenesis. *Parasite Imunol 29(5): 251-258.*

Cincura C, de Lima CMF, Machado PRL, Olivera-F J, Giesby MJ, Lessa MM, Carvalho EM (2017). Mucosal Leishmaniasis: A Retrospective Study of 327 Cases from an Endemic Area of *Leishmania (Viannia) braziliensis. Am J Trop Med Hyg 97(3): 761-766.*

Colineau L, Lambert U, Fornes O, Wasserman WW, Reiner NE (2018). c-Myc is a novel *Leishmania* virulence factor by proxy that targets the host miRNA system and is essential for survival in human macrophages. *J Biol Chem 293(33): 12805-12819.*

Colineau L, Clos J, Moon KM, Foster LJ, Reiner NE (2017). *Leishmania donovani* chaperonin 10 regulates parasite internalization and intracellular survival in human macrophages. *Med Microbiol Immunol 206: 235-257.*

Conceicao-Silva F, Leite-Silva J, Morgado FN (2018). The Binomial Parasite-Host Immunity in the Healing Process and in Reactivation of Human Tegumentary Leishmaniasis. *Frontiers in Microbiology , 9 (1308).*

Cooper AM, Melby PC, Christopher LK, Neva F, Sacks DL (1994). T-Cell Responses to Infected Autologous Monocytes in Patients with Cutaneous and Mucocutaneous Leishmaniasis. *Clinical and Diagnostic Laboratory Immunology, 1; 304-309.*

Coughlan S, Taylor AS, Feane E, Sanders M, Schonian G, Cotton JA, Downing T (2017). *Leishmania naiffi* and *Leishmania guyanensis* reference genomes highlight genome structure and gene evolution in the *Viannia* subgenus. *R Soc Open Sci 5: 172212.*

Coutinho-Abreu, I. V., & Ramalho-Ortigao, M. (2010). Transmission blocking vaccines to control insect-borne diseases - A review. *Memorias Do Instituto Oswaldo Cruz*, *105*(1), 1–12. https://doi.org/10.1590/S0074-02762010000100001

Croft, S. L., Sundar, S., & Fairlamb, A. H. (2006). Drug Resistance in Leishmaniasis Drug Resistance in Leishmaniasis. *Society*, *19*(1), 111–126.

https://doi.org/10.1128/CMR.19.1.111

de Almeida ME, Steurer FJ, Koru O, Herwalkd BL, Pieniazek NJ, da Silva AJ (2011). Identification of *Leishmania* spp. by Molecular Amplification and DNA Sequencing Analysis of a Fragment of rRNA Internal Transcribed Spacer 2. *J Clin Microbiol 49: 3143-3149.*

de Brito RCF, Cardoso JMD, Reis LES, Vieira JF, Mathias FAS, Roatt BM, Aguiar-Soares RDDO, Ruiz JC, Resende DM, Reis AB (2018). Peptide Vaccines for Leishmaniasis. *Frontiers in Immunology 9: 1043.*

de Oliveira Ramos Pereira, L., Maretti-Mira, A. C., Rodrigues, K. M., Lima, R. B., de Oliveira-Neto, M. P., Cupolillo, E., … de Oliveira, M. P. (2013). Severity of tegumentary leishmaniasis is not exclusively associated with Leishmania RNA virus 1 infection in Brazil. *Memorias Do Instituto Oswaldo Cruz*, *108*(5), 665–667. https://doi.org/10.1590/0074-0276108052013021

Diaz NL, Zerpa O, Ponce LV, Convit J, Rondon AJ, Tapia FJ (2002). Intermediate or chronic cutaneous leishmaniasis: leukocyte immunophenotypes and cytokine characterisation of the lesion. *Experimental Dermatology 11, 34-41.*

Dowlati Y (1996). Cutaneous leishmaniasis: clinical aspect. *Clin Dermatol 14: 425-31.*

Drini, S., Criscuolo, A., Lechat, P., Imamura, H., Skalick??, T., Rachidi, N., … Sp??th, G. F. (2016). Species- and Strain-Specific Adaptation of the HSP70 Super Family in Pathogenic Trypanosomatids. *Genome Biology and Evolution*, *8*(6), 1980–1995. https://doi.org/10.1093/gbe/evw140.

Ehrlich A, Castilho TM, Goldsmith-Pestana K, Chae WK, Bothwell ALM, Sparwasser T, McMahon-Pratt D (2019). The Immunotherapeutic Role of Regulatory T Cells in *Leishmania (Viannia) panamensis* Infetion. *J Immunol 193; 2961-2970*

Elmahallawy, E. K., Sampedro Mart??nez, A., Rodriguez-Granger, J., Hoyos-Mallecot, Y., Agil, A., Navarro Mari, J. M., & Gutierrez Fern??ndez, J. (2014). Diagnosis of leishmaniasis. *Journal of Infection in Developing Countries*, *8*(8), 961–972. https://doi.org/10.3855/jidc.4310.

Ennes-Vidal V, Vitorio BS, Menna-Barreto FS, Pitaluga AN, Goncalves da Silva SA, Branquinha MH, Santos ALS, Avila-Levy CM (2019). Calpains of *Leishmania braziliensis:* genome analysis, differential expression and functional analysis. *Mem Inst Oswaldo Cruz 114: e1090147.*

Eren RO, Reverte M, Rossi M, Hartley MA, Castiglioni P, Prevel F, Martin R, Desponds C, Lye LF, Drexler SK, Reith W, Beverley SM, Ronet C, Fasel N (2016). Mammalian Innate Immune Response to a *Leishmania*-Resident RNA Virus Increases Macrophage Survival to Promote Parasite Persistence. *Cell Host Microbe, 20(3): 318-328.*

Fagundes-Silva GA, Romero GAS, Cupolillo E, Yamashita EPG, Gomes-Silva A, Guerra JAO, Da-Cruz AM (2015). *Leishmania (Viannia) naiffi:* rare enough to be neglected? *Mem Inst*

Oswaldo Cruz 110(6): 797-800.

Galvao EL, Rabello A, Cota GF (2017). Efficacy of azole therapy for tegumentary leishmaniasis: A Systematic review and meta-analysis. *PLoS ONE 12(10): e0186117.*

Gaze ST, Dutra WO, Lessa M, Lessa H, Guimaraes LH, de Jesus AR, Carvalho LP, Machado P, Carvalho EM, Gollob KJ (2006). Mucosal Leishmaniasis Patients Display An Activated Inflammatory T-cell Phenotype Associated with a Nonbalanced Monocyte Population. *Scandinavian Journal of Immunology 63, 70-78.*

Gibson, M. E. (1983). The identification of kala-azar and the discovery of Leishmania Donovani. *Medical History*, *27*(2), 203–213. https://doi.org/10.1017/S0025727300042691

Ginouvès, M., Simon, S., Bourreau, E., Lacoste, V., Ronet, C., Couppié, P., … Prévot, G. (2016). Prevalence and distribution of leishmania RNA virus 1 in leishmania parasites from French Guiana. *American Journal of Tropical Medicine and Hygiene*, *94*(1), 102–106. https://doi.org/10.4269/ajtmh.15-0419.

Giudice A, Camada I, Leopolda PTG, Pereira JMB, Riley LW, Wilson ME, Ho JL, Riberio de Jesus A, Carvalho EM, Almeida RP. (2007). Resistance of *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis* to nitric oxide correlates with disease severity in Tegumentary Leishmaniasis. *BMC Infectious Diseases 7:7; 1-12.*

Gomes R, Oliveira F (2012). The immune response to sand fly salivary proteins and its influence on *Leishmania* immunity. *Frontiers in Immunology* 3 (110): 1-8.

Grybchuk D, Kostygov AY, Macedo DH, d-Avila-Levy CM, Yurchenko V (2018). RNA viruses in trypanosomatic parasites: a historical overview. *Mem Inst Oswaldo Cruz, 113(4): e170487.*

Guerra JAO, Maciel MG, Guerra MVF, Talhari AC, Prestes SR, Fernandes MA, Da-Cruz AM, Martins A, Coelho LIARC, Romero GAS, Barbosa MGV (2015). Tegumentary leishmaniasis in the State of Amazonas: what have we learned and what do we need? *Rev Soc Braz Med Trop 48(S1): 12-19.*

Guilbride L, Myler PF, Stuart K (1992). Distribution and sequence divergence of LRV1 viruses among different *Leishmania* species. *Molecular and Biochemical Parasitology 54; 101-104.*

Guimaraes, L. H., Queiroz, A., Silva, J. A., Silva, S. C., Magalh??es, V., Lago, E. L., … Schriefer, A. (2016). Atypical Manifestations of Cutaneous Leishmaniasis in a Region Endemic for Leishmania braziliensis: Clinical, Immunological and Parasitological Aspects. *PLoS Neglected Tropical Diseases*, *10*(12), 1–14. https://doi.org/10.1371/journal.pntd.0005100.

Hajjaran H, Mahdi M, Mohebali M, Samimi-Rad K, Ataei-Pirkooh A, Kazemi-Rad E, Naddaf SR, Raoofian R. Detection and molecular identification of leishmania RNA virus (LRV) in Iranian *Leishmania* species. *Arch Virol 161: 3385-3390.*

Harkins, K. M., Schwartz, R. S., Cartwright, R. A., & Stone, A. C. (2016). Phylogenomic

reconstruction supports supercontinent origins for Leishmania. *Infection, Genetics and Evolution*, *38*(December 2015), 101–109. https://doi.org/10.1016/j.meegid.2015.11.030

Hartley, M.-A., Ronet, C., Zangger, H., Beverley, S. M., & Fasel, N. (2012). Leishmania RNA virus: when the host pays the toll. *Frontiers in Cellular and Infection Microbiology*, *2*(July), 1–15. https://doi.org/10.3389/fcimb.2012.00099

Hartley, M. A., Kohl, K., Ronet, C., & Fasel, N. (2013). The therapeutic potential of immune cross-talk in leishmaniasis. *Clinical Microbiology and Infection*, *19*(2), 119–130. https://doi.org/10.1111/1469-0691.12095.

Herwaldt BL (1999). Leishmaniasis. *The Lancet 354: 1191-1199.*

Herwaldt BL, Arana BAQ, Navin TR (1992). The natural history of cutaneous leishmaniasis in Guatemala. *J Infect Dis 165: 518-27.*

Holakuyee, M., Mandavi, M., Hassan, Z. M., & Abolhassani, M. (2012). Heat shock proteins enriched-promastigotes of leishmania major inducing Th2 immune response in BALB/c Mice. *Iranian Biomedical Journal*, *16*(4), 209–217. https://doi.org/10.6091/ibj.1098.2012

Hombach, A., Ommen, G., MacDonald, A., & Clos, J. (2014). A small heat shock protein is essential for thermotolerance and intracellular survival of Leishmania donovani. *Journal of Cell Science*, *127*(21), 4762–4773. https://doi.org/10.1242/jcs.157297.

Isnard A, Shio MT, Olivier M (2012). Impact of *Leishmania* metalloproteasae GP63 on macrophage signaling. *Frontiers in Cellular and Infection Microbiology 2: 72.*

Ito, M. M., Catanhêde, L. M., Katsuragawa, T. H., da Silva Junior, C. F., Camargo, L. M. A., Mattos, R. de G., & Vilallobos-Salcedo, J. M. (2015). Correlation between presence of Leishmania RNA virus 1 and clinical characteristics of nasal mucosal leishmaniosis. *Brazilian Journal of Otorhinolaryngology*, *81*(5), 533–540. https://doi.org/10.1016/j.bjorl.2015.07.014

Ives, A., Ronet, C., Prevel, F., Ruzzante, G., Fuertes-, S., Schutz, F., … Masina, S. (2012). NIH Public Access, *331*(6018), 775–778. https://doi.org/10.1126/science.1199326.Leishmania

Jara, M., Valencia, B. M., Adaui, V., Alba, M., Lau, R., Arevalo, J., … Boggild, A. K. (2016). Quantitative kinetoplast DNA assessment during treatment of mucosal leishmaniasis as a potential biomarker of outcome: A pilot study. *American Journal of Tropical Medicine and Hygiene*, *94*(1), 107–113. https://doi.org/10.4269/ajtmh.15-0514.

Jeziorski E, Dereure J, Bullen GM, Blanchet C, Ludwig C, Costes V, Rodiere M. (2015). Mucosal relapse of visceral leishmaniasis in achild treated with anti-TNFα. *International Journal of Infectious Diseases 33, 135-136.*

Kerr, S. F. (2000). Palaearctic Origin of Leishmania. *Memorias Do Instituto Oswaldo Cruz*, *95*(1–2), 75–80. https://doi.org/10.1590/S0074-02762000000100011

Khamesipour, A., Dowlati, Y., Asilian, A., Hashemi-Fesharki, R., Javadi, A., Noazin, S., &

Modabber, F. (2005). Leishmanization: Use of an old method for evaluation of candidate vaccines against leishmaniasis. *Vaccine*, *23*(28), 3642–3648. https://doi.org/10.1016/j.vaccine.2005.02.015.

Kleschenko Y, Grybchuk D, Matveeva NS, Macedo DH, Ponirovsky EN, Lukashev AN, Yurchenko V. (2019). Molecular Characterization of *Leishmania RNA virus* 2 in *Leishmania major* from Uzbekistan. *Genes 10, 830.*

Krobitsch, S., Brandau, S., Hoyer, C., Schmetz, C., Hu, A., & Clos, J. (1998). Leishmania donovani Heat Shock Protein 100 We report the cloning and molecular analysis of the, *273*(11), 6488–6494.

Laskay, T., Van Zandbergen, G., & Solbach, W. (2003). Neutrophil granulocytes - Trojan horses for Leishmania major and other intracellular microbes? *Trends in Microbiology*, *11*(5), 210–214. https://doi.org/10.1016/S0966-842X(03)00075-1.

Lee SE, Suh JM, Scheffter S, Patterson JL, Chung K (1996). Identification of a Ribosomal Frameshift in *Leishmania* RNA Virus 1-4. *J. Biochem, 120, 22-25.*

Lessa MM, Lessa HA, Castro TWN, Oliveira A, Scherifer A, Machado P, Carvlaho EM (2007). Mucosal leishmaniasis: epidemiological and clinical aspects. *Rev Bras Ottorinolaringol 73(6): 843-847.*

Lye LF, Owens K, Shi H, Murta SMF, Vieira AC, Turco SJ, Tschudi C, Ullu E, Beverley SM. (2010). Retention and Loss of RNA Interference Pathways in Trypanosomatid Protozoans. *PLoS Pathogens*, 6(10): e1001161.

Macbeth KJ, Patterson JL (1998). Overview of the *Leishmaniavirus* Endoribonuclease and Functions of Other Endoribonucleases Affecting Viral Gene Expression. *The Journal of Experimental Zoology, 282: 254-260.*

Macedo, D. H., Menezes-Neto, A., Rugani, J. M., Rocha, A. C., Silva, S. O., Melo, M. N., … Soares, R. P. (2016). Low frequency of LRV1 in Leishmania braziliensis strains isolated from typical and atypical lesions in the State of Minas Gerais, Brazil. *Molecular and Biochemical Parasitology*, *210*(1–2), 50–54. https://doi.org/10.1016/j.molbiopara.2016.08.005.

Madeira da Silva L, Owens KL, Murta SMF, Beverley SM (2009). Regulated expression of the *Leishmania major* surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins. *PNAS 106(18): 7583-7588.*

Maretti-Mira AC, Rodrigues KMP, Oliveira-Neto MP, Pirmez C, Craft N. (2011). MMP-9 activity is induced by *Leishmania braziliensis* infection and corrleates with mucosal leishmaniasis. *Acta Tropica 119; 160-164.*

Marshall S, Kelly PH, Singh BK, Pope RM, Kim P, Zhanbolat B, Wilson ME, Yao C (2018). Extracellular release of virulence factor major surface protease via exosomes in *Leishmania infantum* promastigotes. *Parasites & Vectors 11: 355.*

Martinez DY, Verdonck K, Kaye PM. (2018). Tegumentary leishmaniasis and coinfections other than HIV. *PLoS NTDs 12: e0006125.*

Maspi, N., Abdoli, A., & Ghaffarifar, F. (2016). Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review. *Pathogens and Global Health, 110*(6), 247–260. https://doi.org/10.1080/20477724.2016.1232042.

McCall LI, Matlashewski G (2012). Involvement of the *Leishmania donovani* virulence factor A2 in protection against heat and oxidative stress. *Experimental Parasitology 132: 109-115.*

McCall LI, Matlashewski G (2010). Localization and induction of the A2 virulence factor in *Leishmania:* evidence that A2 is a stress response protein. *Molecular Microbiology 77(2): 518-530.*

Miranda-Verastegui C, Llanos-Cuentas A, Arevalo I, Ward BJ, Matlashewski G (2005). Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antimoniate in the treatment of cutaneous leishmaniasis in Peru. *Clin Infect Dis 40 (10): 1395-403.*

Miranda-Verastegui C, Tulliano G, Gyorkos TW, Calderon W, Rahme E, Ward B, Cruz M, Llanos-Cuentas A, Matlashewski G (2009). First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiqumod in combination with pentavalent antimony. *PLoS Negl Trop Dis 3(7);e491.*

Moen, M. D., Lyseng-Williamson, K. A., & Scott, L. J. (2009). Liposomal amphotericin B: A review of its use as empirical therapy in febrile neutropenia and in the treatment of invasive fungal infections. *Drugs*, *69*(3), 361–392. https://doi.org/10.2165/00003495-200969030-00010

Momen, H., & Cupolillo, E. (2000). Speculations on the Origin and Evolution of the Genus Leishmania. *Memorias Do Instituto Oswaldo Cruz*, *95*(4), 583–588. https://doi.org/10.1590/S0074-02762000000400023.

Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G (2012). Three new sensitive and specific heat-shock protein 70 PCRs for glocal Leishmania species identification. *J Clin Microbiol Infect Dis 31; 1453-1461.*

Moreira RB, Pirmez C, de Oliveira-Neto MP, Aguiar LS, Goncalves AJS, Pereira LOR, Abreu L, De Oliveira MP (2017). AIM2 inflammasome is associated with disease severity in tegumentary leishmaniasis caused by *Leishmania (V.) braziliensis. Parasite Immunology 39; 312435.*

Morizot G, Kendjo E, Mouri O, Thellier M, Perignon A, Foulet F, Cordoliani F, Bourrat E, Laffitte E, Alcaraz I, Bodak N, Ravel C, Vray M, Grogl M, Mazier D, Caumes E, Lachaud L, Buffet PA, Cutaneous Leishmaniasis French Study Group (2013). Travelers with cutaneous leishmaniasis is cured without systemic therpy. *Clin Infect Dis 57(3): 370-80.*

Mottram, J. C., Coombs, G. H., & Alexander, J. (2004). Cysteine peptidases as virulence factors of Leishmania. *Current Opinion in Microbiology*, *7*(4), 375–381.

https://doi.org/10.1016/j.mib.2004.06.010

Mysuria S. *Leishmania* RNA Virus: Mere Endosymbiont or Major Player in *Leishmania*-Associated Disease? JEMI-PEARLS 2018; 3: 61-68.

Nadim, A., Javadian, E., & Mohebali, M. (1997). The experience of leishmanization in the Islamic Republic of Iran. *Eastern Mediterranean Health Journal*.

Nalcaci M, Karakus M, Yilmaz B, Demir S, Ozbilgin A, Ozbel T, Toz S. (2019). Detection of *Leishmania* RNA virus 2 in *Leihmania* species from Turkey. *Trans R Soc Trop Med Hyg 113: 410-417.*

Nicodemo AC, Amato VS, Miranda AM, Floeter-Winter MF, Zampieri RA, Fernades ER, Duarte MIS (2012). Are the severe injuries of cutaneous leishmaniasis caused by an exacerbted Th1 response? *Parasite Immunology 34: 440-443.*

Nogueira MF, Goto H, Sotto MN, Cuce LC. (2008). Cytokine Profile in Montenegro Skin Test of Patients with Localized Cutaneous and Mucocutaneous Leishmaniasis. *Rev Inst Med Trop S. Paulo 50(6): 333-337.*

Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, Andrade CA (2011). Systematic review of the addverse of cutaneous leishmaniasis treatment in the New World. *Act ropica 118: 87-96.*

Olivier, M. (2011). Host-pathogen interaction: Culprit within a culprit. *Nature*, *471*(7337), 173–174. https://doi.org/10.1038/471173a.

Olivier, M., Atayde, V. D., Isnard, A., Hassani, K., & Shio, M. T. (2012). Leishmania virulence factors: Focus on the metalloprotease GP63. *Microbes and Infection*, *14*(15), 1377–1389. https://doi.org/10.1016/j.micinf.2012.05.014

Patterson, J. L. (2017). Role of LRV1 and RNAi in the Pathogenesis of Leishmania. *Trends in Parasitology*, *33*(2), 76–78. https://doi.org/10.1016/j.pt.2016.11.012

Poinar, G Jr., Poinar, R. (2004). Paleoleishmania proterus n. gen., n. sp., (Trypanosomatidae: Kinetoplastida) from Cretaceous Burmese Amber. *Protist*, *155*(September), 305–310. https://doi.org/S1434-4610(05)70187-5 [pii]\r10.1078/1434461041844259

Ponte-Sucre, A., Díaz, E., & Padrón-Nieves, M. (2013). Drug resistance in leishmania parasites: Consequences, molecular mechanisms and possible treatments. *Drug Resistance in Leishmania Parasites: Consequences, Molecular Mechanisms and Possible Treatments*, 1–459. https://doi.org/10.1007/978-3-7091-1125-3

Reithinger, R., & Dujardin, J. C. (2007). Molecular diagnosis of leishmaniasis: Current status and future applications. *Journal of Clinical Microbiology*, *45*(1), 21–25. https://doi.org/10.1128/JCM.02029-06

Reithinger, R., Dujardin, J., Louzir, H., Pirmez, C., Alexander, B., Brooker, S., … Lockwood, D. N. J. (2007). Cutaneous leishmaniasis. *Clinics in Dermatology*, *25*(2), 203–11.

https://doi.org/10.1016/j.clindermatol.2006.05.008

Rogers, M. E., Chance, M. L., & Bates, P. A. (2002). The role of promastigote secretory gel in the origin and transmission of the infective stage of Leishmania mexicana by the sandfly Lutzomyia longipalpis. *Parasitology, 124*(5), 495–507. https://doi.org/10.1017/S0031182002001439.

Ro YT, Scheffter SM, Patterson JL (1997). Hygromycin B Resistance Mediates Elimination of *Leishmania* Virus from Persistently Infected Parasites. *Journal of Virology, 71(12); 8991-8998.*

Roberts MTM (2006). Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *British Medical Bulletin, 75 and 76: 115-130.*

Rossi M, Castiglioni P, Hartley MA, Eren RO, Prevel F, Desponds C, Utzschneider DT, Zehn D, Cusi MG, Kuhlmann M, Beverley SM, Ronet C, Fasel N (2017). Type I interferons induced by endogenous or exogenous viral infections promote metastasis and relapse of leishmaniasis. *PNAS, 114(19): 4987-4992.*

Ruiz JH, Becker I (2007). CD8 cytotoxic T cells in cutaneous leishmaniasis. *Parasite Immunology 29; 671-678.*

Saberi R, Fakhar M, Mohebali M, Anvari D, Gholami S (2019). Global status of synchronizing Leishmania RNA virus in Leishmania parasites: A systematic review with meta-analysis. *Transbound Emerg Dis; 001:1-8.*

Saiz M, Llanos-Cuentasa A, Echevarria J, Roncal N, Cruz M, Tupayachi Muniz M, Lucas C, Wirth DF, Scheffter S, Magill AJ, Patterson JL. Short Report: Detection of *Leishmaniavirus* in Human Biopsy Samples of Leishmaniasis from Peru. *Am J Trop Med Hyg 58(2): 192-194.*

Salinas, G., Zamora, M., Stuart, K., & Saravia, N. (1996). Leishmania Viruses in Leishmanja Subgenus of the Viannia, *54*(4), 425–429.

Savoia, D. (2015). Recent updates and perspectives on leishmaniasis. *Journal of Infection in Developing Countries*, *9*(6), 588–596. https://doi.org/10.3855/jidc.6833

Scheffter, S. M., Ro, Y. T., Chung, I. K., & Patterson, J. L. (1995). The complete sequence of Leishmania RNA virus LRV2-1, a virus of an Old World parasite strain. *Virology*. https://doi.org/10.1006/viro.1995.1456

Shaw, J., Pratlong, F., Floeter-Winter, L., Ishikawa, E., Baidouri, F. El, Ravel, C., & Dedet, J. P. (2015). Characterization of leishmania (leishmania) waltoni n.sp. (kinetoplastida: Trypanosomatidae), the parasite responsible for diffuse cutaneous leishmaniasis in the Dominican Republic. *American Journal of Tropical Medicine and Hygiene*, *93*(3), 552–558. https://doi.org/10.4269/ajtmh.14-0774.

Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HDFH, Presber W, Jaffe CL (2003). PCR diagnosis and characterization of Leishmania in local and imported clinical

samples. *Diagn Microbiol Infect Dis 47(1): 349-358.*

Shio MT, Christian JG, Jung JY, Chang KP, Olivier M (2015). PKC/ROS-Mediated NLRP3 Inflammasome Activation Is Attenuated by *Leishmania* Zinc-Metalloprotease during Infection. *PLoS Negl Trop Dis 9(6): e0003868.*

Silva-Almeida, M., Pereira, B. A., Ribeiro-Guimarães, M., & Alves, C. (2012). Proteinases as virulence factors in Leishmania spp. infection in mammals. *Parasites & Vectors*, *5*(1), 160. https://doi.org/10.1186/1756-3305-5-160

Singh, N., Kumar, M., & Singh, R. K. (2012). Leishmaniasis: Current status of available drugs and new potential drug targets. *Asian Pacific Journal of Tropical Medicine*, *5*(6), 485–497. https://doi.org/10.1016/S1995-7645(12)60084-4

Solomon, M., Pavlotzky, F., Barzilai, A., & Schwartz, E. (2013). Liposomal amphotericin B in comparison to sodium stibogluconate for Leishmania braziliensis cutaneous leishmaniasis in travelers. *Journal of the American Academy of Dermatology*, *68*(2), 284–289. https://doi.org/10.1016/j.jaad.2012.06.014.

Soulat D, Bogdan C (2017). Function of Macrophages and Parasite Phosphatases in Leishmaniasis. *Front Immunol 8: 1838.*

Sousa, A. Q., Frutuoso, M. S., Moraes, E. A., Pearson, R. D., & Pompeu, M. M. L. (2011). High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to leishmania (vianna) Braziliensis. *Clinical Infectious Diseases*, *53*(7), 693–695. https://doi.org/10.1093/cid/cir496.

Souza MA, Almeida TM, Casstro MCAB, Oliveira-Mendes AP, Almeida AF, Oliveira BC, Rocha LF, Medeiros ACR, Brito MEF, Dessein AJJ, Pereira VRA (2016). American tegumentary leishmaniasis: mRNA expression for Th1 and Treg mediators are predominant in patients with recent active disease. *Immunobiology 221: 253-259.*

Spath GF, Epstein L, Leader B, Singer SM, Avila HA, Turco SJ, Beverley SM (2000). Lipophosphoglycan is a virulence fctor distrinct from related glycoconjugates in the protozoan parasite *Leishmania major. PNAS 97 (16): 9258-9263.*

Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B (2013). *Leishmania* expressed lipophosphoglycan interact with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. *Clinical and Experimental Immunology 172: 403-409.*

Steinke, J. W., & Borish, L. (2006). 3. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology*, *117*(2 SUPPL. 2), 441–445. https://doi.org/10.1016/j.jaci.2005.07.001

Steverding, D. (2017). The history of leishmaniasis. *Parasites & Vectors*, *10*(1), 82. https://doi.org/10.1186/s13071-017-2028-5

Suárez, M., Valencia, B. M., Jara, M., Alba, M., Boggild, A. K., Dujardin, J. C., … Adaui, V.

(2015). Quantification of leishmania (Viannia) kinetoplast DNA in ulcers of cutaneous leishmaniasis reveals inter-site and intersampling variability in parasite load. *PLoS Neglected Tropical Diseases*, *9*(7), 1–14. https://doi.org/10.1371/journal.pntd.0003936.

Sukla S, Roy S, Sundar S, Biswas S. (2017). Leptomonas seymouri narna-like virus 1 and not leishmaniaviruses detected in kala-azar samples from India. *Arch Virol 162: 3827-3835.*

Sundar, S., & Olliaro, P. L. (2007). Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. *Therapeutics and Clinical Risk Management*, *3*(5), 733–740.

Tarr, P. I., Aline, R. F., Smiley, B. L., Scholler, J., Keithly, J., & Stuart, K. (1988). LR1: a candidate RNA virus of Leishmania. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(24), 9572–5. https://doi.org/10.1073/pnas.85.24.9572.

Teles GC, Fonseca FR, Jacirema M, Goncalves F (2019). American Tegumentary Leishmaniasis in the Brazilian Amazon from 2010 to 2014. *Rev Inst Med Trop Sao Paulo 61: e22.*

Thomazelli APFS, Tomiotto-Pellissier F, Miranda-Sapla M, Santos da Silva S, Alvarenga DS, Panis C, Cataneo AHD, Bordignon J, Silveira GF, Yamauchi LC, Rubro de Sa JPS, Pavanelli WR, Conchon-Costa I (2018). Concanavalin-A displays leishmanicidal activity by inducing ROS production in human peripheral blood mononuclear cells. *Immunopharmacology and Immunotoxicology 40(5): 387-392.*

Tripathi, P., Singh, V., & Naik, S. (2007). Immune response to leishmania: Paradox rather than paradigm. *FEMS Immunology and Medical Microbiology*, *51*(2), 229–242. https://doi.org/10.1111/j.1574-695X.2007.00311.x

Tsokana, C. N., & Athanasiou, L. V. (n.d.). World â€^{TM} s largest Science , Technology & Medicine Open Access book publisher Molecular Diagnosis of Leishmaniasis , Species Identification and Phylogenetic Analysis.

Tuon FF, Fernandes ER, Duarte MIS, Amato VS (2012). Expression of TLR2 and TLR4 in Lesions Of Patients With Tegumentary American Leishmaniasis. *Rev Inst Med Trop Sau Paulo 54(3): 159-163.*

U.S Food & Drug. (2014). Impavaido. Center for Drug Evaluation and Research. https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/204684s000lbl.pdf

Valencia, B., Jara, M., Adaui, V., Chantry, M., Alba, M., Ramos, A., … Boggild, A. K. (2014). Controversial role of leishmania RNA virus as a determinant of pathogenicity in human leishmaniasis. *International Journal of Infectious Diseases*, *21*, 165–166. https://doi.org/10.1016/j.ijid.2014.03.767

Valencia, B. M., Miller, D., Witzig, R. S., Boggild, A. K., & Llanos-Cuentas, A. (2013). Novel Low-Cost Thermotherapy for Cutaneous Leishmaniasis in Peru. *PLoS Neglected Tropical Diseases*, *7*(5), 1–9. https://doi.org/10.1371/journal.pntd.0002196

Valencia, B. M., Veland, N., Alba, M., Adaui, V., Arevalo, J., Low, D. E., … Boggild, A. K.

(2012). Non-Invasive Cytology Brush PCR for the Diagnosis and Causative Species Identification of American Cutaneous Leishmaniasis in Peru. *PLoS ONE*, *7*(11), 3–8. https://doi.org/10.1371/journal.pone.0049738

Van der Auwera, G., Bart, A., Chicharro, C., Cortes, S., Davidsson, L., Di Muccio, T., … Chiodini, P. L. (2016). Comparison of Leishmania typing results obtained from 16 European clinical laboratories in 2014. *Eurosurveillance*, *21*(49), 1–11. https://doi.org/10.2807/1560-7917.ES.2016.21.49.30418.

Vargas-Inchaustegui DA, Hogg AE, Tulliano G, Llanos-Cuentas A, Arevalo J, Endsley JJ, Soong L. (2010). CXCL10 Production by Human Monocytes in Response to *Leishmania braziliensis* Infection. *Infection and Immunity, 78(1); 301-308.*

Vieira-Goncalves R, Fagundes-Silva GA, Heringer JF, Fantinatti M, Da-Cruz AM, Oliveira-Neto MP, Guerra JAO, Gomes-Silva A (2018). First report of treatment failure in a patient with cutaneous leishmaniasis infected by *Leishmania (Viannia) naiffi* carrying *Leishmania RNA virus:* a fortuitous combination? *Journal of the Brazilian Society of Tropical Medicine 52: e20180323.*

Weinkopff T, MAriotto A, Simon G, Torre YHL, Auderset F, Schuster S, Zangger H, Fasel N, Barral A, Tacchini-Cottier F (2013). Role of Toll-Like Receptor 9 Signaling in Experimental *LEishmania braziliensis* Infection. *Infection and Immunity, 81(5): 1575-1584.*

Widmer G, Dooley S (1995). Phylogenetic analysis of *Leishmania* RNA virus and *Leishmania* suggests ancient virus-parasite association (1995). *Nucleic Acids Research 23(12): 2300-2304.*

World Health Organization. (2010). Control of the leishmaniases. *World Health Organization Technical Report Series*, (949), 22–26. https://doi.org/10.1038/nrmicro1766.

Zamora M, Sacks GL (2000). Phylogenetic analysis of the 5' subterminal region of isolates of *Leishmania* RNA Virus-1. *Annals of Tropical Medicine & Parasitology, 94(2): 123-133.*

Zangger, H., Hailu, A., Desponds, C., Lye, L. F., Akopyants, N. S., Dobson, D. E., … Fasel, N. (2014). Leishmania aethiopica Field Isolates Bearing an Endosymbiontic dsRNA Virus Induce Pro-inflammatory Cytokine Response. *PLoS Neglected Tropical Diseases*, *8*(4), 1–10. https://doi.org/10.1371/journal.pntd.0002836.

Zhang WW, Miranda-Verastegui C, Arevalo J, Ndao M, Ward M, Llanos-Cuentas A, Matlashewski G. (2006). Development of a Genetic Assay to Distinguish between Leishmania Viannia Species on the Basis of Isoenzyme Differences. *Clin Infect Dis 42; 801-809.*

Zijlstra, E. E., Musa, A. M., Khalil, E. A. G., El Hassan, I. M., & El-Hassan, A. M. (2003). Post-kala-azar dermal leishmaniasis. *Lancet Infectious Diseases*, *3*(2), 87–98. https://doi.org/10.1016/S1473-3099(03)00517-6