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LEISHMANIA: METABOLOMICS, BIOCHEMICAL AND CLINICAL STUDIES

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Dedication

Lovingly to my wife Lamia, my sons; Ahmed and Yamin, and my parents who love, respect, support and appreciate me…

Shawgi

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Abbreviations

DNA	Deoxyribonucleic Acid
kDNA	Kinetoplast DNA
CL	Cutaneous Leishmaniasis
MCL	Mucocutaneous Leishmaniasis
VL	Visceral Leishmaniasis
PKDL	Post Kala-azar Dermal Leishmaniasis
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
DCL	Diffuse Cutaneous Leishmaniasis
IL	Interleukin
APC	Antigen Presenting Cell
DC	Dendritic Cell
CD	Cluster of Differentiation
Th	Helper T Lymphocyte
IFN-γ	Gamma Interferon
TNF-α	Tumor Necrosis Factor-alpha
NK	Natural Killer Cells
iNOS	Inducible Nitric Oxide Synthase
NO	Nitric Oxide
NADPH	Nicotinamide Adenine Dinucleotide Reduced
ROS	Reactive Oxygen Speceis
RNS	Reactive Nitrogen Speceis
MHC	Major Histocompatibility Complex
RPMI	Rosewell Park Memorial Institute
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
6-AN	6-aminonicotinamide
PPP	Pentose Phosphate Pathway
HILIC-MS	Hydrophobic interaction liquid chromatography-Mass Spectrometry
NAD	Nicotinamide Adenine Dinucleotide
PRPP	5-phospho-α-D-ribose 1-diphosphate

Na	Nicotinate
NaAD	Nicotinate Adenine Dinucleotide
G6P	Glucose 6-phosphate
R5P	Ribose 5-phosphate
6PGDH	6-phosphogluconate Dehydrogenase
$T(SH)_2$	Reduced Glutathione
NAm	Nicotinamide
6-ANAD	6-aminonicotinamide Adenine Dinucleotide
6-ANADP	6-aminonicotinamide Adenine Dinucleotide Phosphate
PGI	Phosphoglucose Isomerase
ATP	Adenine Trinucleotide Phosphate
ADP	Adenine Dinucleotide Phosphate
MB	Methylene Blue
MG	Malachite Green
AmB	Amphotericin B
HIFCS	Heat Inactivated Foetal Calf Serum
IC_{50}	Inhibitory Concentration 50
FIC	Fraction Inhibitory Concentration
NaMN	Nicotinic Acid Mononucleotide
UTP	Uridine Triphosphate
UDP	Uridine Diphosphate
UMP	Uridine Monophosphate
IMP	Inosine Monophosphate
XMP	Xanthine Monophosphate
OMP	Ortidine Monophosphate
FITC	Fluorescein Isothiocyanate
FC	Fluorescein Cadaverine
PVDF	Polyvinylidene Difluoride
RA	Rheumatoid Arthritis
AS	Ankylosing Spondylitis
PsA	Psoriatic Arthritis
PBMC	Peripheral Blood Mononuclear Cell

HLA	Human Leukocyte Antigen
RNA	Ribonucleic Acid
ssu rRNA	Small Subunit Ribosomal RNA
ACPA	Anticitrullinated Protein Antibody
CCP	Cyclic Citrullinated Peptide
RF	Rheumatoid Factor
PAD	Peptidyarginine Deiminase
Treg	Regulatory T Lymphocyte
TLR	Toll-Like Receptor
TCR	T cell Receptor
BD	Biological Drug

Abstract

Background

Leishmaniases is a group of diseases with clinical presentations ranging from self-healing cutaneous (CL) to destructive mucocutaneous (MCL), and visceral (VL) forms. VL represents a major health problem worldwide and the antileishmanial drugs used for its treatment have toxic side effects and the parasites have developed resistance to them therefore, new compounds and drug targets are sought. In this thesis I have probed *Leishmania* and leishmaniasis in biochemical and clinical studies. For the biochemical part, in one study I analysed the effect of the niacin analogue, 6-AN on *Leishmania* parasite growth and metabolism using the metabolomics technology. The rational was that, the pentose phosphate pathway (PPP), the major provider of antioxidants, has been reported as a target of 6-AN and *Leishmania* PPP enzymes have significant differences compared to those of the mammals, thus PPP might represent a good target. The other biochemical study was conducted to detect and characterize the *Leishmania* transglutaminase (TGase) enzymes that catalyze the irreversible protein crosslinking by creating chemical- and proteolytic-resistant isopeptide bonds within or between proteins/peptides. Therefore, if these enzymes are fundamental for *Leishmania* parasites, they could represent another promising drug target. TGase activity has been detected in some *Leishmania* species however, the protein TGase has not been purified, cloned, and characterized. The second part of the thesis is more clinical, evaluating in detail by advanced molecular techniques and cytokine analysis the prevalence of asymptomatic/subclinical *Leishmania* infections in autoimmune rheumatic patients treated with immunosuppressive biological drugs (anti-TNF-α antibodies, modulators of T lymphocyte activity, or anti-IL-6 receptor antibodies) and living in *Leishmania* endemic foci in Italy, thus representing a major disease re-emergence.

Methods

In the 6-AN study, *L. mexicana* M379 and *L. infantum* PCM5 promastigotes were treated with 7.8 mM 6-AN and 2.17% DMSO for 24 hours. After vitality, infectivity of 6-AN-treated promastigotes to mouse macrophages, and 6-AN interactions with oxidizing compounds were also studied. Small metabolites were extracted and

analysed by pHILIC-LC-MS in polarity switching mode and data were analysed with IDEOMv19 and MetaboAnalyst 3.0.

In the TGase study, the enzyme activity was detected *in vivo* in canine *L. infantum* promastigotes after their incubation with fluorescein (FITC)-cadaverine (FC). Parasite smears were prepared, fixed and washed for 15 minutes at -20°C in methanol. A Nikon Microphot FXA fluorescent microscope was used to detect the specific incorporation of FC into parasite proteins. In addition, parasite extracts were incubated for 1 hour at 30°C with 4 mM FC, with or without dimethyl casein and the fluorescence was visualized in the SDS-PAGE gel using Molecular Imager Pharos FX imaging system (Bio-Rad). Further, TGase activity was measured in microwell plates using Sigma TGase activity assay Kit in parasite lysates and after precipitation with ammonium sulfate (AS). Western blot of human *L. infantum* promastigote whole lysate and canine *L. infantum* total extract and 15 and 45% AS protein fractions with TGase activity as well as immunocytochemical analysis of canine *L. infantum* were performed to detect *Leishmania* protein transglutaminase using rabbit polyclonal antibodies (pAbs, orb2986) against human TGase 2 and HRP-conjugated Goat anti-Rabbit antibodies or FITC-labelled anti-rabbit antibodies respectively. The blots were visualized by ECL system and the immunocytochemistry was revealed using a Nikon Microphot FXA fluorescent microscope.

In the clinical study, *Leishmania* qualitative PCR and real-time PCR were performed on DNA extracted from PBMCs from 50 autoimmune rheumatic (rheumatoid arthritis (RA), Ankylosing spondylitis (AS) and Psoriatic arthritis (PsA)) patients treated with immunosuppressive biologic drugs for at least 5 years. Genomic DNA extracted from *L. infantum* cultured promastigotes and from PBMCs from 50 healthy subjects were used as reference positive control and negative control respectively. Plasma cytokine concentrations were also measured in plasma from *Leishmania* DNA-positive and -negative rheumatic patients as well as from the healthy control group.

Results

In both *L. mexicana* and *L. infantum*, 6-AN caused significant depletion of phosphoribosylpyrophosphate (PRPP) and nicotinate (Na) and as a result purine and pyrimidine nucleotides were reduced and their nucleobases accumulated. Glutathione, ribose-5-phosphate, 6-phosphogluconate levels and downstream PPP

intermediates were similar to controls. For *L. infantum*, it was possible to analyse NAD^+ and NADPH, which were found decreased together with the PPP intermediate D-sedoheptulose 7-phosphate. Moreover, 6-AN treatment caused a marked elongation in parasite body. 6-AN in combination with the oxidizing compounds has additive effects against *Leishmania* and did not affect the infectivity of the treated promastigotes to mouse macrophages.

TGase activity was detected and confirmed in human and canine *L. infantum* promastigotes. Incubation of cultured promastigotes with FC resulted in a green intracellular fluorescing with clear non-fluorescing background indicating the presence of TGase activity in these parasites. In addition, incubation of promastigote lysates with FC and dimethyl casein revealed fluorescent endogenous as well as dimethyl casein protein bands in the SDS-PAGE gel confirming the presence of an active TGase. The canine protein TGase precipitated at 15 and 45% AS saturations and furthermore, the enzyme activity measured in 45% AS fraction using microplate assay was found Ca^{2+}-dependent and inhibited by 10mM GTP. The immunocytochemical analysis showed specific green fluorescence of detected TGase and the blots revealed calculated protein bands; one at position 74.6 KDa for the canine strain and two; a major one at position 55.34 KDa and a minor one at position 65.87 KDa for the human strain.

Eighteen out the 50 (36%) autoimmune rheumatic patients were positive for *Leishmania* DNA by conventional and/or quantitative PCR with a detection of high parasite burdens (1 to 136 parasite/ml in 4 patients, 1.000 to 40.000 in 11 patients and over 1.000.000 in 3 patients). Patients that were taking a steroid in association with the biological drug showed a higher positivity for circulating *L. infantum* kDNA than those given the biological drug only ($p<0.05$). No statistical difference observed in relation to the ownership of a dog and the type of biological drug administered. Pro-inflammatory IL-1, IL-6, IL-12(p70), IL-7, IL-15, IFN-γ and TNF-α; anti-inflammatory IL-4, IL-13; and regulatory IL-10 cytokines were markedly elevated in all autoimmune rheumatic patients with additional increases in inflammatory mediators in autoimmune rheumatic patients positive for *Leishmania* DNA.

Conclusions

In mammals 6-AN is converted to abnormal 6-ANAD/P by NAD^+ glycohydrolase, however, in *Leishmania* its toxicity is only seen in millimolar range, in which 6-AN is responsible for the depletion of cellular phosphoribosyl pyrophosphate (PRPP) content probably in the Preiss-Handler NAD^+ salvage pathway, resulting in depletion of nucleotides required for nucleic acid biosynthesis. The marked elongation in the 6-AN-treated parasite bodies confirms nucleotide starvation. *Leishmania* NAD^+ glycohydrolase might decompose NAD^+ but might not catalyze exchange reactions, as found in other microrganisms, however, combined ^{13}C-glucose labeling and flux analysis might be useful to ascertain the fate and action mechanism of 6-AN in *Leishmania.* In addition, PRPP synthetase should also be a good target for new potential drugs against leishmaniasis pointing to the growth-inhibitory effect of PRPP depletion.

The presence of an active TGase in *Leishmania* was confirmed by detecting *in vivo* and *in vitro* a Ca^{2+}-dependent transamidation in canine *L. infantum* promastigotes. The inhibition of this TGase by GTP suggests that regulation of the activity of this enzyme might be Ca^{2+}/GTP regulated. Furthermore, the precipitation of the protein TGase at the low 15% AS saturation, suggests that this enzyme might be membrane-associated. In addition, the specific Western blots and immunocytochemical detections of protein indicate that the TGase pAbs (orb2986) could permit affinity chromatography purification of this *Leishmania* enzyme.

The high *Leishmania* parasitaemia detected in PBMC fractions from autoimmune rheumatic patients suggests that treatment with biologic drugs can lead to cryptic VL or *Leishmania* infection in a latent phase which may progress to full VL course in the setting of immunosuppression. In addition, taking a steroid drug in addition to a biological therapy is strongly associated with an increased risk of being positive for *L. infantum* kDNA. Molecular screening using the easy-to-obtain and-prepare PBMC fractions and cytokine analysis should be taken into account before treating autoimmune rheumatic patients with biologic drugs. Moreover, extension of this research even in rural and suburban areas may as well add new knowledge to map the spread of *L. infantum* infection.

Sommario

Background

Le leishmaniosi sono un gruppo di malattie parassitarie, comprendenti una forma viscerale (VL) che colpisce gli organi interni, fatale se non trattata, una grave forma muco-cutanea (MCL), che può portare a mutilazione delle mucose di naso, bocca e gola e una forma cutanea (CL) più lieve, che può guarire anche senza cure. La VL è un grave problema in molte zone del mondo, soprattutto a clima tropicale e subtropicale; i farmaci attuali possono dare effetti collaterali di tossicità, nonché generare fenomeni di resistenza, per cui è costante la ricerca di nuovi composti e l'individuazione di nuovi "drug target". In questa tesi ho voluto approfondire la patologia sia dal punto di vista biochimico che da quello clinico. Lo studio biochimico consta di due parti. La prima è l'analisi dell'effetto antiproliferativo dell'analogo della nicotinamide, 6-AN, sul parassita *Leishmania*, e degli effetti sul suo metabolismo mediante la tecnica dell'analisi metabolomica. Il razionale dello studio è che essendo riportato che il bersaglio principale della 6-AN è la via dei pentosi fosfato (PPP), via metabolica necessaria al sistema di difesa antiossidante della cellula e i cui enzimi in questa famiglia di parassiti, presentano differenze significative con quelli di mammifero, la stessa via potrebbe rappresentare un buon bersaglio di farmaci leishmanicidi. Un secondo potenziale bersaglio potrebbe essere l'enzima transglutaminasi (TGase), catalizzante la formazione di legami isopeptidici intra- e inter-molecolari, resistenti alla proteolisi. In alcune specie di *Leishmania* ne è stata identificata l'attività, la cui inibizione si è dimostrata dannosa per il parassita. Perciò la seconda parte dello studio biochimico ha riguardato la sua ricerca e caratterizzazione in *L. infantum,* allo scopo di tentarne la purificazione, importante per il successivo clonaggio. Nello studio clinico della tesi, si è valutata la prevalenza di parassitosi da *Leishmania,* asintomatiche/subcliniche, mediante tecniche molecolari, in pazienti affetti da patologie reumatiche autoimmuni, trattati con farmaci biologici, immunosoppressori (anticorpi anti-TNF-α, modulatori dell'attività di linfociti T o anticorpi anti-recettore di IL-6) correlandola con l'analisi di specifiche citochine. E' stata anche fatta una correlazione con la zona di residenza, considerando che in Italia la zona di endemicità si sta espandendo.

Metodologia

Nello studio con 6-AN, promastigoti dei ceppi di *L. mexicana* M379 e di *L. infantum* PCM5 sono stati trattati per 24 ore con 6-AN 7,8 mM e DMSO 2.17%. Oltre alla vitalità è stata anche studiata l'infettività dei promastigoti trattati con 6-AN, su macrofagi murini e l'effetto combinato di 6-AN e agenti ossidanti. Dopo estrazione, i metaboliti più piccoli sono stati analizzati mediante pHILIC-LC-MS, in modalità "polarity switching mode", i dati sono stati ulteriormente analizzati mediante IDEOMv19 e MetaboAnalyst 3.0.

Nello studio sulla TGase, l'attività è stata saggiata *in vivo* in promastigoti di un ceppo canino di *L. infantum,* dopo incubazione con fluoresceina-cadaverina (FC). Strisci sono stati fissati e lavati con metanolo a -20°C per 15 minuti e l'incorporazione di FC è stata analizzata con microscopio a fluorescenza Nikon Microphot FXA. In altri esperimenti il lisato cellulare è stato incubato per 1 ora a 30°C con FC 4 mM, in presenza o assenza di dimetil-caseina e la fluorescenza visualizzata su gel, dopo SDS-PAGE, con Molecular Imager Pharos FX imaging system (Bio-Rad). L'attività enzimatica è stata anche saggiata in micropiastra col TGase activity assay Kit (Sigma) sia sul lisato cellulare che dopo frazionamento con solfato d'ammonio (AS). Anticorpi policlonali contro TGase 2 umana (pAbs, orb2986) e anticorpi secondari coniugati con FITC sono stati utilizzati nell'analisi immunocitochimica di *L. infantum* canina mentre anticorpi secondari HRP-coniugati sono stati utilizzati per Western Blot con lisati cellulari di *L. infantum* sia canina che umana e frazioni proteiche ottenute dal ceppo canino dopo precipitazione con AS 15 e 45%, visualizzati col sistema ECL.

Nello studio clinico si sono utilizzate PCR qualitativa e real-time, specifiche per *Leishmania,* su DNA estratti da PBMC di 50 pazienti affetti da patologie reumatiche autoimmuni (artrite reumatoide, RA, spondilite anchilosante, AS, e artrite psoriasica, PsA) trattati con farmaci immunosoppressori per almeno 5 anni. Come controlli negativi si sono utilizzati DNA estratti da 50 soggetti sani mentre come controllo positivo il DNA estratto da *L. infantum.* Ulteriormente si sono quantificate le citochine seriche sia nei pazienti con DNA positivo e negativo per *Leishmania* che nel gruppo di controllo.

Risultati

Sia in *L. mexicana* che in *L. infantum,* 6-AN ha causato una diminuzione significativa di fosforibosil-pirofosfato (PRPP) e nicotinato (Na) e, come conseguenza, di nucleotidi purinici e pirimidinici mentre le nucleobasi sono aumentate. I livelli di glutatione, ribosio-5-fosfato, 6-fosfogluconato e intermedi PPP a valle erano simili ai controlli non trattati. Solo con *L. infantum* si è riusciti ad analizzare NAD^+ e NADPH, che insieme all'intermedio PPP D-sedoeptulosio 7-fosfato, sono stati trovati diminuiti. 6-AN ha inoltre causato un allungamento marcato del corpo cellulare del parassita. Sembra che l'effetto anti-proliferativo di 6-AN sia additivo con gli agenti ossidanti utilizzati mentre non abbia influenza sull'infettività dei macrofagi.

Si è confermato che *L. infantum* possiede attività TGasica, ne è stata rivelata l'attività in promastigoti sia del ceppo umano che di quello canino. Infatti, incubati in coltura con FC, essi hanno presentato fluorescenza verde intracellulare, su sfondo chiaro non fluorescente. Inoltre, dopo incubazione dei lisati cellulari con FC +/- dimetilcaseina e SDS-PAGE, è stato possibile rivelare bande fluorescenti corrispondenti sia alla dimetilcaseina che a substrati endogeni. Dopo precipitazione con AS due frazioni hanno mostrato attività: 15 e 45%, quest'ultima ha rivelato, mediante saggio su micropiastra, attività Ca^{2+}-dipendente e inibita da GTP 10mM. In aggiunta, si è evidenziata fluorescenza mediante analisi immunocitochimica e l'Western Blot trattato con gli stessi anticorpi ha mostrato una banda di 74.6 KDa per il ceppo canino e due per il ceppo umano, di 55.34 KDa e 65.87 KDa rispettivamente.

Mediante PCR convenzionale e/o quantitativa, è stata riscontrata positività per il DNA di *Leishmania* in 18 dei 50 (36%) pazienti con patologie reumatiche autoimmuni analizzati, con reperimento di un'alta carica parassitaria (1-136 parassiti/ml in 4 pazienti, 1.000- 40.000 in 11 pazienti e > 1.000.000 in 3 pazienti). I pazienti in trattamento sia con steroidi che con farmaci biologici hanno mostrato una più alta positività per kDNA di *L. infantum* rispetto a quelli trattati solo con farmaci biologici ($p<0.05$). Non si sono evidenziate invece significative differenze tra possessori o meno di cani, e in riferimento al tipo di farmaco biologico somministrato. Rispetto ai soggetti sani, in tutti i pazienti reumatologici autoimmuni in trattamento con farmaci immunosoppressori, erano significativamente elevate le citochine pro-infiammatorie IL-1, IL-6, IL-12(p70), IL-7, IL-15, IFN-γ e TNF-α, quelle anti- infiammatorie IL-4 e IL-

13 e la regolatoria IL-10, ma un aumento maggiore era presente nei pazienti positivi per la presenza di DNA di *Leishmania*.

Conclusioni

Nei mammiferi la 6-AN è metabolizzata, da una NAD^+ glicoidrolasi, a 6-ANAD/P, responsabile della sua tossicità; in *Leishmania* la tossicità è presente solo in concentrazione millimolare e porta, verosimilmente tramite la via metabolica Preiss-Handler, di sintesi del NAD^+, a deplezione del contenuto cellulare di PRPP, che causa a sua volta il depauperamento in nucleotidi necessario alla biosintesi degli acidi nucleici. Il marcato allungamento del corpo cellulare dei parassiti trattati con 6-AN, ricorda proprio la sofferenza (starvation) indotta da deprivazione di nucleotidi. In *Leishmania* la NAD^+ glicoidrolasi probabilmente idrolizza il NAD^+ ma non catalizza lo scambio tra nicotinamide e 6-AN, come avviene anche in altri microrganismi. Futuri studi con analisi di flusso dopo marcatura con ^{13}C-glucosio potrebbero chiarire destino metabolico e meccanismo d'azione del 6-AN in *Leishmania.* Dai risultati ottenuti salta all'occhio come la PRPP sintetasi potrebbe essere un buon "target" per nuovi potenziali farmaci per le leishmaniosi, data l'inibizione della crescita provocata dalla massiva deplezione in PRPP.

Si è rivelata *in vivo* and *in vitro,* una transamidazione, che conferma la presenza in *Leishmania* di una TGase attiva, dimostratasi Ca^{2+}-dipendente in promastigoti di *L. infantum* canina, e inibita da GTP. La precipitazione della proteina a basse saturazioni di AS (15%) suggerisce che l'enzima potrebbe essere associato alla membrana. Ulteriormente, l'identificazione, tramite gli anticorpi pAbs (orb2986) contro TGase, di bande specifiche in Western blot e, in cellule, di marcatura localizzata, suggerisce che questi anticorpi potrebbero essere utili alla purificazione dell'enzima, mediante cromatografia d'affinità.

L'alta parassitemia da *Leishmania* trovata nelle frazioni PBMC preparate da sangue di pazienti affetti da patologie reumatiche autoimmuni trattati con farmaci biologici, suggerisce che il trattamento con questi farmaci immunosoppressivi possa portare a VL criptica cioè infezione latente, con possibilità di progressione a VL conclamata nel caso il quadro si evolva in uno stato di immunocompromissione. L'assunzione di farmaci steroidei in aggiunta ai biologici è risultata significativamente associata a una maggior positività per *L. infantum* kDNA. I dati suggeriscono che, prima di

somministrare questo tipo di farmaci a pazienti con patologie autoimmuni, si potrebbero introdurre uno screening molecolare su frazioni PBMC, facilmente ottenibili, e l'analisi di citochine. Inoltre l'estensione della ricerca ad aree rurali e suburbane sarebbe utile alla mappatura della distribuzione di ceppi di *Leishmania,* in grado di provocare infezione nell'uomo.

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Chapter 1

1. Overview of *Leishmania*

1.1 General introduction

Leishmaniases are a group of vector-borne diseases caused by obligate intramacrophage protozoa of more than 20 *Leishmania* (L) species of the genus *Leishmania*, family *Trypanosomatidae*, order *Trypanosomatida* and Class Kinetoplastea, and characterized by diversity, complexity, and possession of the kinetoplastid body (a unique form of mitochondrial DNA, kDNA) (Leishman, 1903; Herwaldt, 1999; Desjeux, 2001). On the basis of development in the sandflies, the human-infecting section, *Euleishmania* (Cupolillo *et al*., 2000) has been divided into two subgenera, *Leishmania* and *Viannia*. Development of organisms belonging to the subgenus *Leishmania* is restricted to the anterior portion of the alimentary tract of the *Phlebotomus* sandflies (suprapylarian development), whereas organisms belonging to the subgenus *Viannia* develop in the midgut and hindgut of the sandflies, *Lutzomyia* spp. (peripylarian development) (Lainson *et al*., 1977). *Viannia* contains the complex of *L. braziliensis* (*L. b. brasiliensis*, *L. b guyanensis*, *and L. b panamensis*), whereas the subgenus *Leishmania* contains *L. donovani* complex (*L. d. infantum* in the Mediterranean region, *L. d. donovani* in India, *L. d.* archibaldi in Sudan and *L. d. chagasi* in South America), *L. major, L. tropica*, *L. aethiopica, and L. mexicana* complex (*L. m. mexicana, L. m. amazonensis* and *L. m. panamensis*) (Ashford and Bates, 1998). However, parasites grouped in complexes are under continuous reviewing with descriptions of new cryptic species (Marcili *et al*., 2014; Schönian e*t al*., 2011).

Natural transmission of *Leishmania* is carried out by the bite of infected, tiny 2 to 3 millimeter-long female sandfly (Bates and Rogers, 2004) of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) (Sundar and Rai, 2002). Other modes of transmission do exist although their occurrence is rare. These include direct contact with infected blood (Amela *et al*., 1996), infected organ transplantion (Hernández-Pérez, 1999) mucous, stools or nasal secretions (Manson-Bahr, 1987). Moreover, congenital (Meinecke *et al*., 1999) and sexual (Symmers, 1960) transmission has also been reported.

Leishmaniasis consists of four main clinical syndromes depending on the *Leishmania* species involved; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL, also known as kala-azar) which is fatal if left untreated, and post-kala-azar dermal leishmaniasis (PKDL) (Desjeux, 2004; François *et al.,* 2007) however, most people infected by the parasite do not develop any symptoms at all in their life (WHO, 2016).

CL is the most common form of the disease and it is usually self-limiting. In the Old World CL is caused by *L. major*, *L. tropica*, *L. aethiopica,* and rarely *L. infantum* whereas in the New World it is caused by *L. mexicana* complex with *L. (m) amazonensis* causes diffuse cutaneous leishmaniasis (DCL) (Ashford and Bates, 1998).

MCL is a severely destructive disease in which the parasites disseminate from the site of the initial infection on the skin to mucosal membranes of the nose, mouth, and nose. MCL is caused by *L. braziliensis* complex in Americas with *L. braziliensis braziliensis* causing the most disfiguring form, espundia (Marsden, 1986).

PKDL is a diffuse cutaneous form in which the viscerotropic parasites subtly, disseminate to the skin 6 months to 1 or more years after successful VL treatment. This form is rare in India however; it is frequent in East Africa. PKDL patients are of an epidemiological importance because they act as potential sources of infection (WHO, 2016).

L. donovani is the primary cause of anthroponotic (where humans are the major reservoirs of the parasite) VL in all age groups (François *et al.,* 2007) in the Indian subcontinent and East Africa, and zoonotic VL in Sub-Saharan Africa, especially in Sudan, Ethiopia, Somalia and northern Kenya (Ashford *et al*., 1998). *L. infantum* causes zoonotic (animals are the main reservoir hosts) VL in children and immunosuppressed individuals (François *et al.,* 2007) in the Mediterranean basin, Western Asia and Eastern China whereas *L. chagasi* causes VL in the New World mainly in Brazil, Peru, and Paraguay (Philippe *et al*., 2002). In the zoonotic leishmaniasis, dogs and rodents are the main reservoir hosts and humans are accidently infected (Alvar *et al*., 2004). VL is fatal, with a mortality rate reaching approximately 100% among untreated cases. WHO in 1990 recognized the disease as one of the most important parasitic diseases in the UNDP/World Bank/WHO Special Programme for Research and training in Tropical Disease Research (TDR).

1.2 History of VL

It has been reported that DNA of *L. donovani* was found in Egyptian mummies 4,000 years ago (Zink *et al*., 2006) however, VL was first described in 1824, in Jessore district of Bengal, what is now Bangladesh (Sengupta, 1947) and it has been known in the old world in India since the 19th century by the name black fever or ***Kala-azar*** due to skin darkening (Brahmachari, 1928). Alexander Russell, following an examination of a Turkish patient made the first clinical description in 1756. The disease was then commonly known as "***Aleppo boil***" as it leaves an ugly scar which, remains throughout life (Cunningham, 1885). The disease became known as Leishmaniasis after William Leishman, a Glasgwegian doctor serving with the British Army in India, developed one of the earliest stains of *Leishmania* in 1901. He discovered ovoid bodies in the spleen of a British soldier in Dum Dum, a town near Calcutta. Leishman described this illness as "***dum dum fever***" and published his findings in 1903 (WHO, 2010). Charles Donovan also recognized the parasite using Leishman's stain in other kala-azar patients and published his discovery a few weeks after Leishman. Hence these amastigotes were known as Leishman-Donovan bodies and officially, this species became known as *L. donovani* (WHO, 2010). In 1904 Rogers succeeded in culturing the parasite responsible for VL from splenic aspirates (Dedet *et al*., 1999).

1.3 Epidemiology of *Leishmania*

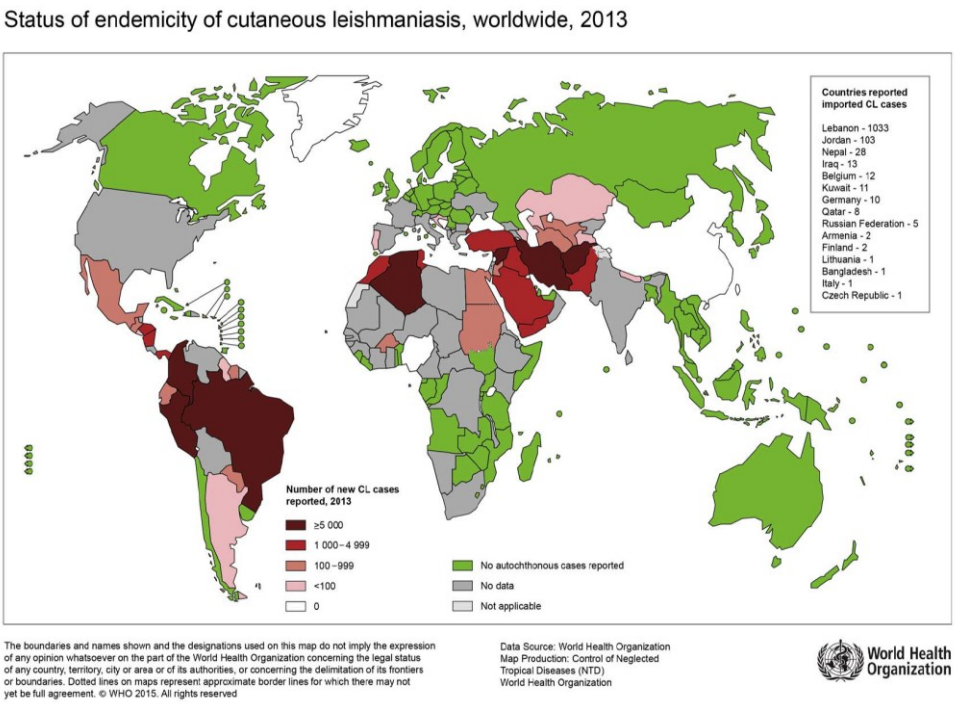
The global geographical distribution of leishmaniasis is worldwide (Fig. 1.1) and limited to the areas where sandflies are found. The insect vectors are present in all continents except Australia and Antarctica due to the lack of suitable vector breeding sites (Santaŕem and Cordeiro-da-Silva, 2007; Manson-Bahr, 1987). The disease is endemic in tropical and subtropical regions of 88 countries 72 out of which are developing ones (WHO, 1998). About 350 million are believed to be at risk of having leishmaniasis. There are an estimated 12 million cases worldwide (Salotra *et al.,* 2001) with approximately, 0.2 to 0.4 million VL cases and 0.7 to 1.2 million CL cases occurring each year (Alvar *et al*., 2012). Cutaneous forms represent 50 to 75% of all new leishmaniasis cases and the majority of these cases occur in Afghanistan, Algeria, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia and Syria. About 90% of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil and Peru (WHO, 2016).

Although confirmed cases of VL have been reported from 88 countries, 90% of the new cases occurred in Brazil, Ethiopia, India, Somalia, South Sudan and Sudan (WHO, 2016).

Leishmaniasis ranks second in mortality among tropical diseases (Mathers *et al*., 2007) however, the mortality data of leishmaniasis are extremely sparse and generally reflect deaths that occur in hospitals only although, 20,000 to 40,000 leishmaniasis deaths occur every year (Alvar *et al*., 2012).

The epidemiological data are usually incomplete, and official figures are likely to underestimate the real prevalence of the disease (Thakur, 2000), however, the actual number of cases estimated to be three or five times higher than the number reported as leishmaniasis usually occurs in remote areas where other health problems are predominant (WHO 1993) and the *Leishmania* distribution is obscure and dependent on expert opinion and locative analysis (Pigott *et al*., 2014). Since 1993, the geographical distribution and the number of cases of leishmaniasis have expanded significantly (WHO, 2010). Economic development including climate changes, widespread urbanization, deforestation, settlements expansion, besides migration from rural to urban areas, is responsible for the spread of the sandflies (Pavli and Maltezou, 2010; Dujardin *et al*., 2008; Thakur, 2000). Yet, population movement, lack of control measures, VL/HIV co-infection, and appearance of new feline reservoir hosts in the Mediterranean are the main factors driving the increased incidence of VL (Desjeux, 2004; Ozon *et al*., 1998). Previous, severe VL epidemics have been reported in Southern Sudan, in a context of civil war and famine where VL killed an estimated 100,000 people out of a population of 280,000 between 1984 and 1994 (Seaman *et al.*, 1996), and in India and Bangladesh involving hundreds of thousands of people (WHO, 1996). In 2007, the World Health Organization (WHO) approved strategies for the control of leishmaniasis. Currently, kala-azar elimination program in the endemic countries is remarkably progressing. For example, in Bangladesh as low as 600 new cases were reported in 2014–2015 compared to more than 9000 cases in 2006 (WHO, 2015). According to the WHO plan, by 2020, all VL cases should be identified and treated in the endemic regions of Africa, Europe and Americas and the prevalence should be reduced to below 1/10,000 in the endemic districts of the Indian sub-continent (WHO, 2013).

A.



B.

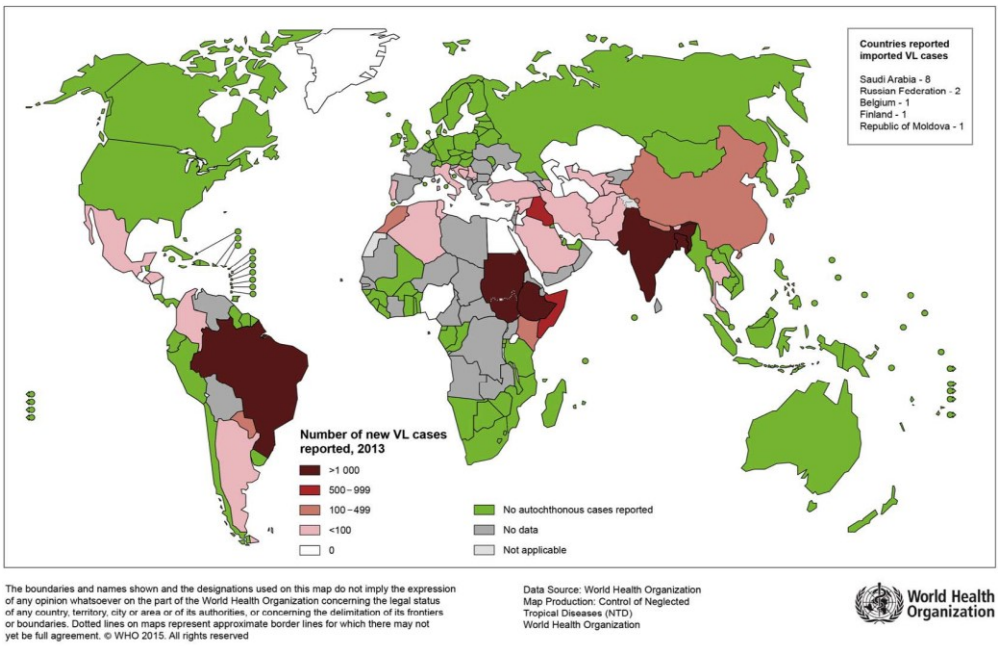


Figure 1.1. The geographic distribution and endemicity map of cutaneous leishmaniasis (A), and visceral leishmaniasis (B) according to WHO 2013 report. (http://www.who.int/entity/leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL_CL_2013.pdf?ua=1).

1.4 Biology of *Leishmania* parasites

Leishmania parasites exist in two forms (dimorphic); amastigote, which is the ovoid, non-flagellated form measuring 3-5 µm in length and contains round or oval nucleus and smaller round- or rod-shaped kinetoplast. In the sand fly and culture medium the parasite is found as promastigote, the elongated (8-15 µm) and flagellated (motile) form (Ross, 1903). Sandfly inoculates infectious metacyclic promastigotes along with the pro-inflammatory saliva components into the host skin while taking a blood meal (Bate, 2007). In the skin the mononuclear phagocytic cells (particularly macrophages) take promastigotes, which transform into obligatory intracellular amastigotes within the phagosomal vacuole and the latter multiply through multiple rounds of binary fission. Infected macrophages rupture when they are heavily parasitized, releasing many amastigotes that are engulfed again by other new cells. Depending on the species of the parasite, amastigote-infected cells may localize to the skin lesion (CL and MCL), or spread to other sites in the body including spleen, liver, and bone marrow (VL) (Sacks and Perkins, 1984). Circulating amastigote-infected macrophages are taken up by sandfly in the blood meal. The decrease in temperature and increase in pH in the insect midgut (Bates and Rogers, 2004) trigger the transformation of the amastigotes into small, sluggish procyclic promastigotes in the midgut of the sandfly. Surrounded by the insect-secreted peritrophic matrix (PM), the procyclic forms divide within the digesting blood (Killick-Kendrick, 1979) while attaching to the membrane via their surface-covering lipophosphoglycan (LPG) to avoid being secreted with the digested blood remnants (Rogers *et al*., 2002). After 2-3 days they transform into large, slender, non-dividing nectomonads and migrate to the foregut of the insect where they differentiate into short, broad leptomonad forms (Bates and Rogers, 2004), which, resume replication, secrete promastigote secretory gel (PSG) and convert to small-bodied with long flagellum, infective metacyclic promastigotes. The PSG interferes with normal blood feeding of the insect by blocking the stomodeal valve (Rogers *et al*., 2002). The insect regurgitates the plug leading to the co-deposition of the parasites into skin (Bate, 2007) initiating a new cycle (Fig. 1.2).

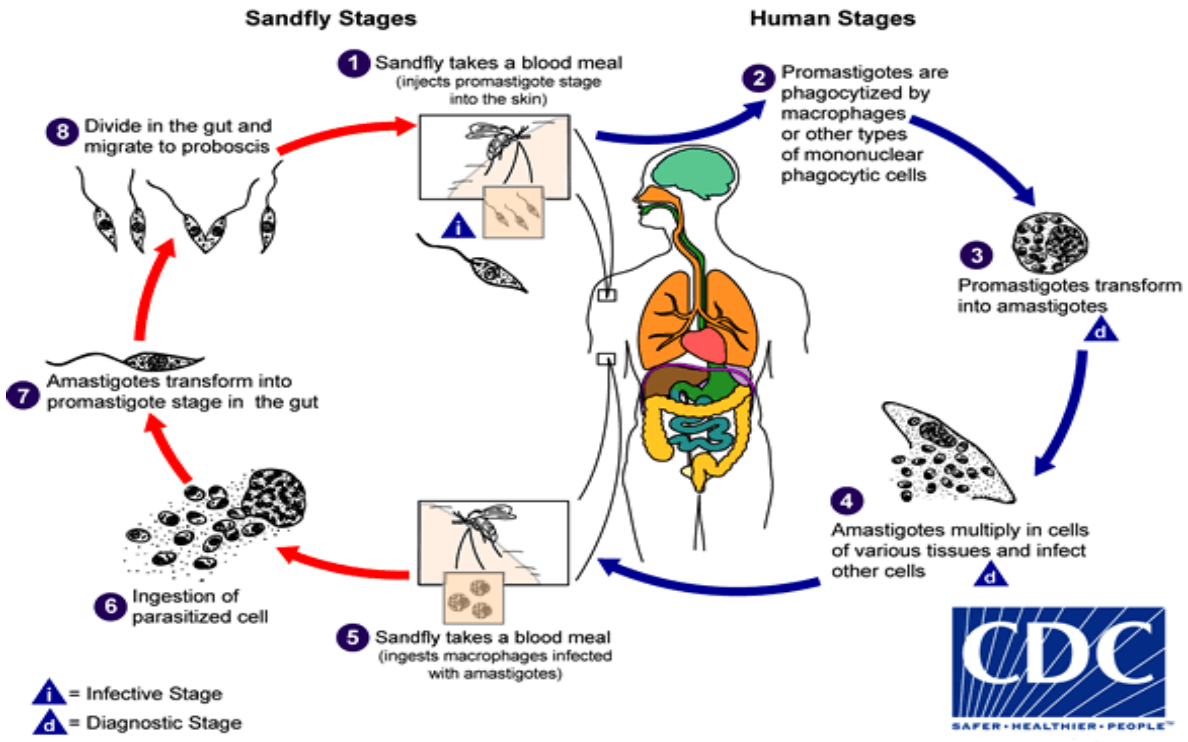


Figure 1.2. The developmental life cycle of *Leishmania* parasites. Adapted from US Centers for Disease Control and Prevention (CDC) (https://www.cdc.gov/parasites/leishmaniasis/biology.html).

1.5 Pathology of leishmaniasis

The outcome of the *Leishmania* infection depends upon the parasite species therefore; CL, MCL, or VL can develop with varying degrees of severity and manifestation.

CL infections can remain subclinical or become clinically evident. Single or multiple lesions ranging from papules, nodules, to ulcerative lesions can appear in the exposed arms, legs, and face (Chappuis *et al*., 2007) however; they are self-limiting over a period of several weeks (Herwaldt, 1999). DCL lesions are usually non-ulcerative and do not have a tendency to self-cure (David and Craft, 2009).

In MCL, the amastigotes disseminate from the skin to the naso-oropharyngeal mucosa causing destruction of these tissues due to hyperactive immune response with possible perforation of nasal septum (Herwaldt, 1999; Reithinger *et al*., 2007). MCL does not heal spontaneously and can be fatal (Chappuis *et al*., 2007).

In VL, the amastigotes disseminate through the lymphatic and vascular systems and infect other monocytes and macrophages in the reticulo-endothelial system (RES); liver, spleen, bone marrow and lymph glands, rarely the intestine, adrenals, kidneys and lungs (Zijlstra *et al*., 1994). Parasite proliferation in these organs produces enlargement (hepatomegaly and splenomegaly) with atrophy or replacement of the normal tissue, and lymphadenopathy therefore *kala-azar* is a systemic granulomatous disease of the RES (Manson-Bahr, 1987). Following incubation period, VL patients present symptoms and signs of persistent systemic infection including fever, fatigue, weakness, decreased appetite, anaemia and weight loss (François *et al*., 2007; Philippe *et al*., 2002). Splenomegaly may be absent in 5% of cases (Nandy, 1998). Enlarged lymph nodes are rarely found in Indian VL patients but are frequent in Sudanese patients (Zijlstra *et al*., 1991). VL symptoms often persist for several weeks to months before patients seek medical care or die from bacterial co-infections, massive bleeding or severe anaemia (François *et al*., 2007).

PKDL develops frequently after successful treatment which is a macular, popular, or nodular rash as a complication of VL in Sudan and more rarely in other East African countries and in the Indian subcontinent (Zijlstra *et al*., 2003). The interval between treated VL and PKDL is 0–6 months in Sudan and 6 months to 3 years in India

(François *et al*., 2007). PKDL cases are highly infectious because the nodular lesions contain many parasites thus act as reservoir hosts (Addy and Nandy, 1992).

1.6 *Leishmania*-HIV co-infection

In the mid-1980s the first cases of VL have been reported in southern Europe in patients infected with human immunodeficiency virus (HIV) (De la Loma *et al*., 1885). In this region, 25-70% of adult patients with VL are co-infected with HIV and it is estimated that 1.5-9% of patients with AIDS will develop leishmaniasis (http://www.who.int/docstore/wer/pdf/1997/wer7208.pdf?ua=1). In addition, a number of CL cases in AIDS patients have been reported in Brazil (Lindoso *et al*., 2009), and PKDL in HIV-infected patients has also been reported (Puig and Pradinaud, 2003). To date, *Leishmania*-HIV co-infection has been reported from 33 countries with most of the cases located in southern Europe. The association of VL and HIV infection clearly confirms the fact that VL is an opportunistic infection in that HIV rapidly activates infection to disease in asymptomatic parasite carriers bringing the severe VL to new geographical areas, and presenting dangerous epidemiology (WHO, 2016 (http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/).

1.7 Immunity to *Leishmania*

Leishmania amastigotes produce variable effects on the elements of the immune system leading to variable host responses and producing a wide spectrum of disease (Manson-Bahr and Apted, 1985). However, the outcome of *Leishmania* infection depends on different factors of which parasite species, host genetic factors, and status of the host immunity are the most important determinants (Bosque *et al*., 2000). Susceptibility to *L. donovani* infection is controlled by polymorphism in *Slc11a1* gene (formerly Nramp1; natural resistance associated macrophage protein 1) that encodes an iron and manganese transporter involved in the activation of macrophage antimicrobial mechanisms (Blackwell *et al*., 2001), polymorphisms in the interlukin (*IL*)-*2Rβ* gene, which is involved in T cell function (Bucheton *et al*., 2006), a major susceptibility locus on chromosome 22q12 (Bucheton *et al*., 2003), polymorphisms within the human *IL-1β* gene (Moravej *et al*., 2012), and in Indian patients polymorphisms in the chemokine receptor 2 (*CXCR2)* gene, which encodes receptors for IL-8 and other chemokines (Mehrotra *et al*., 2011). The early interaction between

promastigotes and the professional antigen presenting cells (APCs), macrophages and dendritic cell (DC) in the skin determine the outcome of the disease (Liu and Uzonna, 2012). The wound inflicted by sandflies induces the infiltration of the blood immune cells particularly neutrophils (Peters *et al*., 2008) which uptake the parasites and facilitate their silent entry into the macrophages (van Zandbergen *et al*. 2004). Free parasites and infected apoptotic neutrophils are phagocytosed by macrophages and consequently the parasites reach their final hosts (Antoine *et al*., 1998). In asymptomatic infections, infected DCs secrete interleukin (IL-12), the pro-inflammatory cytokine that leads to the activation and differentiation of the naïve CD4+ T cells into antigen specific T helper (Th1) cells (type 1 immune response). Th1 cells secrete gamma interferon (IFN-γ), a cytokine that synergizes with macrophage-secreted tumor necrosis factor alpha (TNF-α) to activate macrophages to up-regulate their inducible nitric oxide synthase (iNOS), produce nitric oxide (NO) from L-arginine, and eventually kill the parasites (Liew *et al*., 1990). In addition, DCs trigger early NK cell activities including IFN-γ production and cytotoxicity (Liese *et al*., 2007). In active infections, early production of IL-4 by APCs causes naïve T cell differentiate into Th2 subset (type 2 immune response), which produces the anti-inflammatory cytokines, IL-4 and IL-13, that inhibit the production of NO and divert L-arginine metabolism towards polyamine synthesis by up-regulation of macrophage arginase leading to parasite persistence and proliferation (Iniesta *et al*., 2005). Also, despite the induction of protective immune response, disease develops due to many factors of which a mixed activation of Th1 and Th 2 subsets (Ghalib *et al*., 1995), and the action of suppressive cytokines such as IL-10 (Gordon, 2003), secreted by regulatory CD4+ T cells (Treg) to neutralize the tissue destructive effects of Th 1 response, evident in MCL with high TNF-α and IFN-γ (Bacellar *et al*., 2002). In addition, the recently described IL-27 was shown to attenuate the inflammatory response by improving IL-10 production by Treg cells (Murugaiyan *et al*., 2009). Although antibodies have little if any protective role against *Leishmania* parasite (Ghalib, 1993), there is a marked increase in gamma immunoglobulins (IgG) (Manson-Bahr, 1987), however; IgG-opsonized amastigotes (antigen/antibody complexes) promote IL-10 secretion and inhibit IL-12 production by macrophages (Miles *et al*., 2005).

Leishmania parasites have developed immunomodulatory strategies to disrupt macrophage activation signaling in order to evade killing, survive, and proliferate in these cells. Since the uptake of *Leishmania* promastigotes is a receptor-mediated process, metacyclic promastigotes selectively use complement receptor 3 (CR3) to enter macrophages without triggering reduced nicotinamide adenine dinucleotide (NADPH) oxidase activation and subsequent respiratory burst and production of reactive oxygen species (ROS), which kill intracellular parasites (Sehgal *et al*., 1993). In addition, lipophosphoglycan (LPG), the parasite surface glycolipid delays phagolysosome biogenesis leading to parasite survival (Lodge and Descoteaux, 2005). Moreover, *Leishmania* parasites interfere with antigens loading onto major histocompatibility complex (MHC) class II molecules and subsequent presentations to T cells (Fruth *et al*., 1993; De Almeida *et al*., 2003) as well as inhibiting secretion of CD4+ T cell-activating IL-12 by macrophages (von Stebut *et al*., 1998). On the other hand, VL patients become immunocompromised and subject to secondary infections (Bryceson, 1996) as a result of antigen-encountered T cell exhaustion (gradual loss of function) due to chronic parasite persistence (Wherry, 2011).

1.8 Diagnosis of leishmaniasis

The diagnosis of leishmaniasis is complex because its clinical features are shared by a host of other commonly occurring diseases such as cutaneous mycobacterium infections, leprosy, and skin infections for CL and MCL (David and Craft, 2009) and malaria, typhoid, and tuberculosis for VL (Sundar and Rai, 2002). For clinical diagnosis the WHO established a clinical case definition of VL as persistent fever (two weeks or more) and splenomegaly in a person residing in a VL-endemic area (WHO, 1996).

The laboratory diagnosis is divided into parasite detection, serology and molecular methods. However, only the detection of the parasite can give a final answer in suspected leishmaniasis cases (Singh *et al*., 2006).

1.8.1 Detection and isolation of the parasites

The confirmatory diagnosis of leishmaniasis relies on the microscopic detection of *Leishmania* amastigotes (Leishman-Donovan (LD) bodies) in hemotoxyline/eosin or Geimsa stained smears, prepared from biopsy, wound scraping or nasal mucosa for

CL and MCL, or either tissue, bone marrow, spleen, lymph nodes or liver aspirates, or peripheral blood buffy coat for VL (Singh *et al*., 2000; Liarte *et al*., 2001). Rarely the amastigotes can be demonstrated in the buffy coat of peripheral blood. Such a parasitaemia is common in severely immunocompromised patients such as AIDS (Martinez *et al*., 1993) and patients on immunosuppressive therapy (Maggi *et al*., 2004). Biological material can be aseptically cultured at 24±2°C in several solid or semisolid media such as Novy McNeal Nicole (NNN) rabbit blood agar (Nicolle and Blaizot, 1911; Evans, 1989) or fetal bovine serum (FBS)-supplemented liquid media including, Schneider's medium, RPMI 1640, M199, and HOMEM (Hendricks and Wright, 1979; Berens *et al*., 1976) for the isolation and characterization of the causative agent. Human urine has been successfully used in place of fetal calf serum in *in vitro* culture of *L. donovani* (Singh *et al*., 2000). The patient's sample can also be inoculated into sterile laboratory animals such as hamsters, mice or guinea pigs for parasite recovery (Marsden, 1986).

1.8.2 Serological diagnosis

In CL and MCL, the humoral immune response is extremely poor while hyperimmunoglobulinaemia is a hallmark in VL (Singh *et al*., 2006). Specific and non-specific tests, which detect and measure antibodies in the serum, and methods, which test cell mediated response to *Leishmania* antigen has been described (Lakshmi *et al*., 1984). Of these, several immunological techniques that measure antibody level or detect *Leishmania* antigens were developed for the diagnosis of VL (Zijlstra *et al*., 2001) including Immunofluorescence antibody assay (IFA) (Badaró *et al*., 1983), enzyme-linked immunosorbent assay (ELISA) (Hommel *et al*., 1978), western blot (Jaffe *et al*., 1984), recombinant Kinesin 39 (rK39) antigen detection tests (Badaro *et al*., 1996; Zijlstra *et al*., 1998; Sundar *et al*., 1998), direct agglutination test (DAT) (Harith *et al*., 1986), or detection of *Leishmania* antigen in urine by latex agglutination (KATEX) (Attar *et al*., 2001). The main asset of these tests is non-invasiveness however, antibody-detecting tests cannot differentiate between clinical, subclinical or past infections (Zijlstra and Elhassan, 2001). Formal gel test is a non-specific technique that detects raised immunoglobulins in VL (Boelaert *et al*., 2004)**.** Tests that detect a delayed hypersensitivity reaction of cell-mediated immune response to *Leishmania* parasites such as Leishmanin skin test

(LST) have diagnostic value in CL and MCL (Montenegro, 1926) however, it is usually negative during active VL (Gidwani *et al*., 2009). The rK39 was initially used in ELISA however, because sophisticated methods cannot be employed in the poor areas of endemicity and generally require skilled personnel; immunochromatographic device based on rK39 has been developed (Sundar *et al*., 1998).

1.8.3 Molecular diagnosis

Since the direct visualization of the parasite has certain limitations, indirect ways of detecting the parasite were implemented; most of them are based on the detection of parasite DNA (François *et al.,* 2007). Several polymerase chain reaction (PCR)-based methods for detection of *Leishmania* DNA in a variety of clinical samples such as skin biopsies and smears, bone marrow and lymph node aspirates and peripheral blood with remarkable sensitivities and specificities have been described (Schallig and Oskam, 2002). These methods have a great value in detecting asymptomatic *Leishmania* infections and prognosis (Fukutani *et al*., 2014). Several target sequences such as ribosomal RNA genes, kDNA, mini-exon-derived RNA genes and genomic repeats have been used (Osman, 1998) and maximum sensitivity can be achieved by using multicopy sequences as the PCR target (Lachaud *et al*., 2002). The kDNA is still one of the most appealing targets for primer design and *Leishmania* detection due to the conserved highly repetitive copies of minicircle DNA present in the kinetoplast (Santarém and Cordeiro-da-Silva, 2007). The qualitative conventional PCR involves electrophoresis and visualization of the product by UV light after ethidium bromide staining (Singh *et al*., 2005). In the real-time PCR, DNA products are analysed during their amplification decreasing the risk of contamination by amplicons (Espy *et al*., 2006). In addition, real-time PCR has the advantage of being quantitative which could be useful in the follow-up of treatment allowing for the assessment of the parasite burden (Mary *et al*., 2004). Loop-mediated isothermal amplification (LAMP) represents a promising avenue for which a simple water bath is needed for amplification, and detection can be done visually by using SYBR-green I dye (Reithinger and Dujardin, 2007). PCR-ELISA method based on the capture of PCR amplicons by specific probes immobilized in micro-plate wells and colorimetric visualization has been developed (Reithinger and Dujardin, 2007). More recent methods such as oligochromatography-PCR (OC-PCR) are developed. In this method

the PCR product is visualized in 5 minutes on a dipstick through hybridization with a gold-conjugated probe (Deborggraeve *et al*., 2006).

1.9 Treatment of leishmaniasis

Despite the wide range of studies carried out during quite a long period of time, there is no reliable vaccine against leishmaniasis available at the present moment (Srivastava *et al*., 2016). Thus, currently the only means to treat and control leishmaniasis is by rational medications and vector control.

Leishmaniasis is treated with a variety of remedies and although there are differences between *Leishmania* species, their susceptibility to drugs, and disease manifestations, the same therapeutic agents are used for treatment (Goto and Lindosos, 2010). Pentavalent antimonials (Sb^{v}; sodium stibogluconate and meglumine antimoniate), amphotericin B deoxycholate, and pentamidine have been the first-line drugs used for treatment of both VL and CL in the Old World and New World (Mishra *et al*., 2007; Goto and Lindosos, 2010). For many years, antimonial pentavalent forms have been used as prodrugs, which are converted to biologically active trivalent forms (Sb^{III}) by the parasite glutathione system (Denton *et al*., 2004). The active forms interfere with glycolysis (Berman *et al*., 1987), oxidation of fatty acids (Croft *et al*., 2006), redox balance (Wyllie *et al*., 2004), and DNA topoisomerase 1 (Chakrabort and Majumder, 1988). Unfortunately, antimonials have toxic effects, parenteral treatment course requires several weeks and the parasites have developed some resistance to them (Guerin *et al*., 2002). The antifungal, amphotericin B was introduced in 1960s. Its antileishmanial activity is attributed to its ability to create pores in the parasite membrane affecting its fluidity due to the high affinity of the drug to the membrane ergosterol (Sundar *et al*., 2006) however; the drug has showed unpleasant side effects such as renal toxicity (Cruz et al., 2009) and is expensive (Sundar and Chatterjee, 2006) and the parasites are becoming resistant to it by reducing their drug-binding ergosterol (Mwenechanya *et al*., 2017). AmBisome, a lipid formulation of amphotericin B in which the deoxycholate is replaced by lipid to mask amphotericin B and reduce its toxic effect on tissues has been developed (Hiemenz and Walsh, 1996) and is effective against VL however, it is expensive (Olliaro and Sundar, 2009). Pentamide was used in treatment of VL in India however; it was abandoned due to its severe side effects and low efficacy

(Sundar and Chatterjee, 2006). Second-line drugs include miltefosine, paromomycin, azoles, azithromycin, allopurinol, dapsone, and rifampicin of which miltefosine and paromomycin are mostly used (Goto and Lindosos, 2010). Miltefosine is the first oral antileishmanial agent and was registered in India in 2002 (Sundar and Chatterjee, 2006). It inhibits and interferes with many essential biochemical activities (Luque-Ortega and Rivas, 2007; Lux *et al*., 2000; Rakotomanga *et al*., 2007; Maly *et al*., 1995; Wieder *et al*., 1999). The major limitations of miltefosine are teratogenicity, long half-life, high cost, and gastrointestinal and renal toxicity (Sundar *et al*., 2012). Paromomycin is used as parenteral and topical formulations to treat VL and CL respectively (Sinha *et al*., 2011). Combining miltefosine and paromomycin is advantageous since it enhances the macrophages activation leading to release of TNF-α and NO and subsequent parasite killing (Das *et al*., 2012). Immunotherapeutic treatment of human VL with pro-inflammatory cytokines, IFN-γ (Badaro and Johnson, 1993) and IL-12 (Murray and Hariprashad, 1995) has been utilized however; the excessive immunopathology was feared. Alternatively, IL-10 blockade therapy has been used in combination with conventional treatment and it showed a noticeable success in reducing parasite loads (Singh *et al*., 2014).

Photodynamic and thermotherapy (heat therapy) were developed for local treatment of cutaneous lesions caused by *Leishmania* parasite however, these methods cannot affect disseminated forms of the disease (Kobets *et al*., 2012)

Anyway, since overcoming the drug resistance issue is important (Bhandari *et al*., 2014), it is always necessary to search for new leishmanicidal compounds.

2. Rationale and objectives

Detection of the leishmaniasis infections and prompt, effective treatment are fundamental in the disease control strategies. Unfortunately, many VL cases are cryptic representing potential parasite sources, and most of the current chemotherapeutic agents show toxicity and suffer resistance obstacles thus, new compounds and new drug targets are sought. However, development of new drug formulations is a gigantic task that requires in depth understanding of the parasite biochemistry and biology in order to find parasite pathways in some way different from those of the mammalian hosts. In this work, I have probed *Leishmania* and leishmaniasis in biochemical and clinical studies. For the biochemical part, in one study I analysed the effect of the niacin analogue, 6-AN on *Leishmania* parasite growth and metabolism using the metabolomics technology. The rational was that, the pentose phosphate pathway (PPP), the major provider of antioxidants, has been reported as a target of 6-AN (Kohler *et al*., 1970) and *Leishmania* PPP enzymes have significant differences compared to those of the mammals (Hanau *et al*., 2009; Gupta *et al*., 2011). The other biochemical study was carried out to detect and characterize *Leishmania* transglutaminase (TGase) enzymes that catalyze the irreversible protein crosslinking by creating isopeptide bonds within or between proteins/polypeptides. Since the isopeptide bond shows high resistance to proteolytic and detergent degradation, this enzyme could be one of the parasite's virulence factors in that it crosslinks parasite- or host-derived proteins around the parasites protecting them from the host immunity. Therefore, if these enzymes are fundamental for *Leishmania* parasites, they could represent another promising drug target. TGase activity has been detected in promastigote and amastigote stages of some *Leishmania* species (Brobey and Soong, 2006) however, the protein TGase has not been purified, cloned, and characterized. The second part of the thesis involved a more clinical study that evaluated in detail using advanced molecular techniques and cytokine analysis the prevalence of asymptomatic/subclinical *Leishmania* infections in autoimmune rheumatic patients treated with immunosuppressive biological drugs (anti-TNF-α antibodies, modulators of T lymphocyte activity, or anti-IL-6 receptor antibodies) and living in *Leishmania* endemic foci in Italy. Since TNF-α is the macrophage arm in their

killing of the intracellular parasites, opportunistic VL can occur if this cytokine is neutralized.

The biochemical part

Chapter 2

Metabolomics analysis of *Leishmania* promastigotes treated with 6-aminonicotinamide (6-AN)

2.1 Introduction

2.1.1 General introduction

Treatment of leishmaniasis is a complicated process due to problems associated with drug toxicity and parasite's resistance to current antileishmanial agents, new drugs and targets are needed (Barrett and Croft, 2012).

The pentose phosphate pathway (PPP, Fig. 2.1) has been one of the research areas that could offer new treatment possibilities since it maintains the redox balance in cells. It has two parts, a three-step unidirectional, oxidative one that utilizes glucose 6-phosphate (G6P) and oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) as a cofactor, and produces the reduced NADPH through the catalytic action of the first enzyme, glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and the third enzyme, 6-phosphogluconate dehydrogenase (6PGDH, EC 3.1.1.17) (Dickens and Glock, 1951; Glaser and Brown, 1955).

phosphoribosyl pyrophosphate (PRPP), as well as some carbohydrate intermediates (like those used in aromatic amino acids biosynthesis) which then recycle back to glycolysis (Kovarova and Barrett, 2016). All the enzymes of the PPP are found to be functional in *Leishmania* (Louassini *et al*., 1999). Inhibition by RNA interference of 6-phosphogluconate dehydrogenase (6PGDH) expression, the third enzyme in the oxidative part, is lethal to the bloodstream form of *Trypanosoma brucei*, a closely related parasite (Kerkhoven *et al*., 2013). Glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme in the PPP oxidative part (Ghosh *et al*., 2015) and ribose-5-phosphate isomerase (RPI) (Faria *et al*., 2016), in the non-oxidative part, have also been shown to have important roles in *Leishmania* parasites viability.

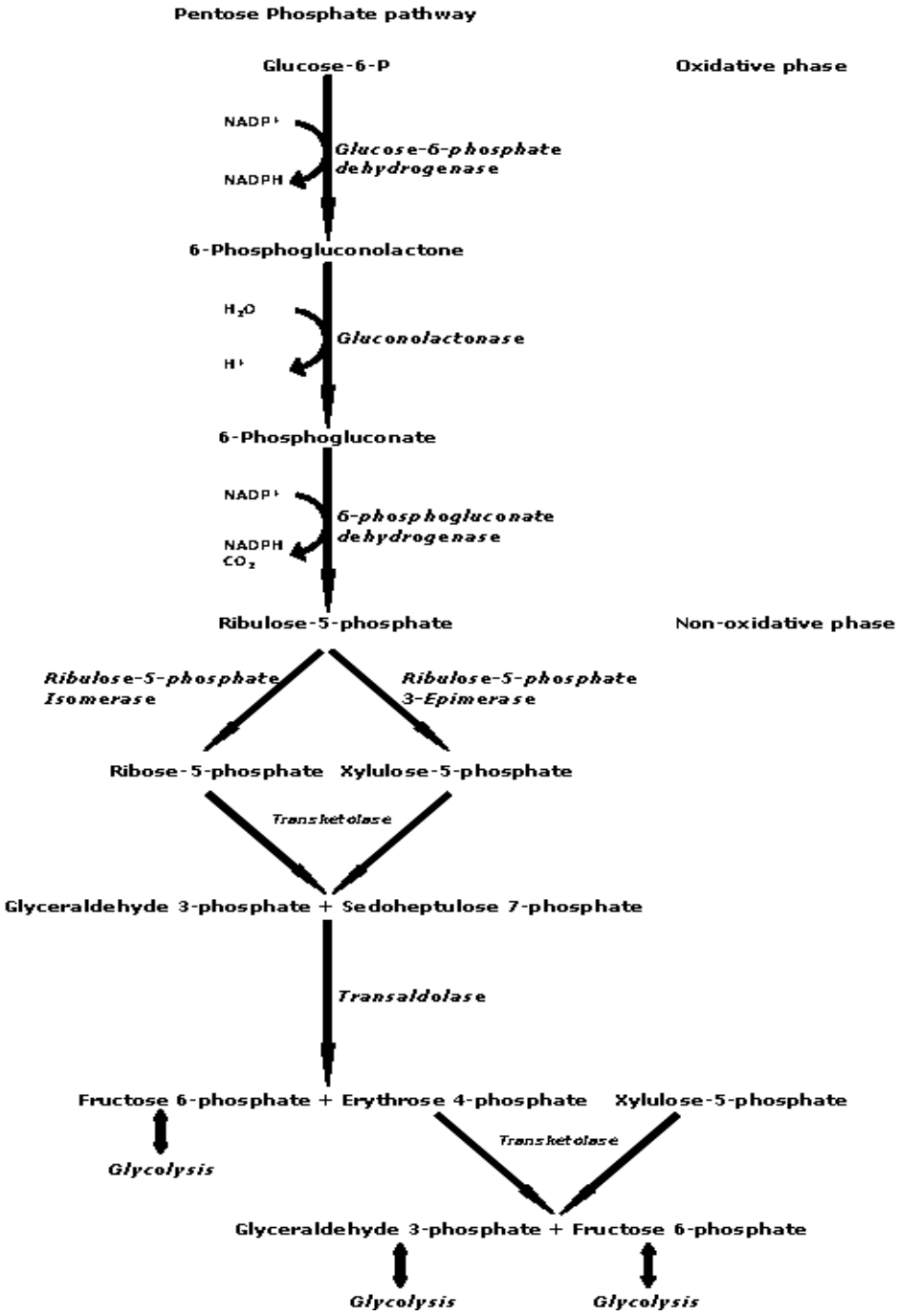


Figure 2.1. A graphical depiction of the pentose phosphate pathway. The oxidative part utilizes glucose 6-phosphate and $NADP^+$ as a cofactor to produce NADPH and ribulose 5-phosphate while the non-oxidative part proceeds to generate ribose 5-phosphate and other sugar intermediates with some of them are recycled in the glycolysis pathway. (https://upload.wikimedia.org/wikipedia/commons/6/61/Pentose_Phosphate_Pathway.png).

NADPH is fundamental in protecting the cells from the oxidative stress and detoxification of xenobiotics by its ability to act as a hydrogen donor (Barrett, 1997). Other enzymatic reactions such as those catalysed by the malic enzyme also produce NADPH (Mottram and Coombs, 1985). The second bidirectional non-oxidative part provides the cells with the ribose-5-phosphate (R5P) sugar essential for purine and pyrimidine nucleotides, and NAD^+ synthesis after being converted to the

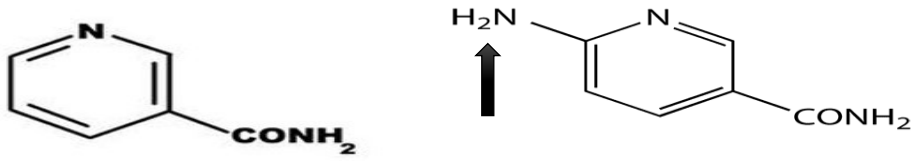
Among leishmanicidal compounds, antimonials are drugs whose action depends on creating oxidative stress, since they impair the antioxidant defence system of the parasite represented by reduced trypanothione [$T(SH)_2$] (Wrenger *et al*., 2013; Mittal *et al*., 2007; Cunningham and Fairlamb, 1995). The NADPH-dependent trypanothione reductase is a pivotal enzyme that maintains trypanothione in its reduced form (Tovar *et al*., 1998).

2.1.2 6-aminonicotinamide (6-AN)

6-AN is a powerful nicotinamide analogue (antimetabolite) (Johnson and McColl, 1955). It differs from nicotinamide (NAm) in that it has an additional amine group at carbon number 6 (Fig. 2.2A), which might interfere with the functional reactions (Dietrich *et al*., 1958). 6-AN is taken up by the mammalian cells and converted to 6-aminoNAD (6-ANAD) and 6-aminoNADP (6-ANADP) through the action of nicotinamide adenine dinucleotide (NAD^+) glycohydrolases (EC 3.2.2.5), which normally hydrolyze NAD/P generating NAm and adenosine diphosphate (ADP)-ribose (Fig. 2.2B). These enzymes exchange 6-AN for NAm generating the non-authentic cofactors (Zatman e*t al.,* 1953). However, microbial NAD^+ glycohydrolases do not catalyze exchange reactions (Zatman *et al*., 1953) and instead, they utilize 6-AN in the Preiss-Handler part of the NAD^+ biosynthesis pathway generating the non-physiological analogue of NAD^+ (Gholson, 1966). 6-ANADP is unable to act as a cofactor due to its inability to carry hydrogen in oxidation/reduction systems thus represents a strong competitive inhibitor of PPP dehydrogenases (Dietrich *et al*., 1958) with 6PGDH 400 times more sensitive to 6-AN than G6PDH and other $NADP^+$-requiring enzymes (Kohler *et al*., 1970). Blocking 6PGDH activity results in an accumulation of 6-phosphogluconate (6-PG), which in turns inhibits the phosphoglucose isomerase (PGI) (Kahana *et al*., 1960) leading to inhibition of glycolysis and subsequent ATP insufficiency too (Street *et al*., 1996). 6-AN is found to

be a chemo and radio sensitizer potentiating the effects of either radiation or drugs in cancer cells possibly, by inhibiting energy-dependent DNA repair processes (Keniry *et al*., 1989; Varnes, 1988; Street *et al*., 1996). 6-AN treatment of murine leukaemia cells resulted in reduced adenosine triphosphate (ATP):adenosine diphosphate (ADP) ratio and NAD^+, depletion of purine and pyrimidine nucleotides and growth inhibition (Hunting *et al*., 1985). In contrary, Kolbe *et al*., (1969) reported no effect on ATP while it was found elevated by Sheffield and Seegmiller, (1980). Reduction in NAD^+ affects NAD^+-requiring enzymes leading to a decrease in poly (ADP)-ribose needed for DNA repair (Berger *et al*., 1982). Administration of 6-AN into animals results in inhibition of haemopoietic systems, central nervous system damage with limb paralysis, and death (Baserqa *et al*., 1956; Sternbergs and Philips, 1958; Morsiani and Soffritti, 1956; Wolf and Cowen, 1959). Clinical use of 6-AN has shown drastic adverse effects. Patients with advanced and disseminated cancers treated with 6-AN at low dose have developed pellagra (nicotinamide deficiency)-like symptoms and at high dose showed neurologic disturbances and ocular signs without reduction in tumor size however, if used in combination with other remedies, 6-AN might be useful (Herter *et al*., 1961; Perlia *et al*., 1961). Topical treatment of psoriasis with 6-AN was successful (Zackheim, 1975).

(A)



Nicotinamide **6-aminonicotinamide**

(B) **1- NAD/P + H_2O ⇌ ADP-ribose/enzyme intermediate+ nicotinamide**

2- ADP-ribose + nicotinamide or 6-AN ⇌ NAD/P or 6-ANAD/P

Figure 2.2. A. The chemical structure of nicotinamine and 6-AN. The arrow denotes the additional amine group at carbon 6 of 6-AN. B. The exchange reactions (transglycosidation) catalysed by NAD^+ glycohydrolases, which hydrolyze NAD/P (B.1) but if nicotinamide or 6-AN is at an inhibitory concentration the reaction will go back generating either NAD/P or 6-ANAD/P (B. 2) (Zatman e*t al.,* 1953).

2.1.3 Metabolomics

Metabolomics analysis is used to study the mode of action of drugs and the mechanism of microorganism resistance to them as well as revising and elucidating new biochemical pathways (Vincent and Barrett, 2015). Metabolite distribution reflects what happens at the genomic and transcriptomic level in cells in response to environmental stimuli and stressors (drug perturbation) (Roessner and Bowne, 2009). Due to the chemical diversity of the metabolites, there is no an ideal universal metabolomics technique that can cover all metabolites of a biological system (Creek *et al*., 2011)**.** Mass spectrometry (MS) coupled to selected chromatographic methods e.g., liquid chromatography (LC-MS) and gas chromatography (GC-MS), is the technique widely used in separation and identification of small-molecule metabolites. Extracted metabolites are separated by the chromatographic technique first, then their levels are detected by MS (metabolite mass and retention time, RT) or nuclear magnetic resonance (NMR) (vibration energy of chemical bonds) (Fig. 2.3) (Vincent and Barrett, 2015). LC is a common system used for untargeted (qualitative) metabolomics analysis because it separates the majority of the cell metabolites. Of these LC methods, hydrophilic interaction liquid chromatography (HILIC) is the popular method to separate polar metabolites (Ramautar and de Jong, 2014). An efficient computer-based program is required for data analysis, which makes metabolomics a meaningful tool.

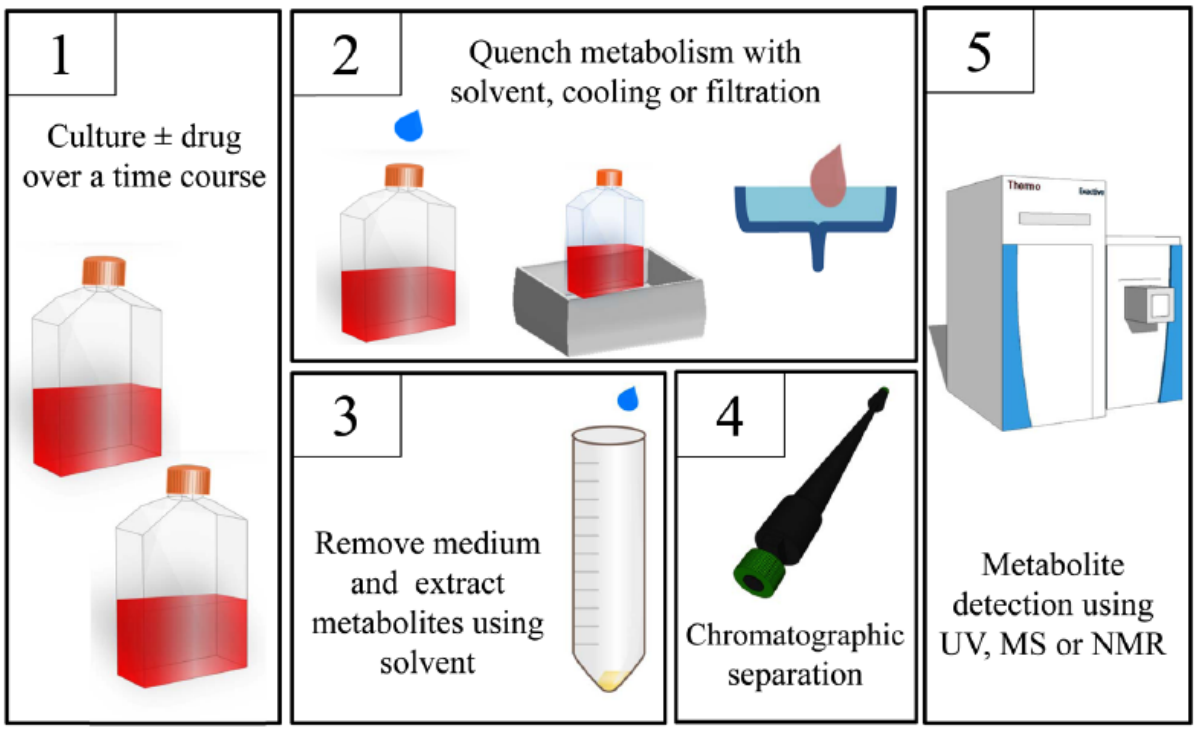


Figure 2.3. Metabolomics work flow (from 1 to 5) for disclosing drug effects and mode of action. Cells are cultured in the presence and absence of the drug. Cells are washed to remove the medium, their metabolism is quenched and small metabolites are extracted. Metabolites are chromatgraphically resolved and detected. Adapted from Vincent and Barrett, (2015).

2.2 Materials and methods

2.2.1 Materials

All compounds were purchased from Sigma-Aldrich, GmbH, Germany. 6-aminonicotinamide (6-AN) was prepared in DMSO to a maximum concentration of 0.729 M. 6-amininicotinic acid (6-ANA) was solved in RPMI medium at 20 mM concentration. Methylene blue (MB), malachite green (MG) and amphotericin B (AmB) were prepared in double distilled water at concentrations of 2.97 mM, 10 mM, 0.27 mM and 30 mM respectively. All the compounds were filter sterilized through 0.22 µm filters.

2.2.2 Parasites and cultures

Three strains were used in this study, human M379 *Leishmania (L) mexicana* (MHOM/GT/2001/U1103), human PCM5 *L. infantum* strains (MCAN/ES/98/LIM-877) and canine MO1 *L. infantum* (from IZSLER in Modena, Italy).

The promastigotes of the strains M379 and PCM5 were routinely cultured in HOMEM medium (pH 7.2) (Berens *et al*., 1976) (GE Healthcare Bioscience, GmbH, Austria) supplemented with 10% heat inactivated foetal calf serum (HIFCS) (Gibco, Paisley, UK), 100U/ml Penicillin, and 100U/ml Streptomycin sulphate in 25cm^2 non-vented flasks (Corning, USA) at 25°C in a humidified incubator under air as a gas phase. The promastigotes of the MO1 strain were cultured in RPMI 1640 medium supplemented with 15% HIFCS and antibiotics as in HOMEM medium.

Axenic amastigotes of *L. mexicana* were prepared and continuously cultivated in Schneider's Drosophila Medium (SDM) (pH 5.5) supplemented with 20% HIFCS and 15µg/ml HEMIN (Bates *et al*., 1992). For the initiation of the amastigote cultivation, promastigotes were seeded in 25cm^2 vented tissue culture flasks (Corning, USA) at a density of 10^6 cells/ml and incubated at 32°C in a humid incubator with 5% CO_2 for one week. The transformed amastigotes were passed through a 26-gauge syringe to separate their clumps before counting and routinely cultured under the same conditions and passaged in a fresh medium once a week.

2.2.3 Antileishmanial activity

To find IC_{50} values against *Leishmania* growth, in 96-well flat-bottom polystyrene plates (Greiner Bio One Ltd), 15.6 mM 6-AN, 10 mM 6-ANA, 100 µM MB, 100 µM

MG, and 2 µM AmB were two-fold serially diluted 11 times in 100 µl volume of complete culture medium/well. One hundred microliters of parasite suspension was added to each well so the final parasite density in each well was either 10^5cells/ml in some experiments or 10^6 cells/ml in some others. The treated parasites were incubated for 72 hours at 25°C. The number of viable cells was then measured either using a hemocytometer, after fixing with 2% formaldehyde in phosphate buffer saline (PBS) pH 7.4, or by Alamar Blue reduction, fluorescence measurement (Lancaster and Fields, 1996). In the latter case 20 µl ($1/10^{th}$ of the total volume in each well) of filter-sterilized Alamar Blue (SIGMA-ALDRICH CHEME, GmbH, Germany) was added to both treated and control cells in plates suitable for fluorescence analysis. After 48-hour (for parasites cultured in HOMEM) or 72-hour (for parasites cultured in RPMI) incubation at 25 °C the reaction was stopped by addition of 50 µl of 3% SDS in PBS (pH 7.4), in each well, for fluorescence measurement at 530 nm excitation and 590 nm emission wavelengths. Treatments were done in triplicate and in several independent experiments, the IC_{50} values were calculated using Prism 6.0 software nonlinear dose-response curve mode.

2.2.4 Compound interactions analysis

The modified fixed-ratio isobologram method was used to ascertain whether the mode action of the compounds is synergistic, indifferent or antagonistic (Fivelman *et al*., 2004; Li RC *et al*., 1993). The IC_{50} values of either 6-AN, MB or MG were determined first. The starting concentration of both MB and MG were 32 times their IC_{50} values. To avoid the effect of the DMSO (solvent) on the parasites, 6-AN was prepared at a concentration 4 times its IC_{50}. Combinations ratios of 6-AN:MB and MB:MG were 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 (combinations below).

Combo	Ratio	Volume required
1	5:0	500µl 6-AN
2	4:1	400µl 6-AN+100µl MB
3	3:2	350µl 6-AN+150µl MB
4	2:3	150µl 6-AN+350µl MB
5	1:4	100µl 6-AN+400µl MB
6	0:5	500µl MB

Combo	Ratio	Volume required
1	5:0	500µl MB
2	4:1	400µl MB+100µlMG
3	3:2	350µl MB+150µl MG
4	2:3	150µl MB+350µl MG
5	1:4	100µl MB+400µl MG
6	0:5	500µl MG

For each ratio, two-fold serial dilutions were done 11 times, and the IC_{50} values were calculated for each compound, then fractional inhibitory concentrations (FIC) were

obtained by dividing the IC_{50} values by the IC_{50} of the drug alone. The isobologram involved plotting one compound FIC values against the other compound's FIC values. Each drug combination experiment was replicated three times.

2.2.5 Microscopic analysis of *L. mexicana* promastigotes treated with 6-AN

Procyclic promastigotes were passaged daily for a week and then seeded at a density of 10^5 cells/ml, in complete HOMEM medium with either 15.6 mM 6AN or 2.14% DMSO alone, and in medium alone, as untreated control. Parasites were incubated at 25°C in a humidified incubator under air as a gas phase for 72 hours. Smears were prepared at a time course; zero, 6, 14, 48, and 72 hours. Smears were fixed in absolute methanol for 30 seconds and then stained for 10 min with 8% Giemsa stain (Fluka Analytical, Sigma-Aldrich, Germany) in Sörensen staining buffer. Slides were then washed with tap water, air dried, and examined under a light microscope using 100X magnification.

2.2.6 Macrophage preparation and infection

On day 0, bone marrow macrophages were prepared from the hind legs of male C57BL/6 mice aged between 8 and 12 weeks old under sterile conditions. Tibias and femurs were cut at both ends and the bone marrow was flushed out in petri dishes using a 5 ml syringe and a 25-gauge needle filled with complete RPMI 1640 (RPMI supplemented with 1% Penicillin/Streptomycin and 10% FBS, cRPMI) medium. Clumps were broken-up by repeatedly sucking up and pushing out the medium. The cell suspension was passed through a 70µm-sterile cell strainer (Greiner Bio-One) into a 50 ml falcon tube and then centrifuged at 300g for 5 min. The supernatant was discarded and the cells were suspended in 10 ml cRPMI, counted and resuspended at a density of 6-7 x 10^6 cells/ml. They were then plated in petri dishes by adding 1 ml of this suspension to a mixture of 2 ml filtered L929 fibroblasts supernatant and 7 ml cRPMI, and incubated at 37°C and 5% CO_2. On day 3, each petri dish was supplemented with 5 ml cRPMI and 2ml L929 supernatant. On day 6, the medium was removed and the petri dishes were washed 3 times in warm cRPMI to remove non-adherent cells. Six milliliters ice cold PBS were added to each dish and left on ice for 1-2 minute. Cells were scrapped from the bottom of the dish, collected in a 50 ml falcon tube, spun at 300g 5 minutes and resuspended at a density of 10^6 cells/ml in

warm cRPMI, then transferred to chamber slides (Lab-Tek® Chamber Slide Products), 200µl/well and incubated for 24 hours at 32°C and 5% CO_2 (Marim *et al*., 2010).

Procyclic *L. mexicana* promastigotes were seeded at 10^5 cells/ml and treated with 15.8 mM 6-AN in complete HOMEM medium in twelve-well tissue culture plates for a time-course incubation; 7, 5, and 3 days, 24 and 8 hours. Parasites treated with 2.14% DMSO (the solvent used to dissolve 6-AN) were used as controls. Before infection the parasites were washed twice in warm cRPMI, re-suspended in cRPMI and counted. Macrophage/parasite at a ratio of 1:4 were incubated in chamber slides for 24 hours at 32°C and 5% CO_2. After that extracellular parasites were removed by washing the slides three times in cRPMI and then macrophages were incubated for 5 days with daily washing and medium replacement. After 5 days, the medium was removed and cells were washed with warm PBS and the plastic chambers were detached. The slides were then fixed while wet in absolute methanol, stained with 8% Giemsa and examined microscopically using the oil immersion lens (100X magnification). This experiment was done in duplicate.

2.2.7 Metabolomics analysis

2.2.7.1 Metabolite extraction

Small molecule metabolites of *Leishmania* promastigotes were prepared for mass spectrometry analysis of the global metabolome according to the method of Creek *et al.* (2011).

Promastigotes of either M379 *L. mexicana* or PCM5 *L. infantum* were seeded at a density of 10^6 cells/ml in 8 non-vented, 75 mm^2 tissue culture flasks, each with a total volume of 20ml. 6-AN (in DMSO) was added to 4 flasks at a final concentration of 7.8 mM. DMSO alone was added to the other 4 flasks at the same percentage (1.07%) used with 6-AN. After 24-hour incubation at 25°C, cells in each flask were counted and transferred at an equal density to a 50-ml falcon tube and then a rapid quenching was carried out in a dry ice/ethanol bath. Tubes were spun down for 10 minutes at 1200g at 4°C, the medium was aspirated and each pellet was suspended in 1 ml ice cold PBS and transferred to eppendorf tube. Tubes were centrifuged at 1200g, 4°C for 5 min, supernatants were removed and 200 µl of extraction solvent (Chloroform:Methanol:Water; 1:3:1) were added to each tube, with a brief mixing.

Then, 500 µl of the extraction solvent were added to an empty tube and was used as a blank. After that, samples were shaken for 1 hour at 4°C, centrifuged at 16,060g, 4°C for 10 min, and supernatants were swiftly transferred to labelled screw-cap mass spectrometry vials. Forty microliters from each sample were pooled together in a new vial and this was taken as a quality control (QC) sample. Finally, air was displaced on the top of the samples with argon gas and tubes were stored at -80°C.

2.2.7.2 Hydrophobic interaction liquid chromatography-Mass Spectrometry (pHILIC-LC-MS)

LC-MS analysis was performed on a Dionex UltMate 3000 RSLC system (Thermo Fisher, Hemel Hempstead, UK) using a ZIC-pHILIC column (150 mm × 4.4 mm, 5µm column, Merck Sequant). *L. mexicana* and *L. infantum* 6-AN, DMSO, and QC samples were maintained at 4°C prior use. Ten microliter sample volume was injected and the column was maintained at 30°C. Mobile phase A consisted of 20 mM ammonium carbonate in water, and B consisted of acetonitrile and the flow rate was of 0.3 ml/min. Gradient elution chromatography was performed starting with 20% solvent A. Within a 15 min time interval, solvent A was increased to 80% and solvent B decreased to 20%, followed by an increase of A to 95% and a decrease of B to 5% within 15 minutes and maintained for 17 minutes. Then the system returned to the initial solvent composition in 17 minutes and left to re-equilibrate under these conditions for 24 minutes. For MS analysis of *L. mexicana* metabolites, a Thermo Orbitrap qExactive machine (Thermo Fisher Scientific, UK) was operated in polarity (positive and negative) switching mode and the MS setting were; resolution 50,000, AGC 106, m/z range 70-1400, sheath gas 40, auxiliary gas 5, sweep gas 1, probe temperature 150°C, capillary temperature 275°C. For *L. infantum* metabolites, an Orbitrap Fusion (Thermo Fisher Scientific) was operated in polarity switching mode and the MS setting were, resolution 120,000, AGC 200,000, m/z range 70–1000, sheath gas 40, auxiliary gas 5, sweep gas 1, probe temperature 150°C and capillary temperature 325°C. For the positive mode ionisation, the source voltage was +4.3 kV whereas, for the negative mode ionisation, the source voltage was -3.2 kV. S-Lens RF Level was 30.00%. Prior to each analysis batch, mass calibration for each polarity was performed. Small metabolites calibration was done by the inclusion of low-mass

contaminants in the standard (Thermo calmix masses). Electrospray ionization was used for both positive and negative modes.

2.2.7.3 Data processing pipeline

For data analysis, IDEOMv19 (http://mzmatch.sourceforge.net/ideom.php) (Creek *et al*., 2012) was used. Here raw files are converted to mzXML files and split polarity by proteowizard (msconvert) (Chambers *et al*., 2012; Holman *et al*., 2015). XCMS is then run to pick peaks and convert them to PeakML format (Smith *et al*., 2006; Tautenhahn *et al*., 2008; Benton *et al*., 2010). A key component of our data processing pipeline is application of the default noise filters of mzMatch.R and IDEOM to obtain a list of monoisotopic peaks representing putative metabolites present in the label-free sample. After processing and deletion of noisy peaks, lipids, fatty acids and peptides (whose identifications are not reliable on this platform), 212 peaks were putatively identified by IDEOM (levels 2 and 3, Metabolomics Standards Initiative; MSI) in *L. mexicana* and 195 in *L. infantum*. A total of 61 metabolites were identified confidently in *L. mexicana* and 62 in *L. infantum* by exact mass and retention time based on authentic metabolite standards (level 1 identification according to the MSI) (Sumner *et al*., 2007). Other identifications remain putative. Additional peaks within the rejected list may contain relevant information for later interpretation. Metabolomics data have been deposited to the EMBL-EBI MetaboLights (Haug *et al*., 2013) database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTBLS443. The complete dataset can be accessed here (http://www.ebi.ac.uk/metabolights/MTBLS443).

2.3 Results

2.3.1 Antileishmanial activity

The effect of 6-AN is leishmaniostatic at high concentrations as seen in growth pattern of *L. mexicana* promastigotes (Fig. 2.4) where parasites treated with 7.8 mM 6-AN (IC_{50}) grew very slowly compared to the DMSO and untreated controls. However, they regained growth after 2 days incubation. Parasites treated with 15.6 mM 6-AN did not grow. *L. mexicana* amastigotes were more resistant to 6-AN therefore, it was difficult to calculate the IC_{50}. Promastigotes of the PCM5 human and MO1 canine *L. infantum* strains were somewhat more sensitive to 6-AN than those of *L. mexicana* strain, showing lower IC_{50} values at 4.3 mM and 1.8 mM (Table 2.1). In contrast, 6-aminonicotinic acid (6-ANA) assayed against IZLER MO1 *L. infantum* strain, had no effect even at 10 mM concentration, which could indicate a failure of the parasite to transport this charged molecule across its membrane.

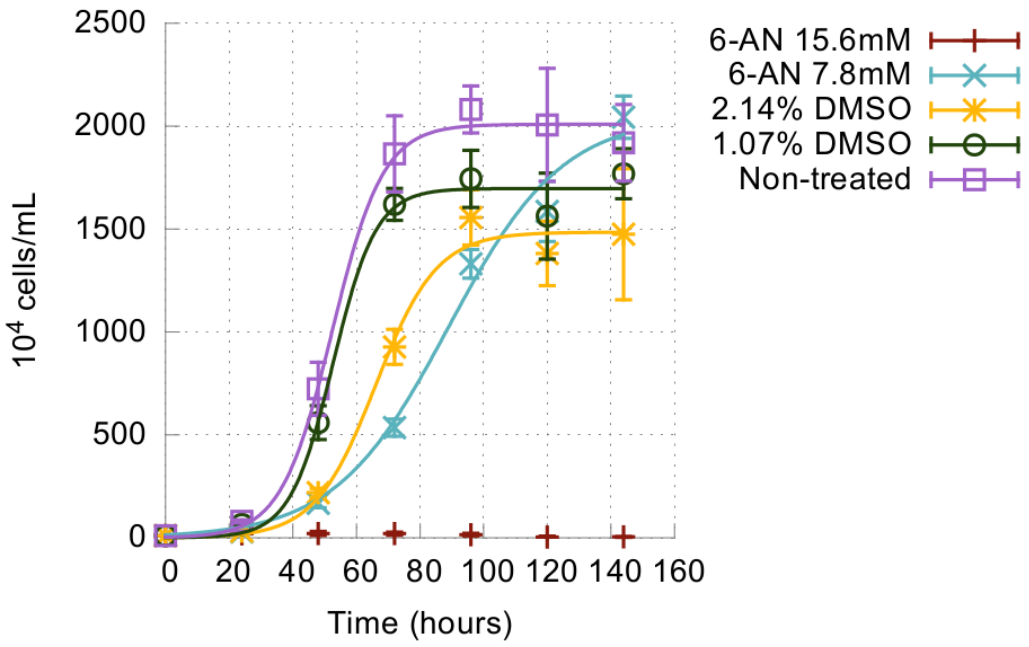


Figure 2.4. 6-AN-treated *L. mexicana* promastigotes growth curve. The graphs are representative of three independent experiments.

Table 2.1. Susceptibility of *L. mexicana* and *L. infantum* promastigotes to 6-aminonicotinamide (6-AN), methylene blue (MB), malachite green (MG) and amphotericin B (AmB). Results are the means ± SD of three independent experiments with each performed in triplicate.

Cell type	Compound	IC_{50}
L. mexicana M379	6-AN	7.8 ± 1.4 mM
	MB	2.8 ± 1.6 µM
	MG	23.5 ± 11.9 µM
	AmB	27 ± 2.3 nM
L. infantum PCM5	6-AN	4.3 ± 1.34 mM
	AmB	24 ± 5.35 nM
L. infantum MO1	6-AN	1.8 ± 0.36 mM

2.3.2 Microscopic analysis of *L. mexicana* promastigotes treated with 6-AN

A leishmaniostatic effect is also evident by the microscopic analysis of *L. mexicana* promastigotes treated with 15.6 mM 6-AN. Smears of procyclic promastigotes of *L. mexicana* treated with 15.6 mM 6AN were prepared at 0, 6, 14, 48, and 72-hour time points. 6-AN-treated promastigotes showed an unusual body elongation (observed in about 50% of the cells) after 6 hours of incubation, while the control cells became long slender nectomonads (late logarithmic forms) after 48-72 hours of incubation (Fig. 2.5).

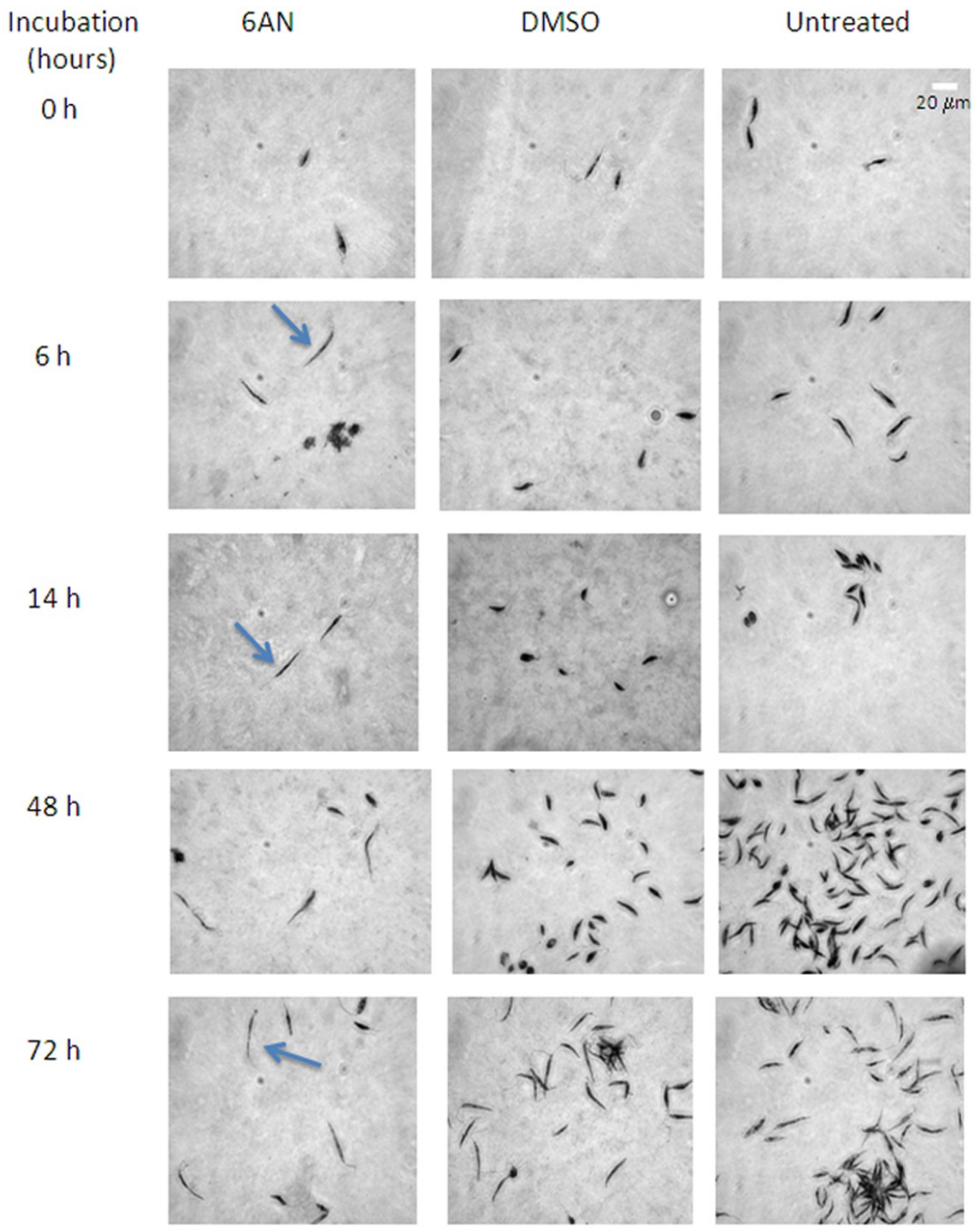


Figure 2.5. General morphology of *L. mexicana* promastigotes treated with 15.6 mM 6-AN, 2.14% DMSO, and untreated controls. Arrows show nectomonad-like elongated parasites seen under a light microscope using 100X magnification. This experiment was replicated three times.

2.3.3 Infectivity of 6-AN-treated *L. mexicana* promastigotes to mouse macrophages

Promastigotes treated with 15.8 mM 6-AN for different times, were used to infect mouse bone marrow derived macrophages. Infectivity of all treated parasites to macrophages was similar to that of the control cells (Fig. 2.6).

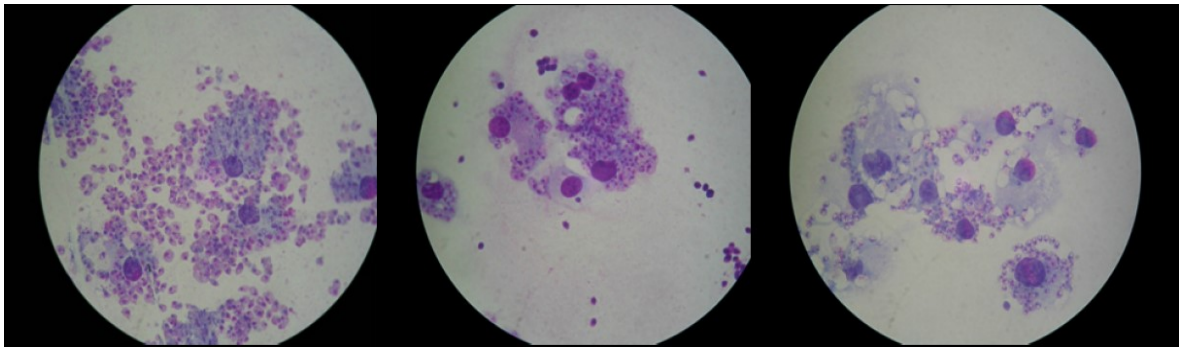


Figure 2.6. *L. mexicana*-infected mouse macrophages. Promastigotes used for infection were untreated (left), treated with 2.14% DMSO (center) and treated with 15.8 mM 6-AN in 2.14% DMSO for 24 hours. Visualization was done under a light microscope using 100X magnification.

2.3.4 Interaction of Methylene Blue with 6-AN or Malachite Green

Although 6AN has relatively poor activity against Leishmania promastigotes, its proposed action against the PPP, with concomitant perturbation of NADPH production and increased sensitivity to oxidative stress, prompted us to determine whether its action was synergistic with stress inducing agents methylene blue and malachite green. By the fixed-ratio isobologram method (Fivelman *et al*., 2004; Li RC *et al*., 1993), it is possible to identify whether the effect of two compounds is additive, when the total fractional maximum effect line (TFME, that is the reciprocal position of the fractional inhibitory effect, FIC, of the two compounds), is a diagonal line connecting the two coordinates (0, 1) and (1, 0), is antagonistic (convex apparent TFME line, that is the points are above the diagonal), or synergistic (concave apparent TFME line, that is the points are distributed below the line). In Fig. 2.7,

representative isobolograms are reported for 6-AN/MB and MG/MB combinations against *L. mexicana* promastigotes. Most of 6-AN and MB combination points are distributed at one corner whereas those of MB and MG are distributed across the line so that all combinations have an additive effect, implying that most probably each compound has different action mechanisms.

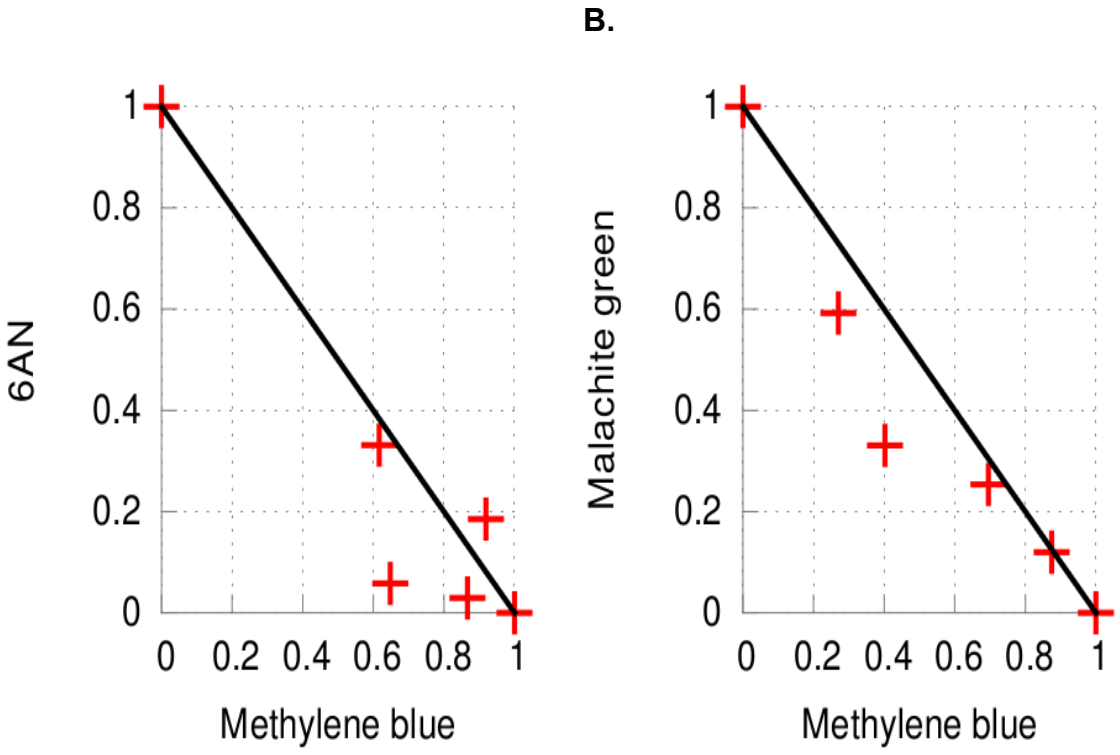


Figure 2.7. Isobolograms of the interaction between 6-AN/MB (A), and MB/MG (B) on *L. mexicana* promastigotes. In the axes the fractional inhibitory concentration (FIC) of 6-AN, methylene blue and malachite green are reported.

2.3.5 Metabolomics profile

Metabolomics analysis was performed on the extracted metabolites of *L. mexicana* strain M379 and *L. infantum* strain PCM5 promastigotes treated, in quadruplicate, for 24 hours with 7.8 (IC_{50}) and 8.6 ($2\times IC_{50}$) mM 6-AN, respectively, at a starting parasite density of 10^6 cells/ml. This incubation time and starting cell density for 6-AN-treated parasites, were chosen in order to extract the metabolites before the cells die or the

control cells, treated with DMSO at percentages equal to those used with 6-AN, enter the stationary phase.

Mass spectral raw data were processed using IDEOM (Creek *et al*., 2012) then filtered manually (as in the methods). The filtered files were then uploaded to MetaboAnalyst 3.0 (Xia *et al*., 2015) to calculate *p* values via t-test and calculate the false detection rates (FDR) using Benjamini–Hochberg correction after having log transformed the data. Any putatively identified metabolite that does not pass the t-test was excluded from the analysis (FDR < 0.05).

2.3.5.1 *L. mexicana*

The 6-AN and DMSO groups were clearly separated according to principal component analysis (PCA) (Fig. 2.8A); 6AN itself is present only in samples of promastigotes treated with 6AN (Fig. 2.8B), as expected. A clear-cut separation is also evident in the heat map of main metabolites that showed differences between 6-AN and DMSO treated *L. mexicana* promastigotes (Fig. 2.9).

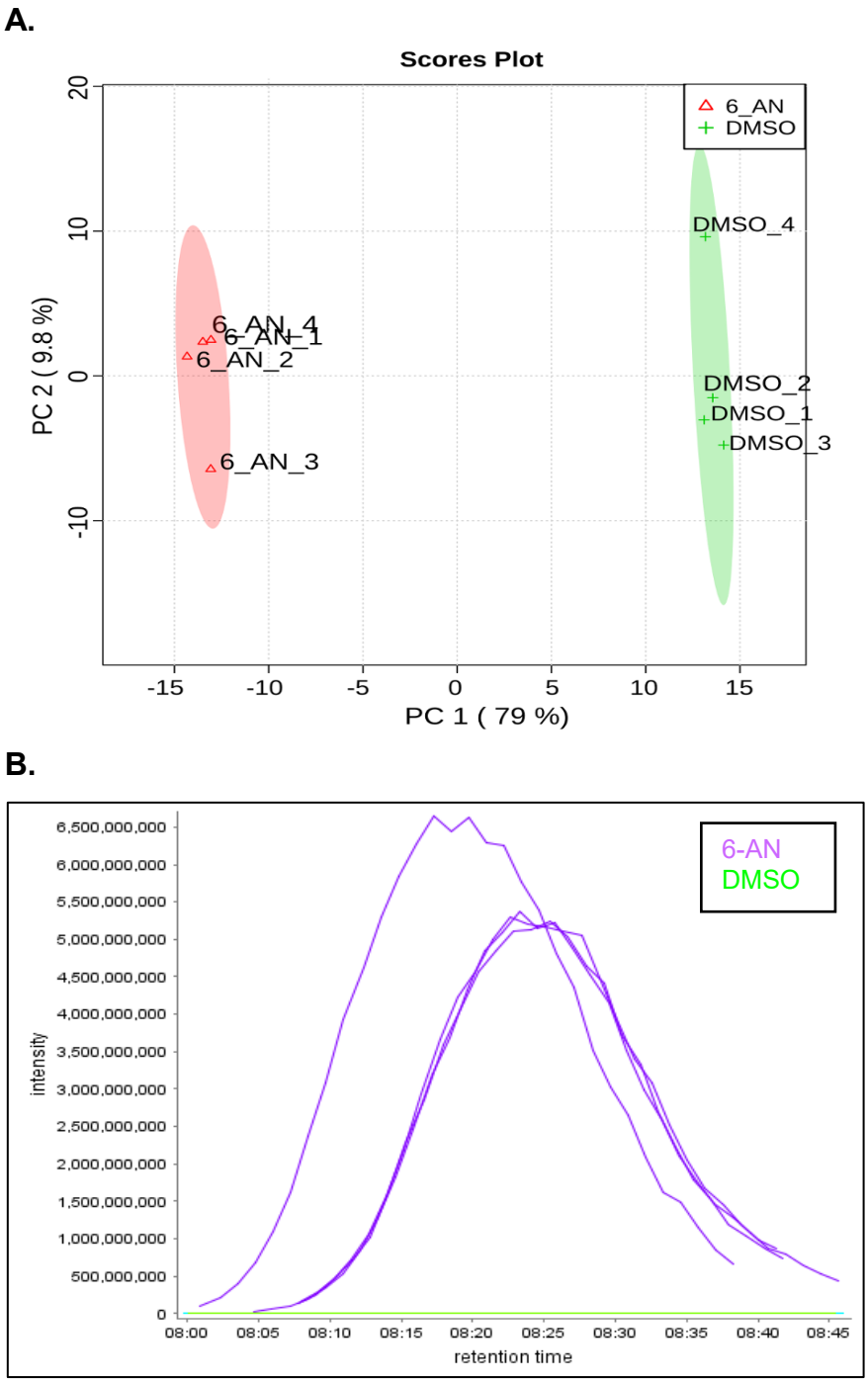


Figure 2.8. A. Principal Component Analysis (PCA) of 6-AN- and DMSO-treated *L. mexicana* samples. 6-AN and DMSO data were uploaded to Metabonalyst; log was transformed prior to PCA (to reduce a biasing effect of high intensity metabolites). The coloured areas denote the 95% confidence interval. B. Absence of 6-AN in DMSO-treated control samples.

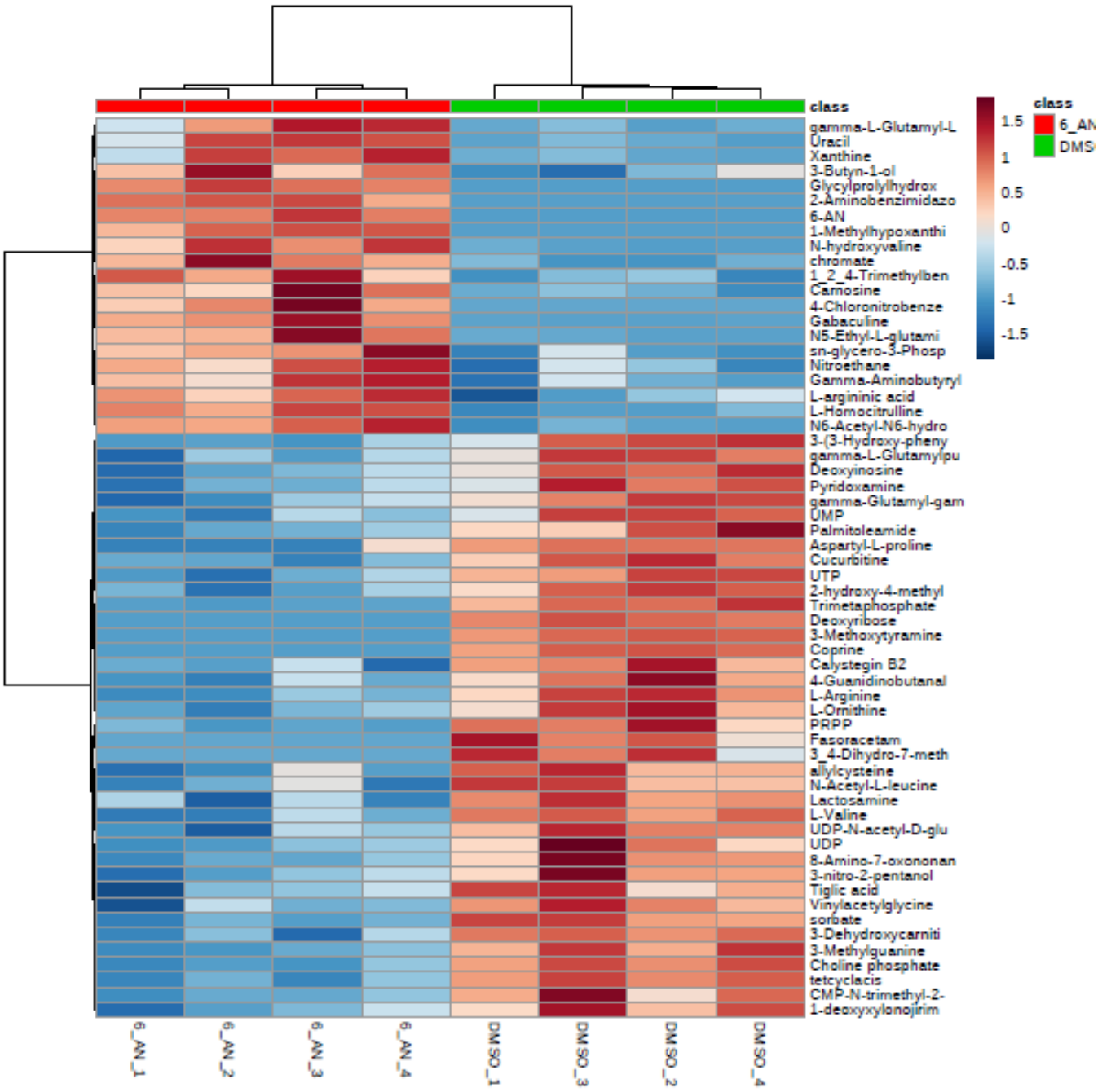


Figure 2.9. The Heat-map of the primary metabolites that differ between 6-AN-treated and control (DMSO-treated) *L. mexicana* promastigotes. The averages of results from four experiments are reported. Red bars depict relative metabolite abundance, while blue bars denote relative metabolite lack.

Glutathione and 6-phosphogluconate (6PG) levels were similar to those of the controls and other downstream carbohydrate intermediates, from the PPP, were unchanged. This indicates that *Leishmania* parasites respond differently to 6-AN than mammalian cells in which accumulation of 6PG has been reported, for example in hepatocytes (Carmona and Freedland, 1990) however, ribose 5-phosphate seems lower in the 6-AN samples (Fig. 2.10).

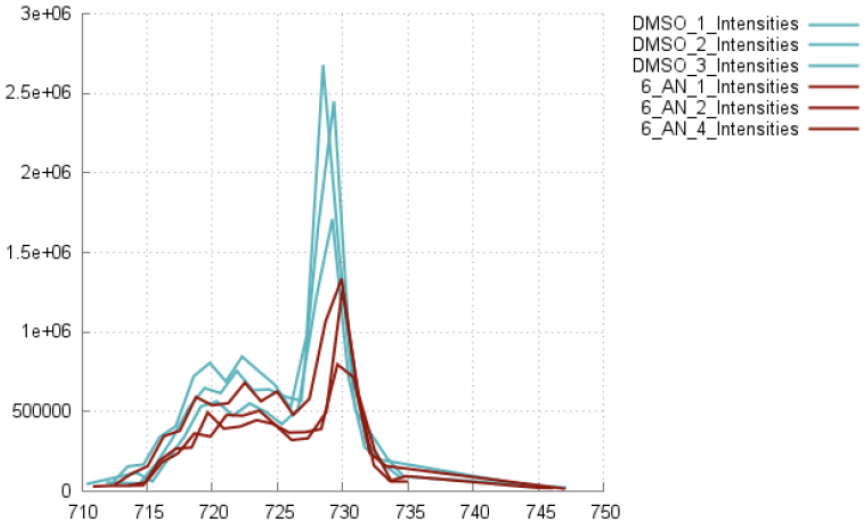


Figure 2.10. Ribose 5-phosphate peaks in 6-AN-treated and -untreated *L. mexicana* promastigotes.

6-AN caused a very marked depletion in the carbohydrate intermediate 5-phospho-□-D- ribose 1-diphosphate (PRPP) and a small (non-significant, FDR=0.09) decrease in nicotinate (Na) (Fig. 2.11A). *Leishmania* parasites are NAD^+ auxotrophic (Gazanion *et al*., 2011). They synthesize NAD^+ from nicotinate, which is a precursor in the Preiss-Handler salvage pathway for NAD^+ biosynthesis, deriving from nicotinamide (NAm) through the activity of nicotinamidase (Fig. 2.11B), which is the obliged step in salvage NAD^+ biosynthesis in these parasites since they do not possess either NAm riboside (NR) kinase or NAm phosphoribosyltransferase, to produce NAm mononucleotide (NMN) (Gazanion *et al*., 2011). Na then combines with PRPP to generate Na mononucleotide (NaMN) in the reaction catalysed by Na

phosphoribosyltransferase. NaMN is then converted to NaAD by NaMN adenylyltransferase, and NaAD to NAD^+ by an ammonia-dependent NAD^+ synthase.

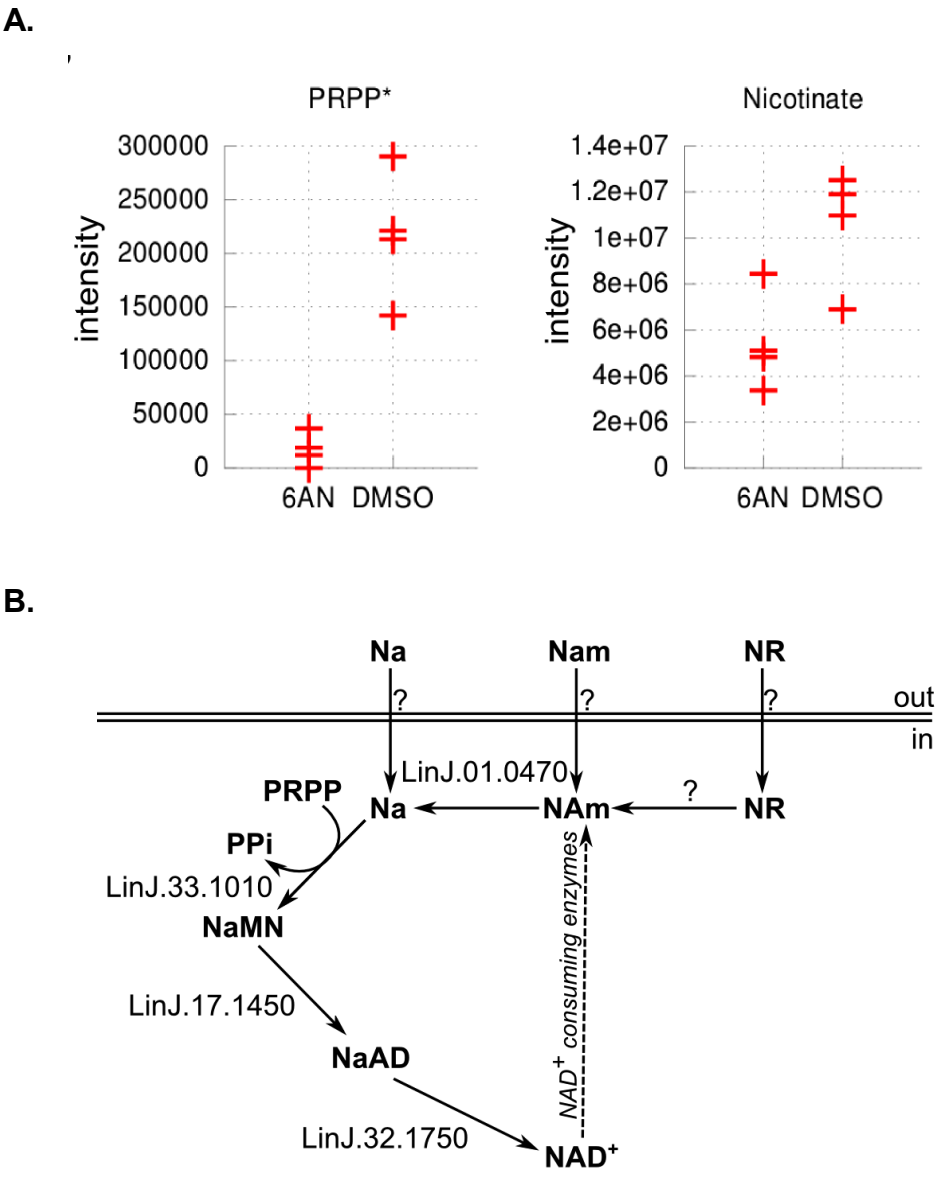


Figure 2.11. A. PRPP and Nicotinate levels in 6-AN-treated parasites and DMSO controls. *: FDR < 0.05. B. The Preiss Handler pathway for NAD^+ synthesis in *Leishmania*. Abbreviations: NAm, nicotinamide, Na, nicotinate, NaMN, nicotinate mononucleotide, NaAD, Nicotinate adenine dinucleotide, NAD, Nicotinamine adenine dinucleotide, 5-phospho-α-D-ribose 1-diphosphate, PRPP, diphosphate, PP (modified from Gazanion *et al*., (2011).

PRPP is also used in the purine and pyrimidine nucleotides syntheses. *Leishmania* lacks the *De novo* purine biosynthetic pathway depending exclusively on salvage pathway while both de novo and salvage pathways exist for pyrimidine synthesis. Purine and pyrimidine nucleotides (UTP, UDP, and UMP), nucleotide derivatives like UDP-glucose and UDP-N-acetyl-glucosamine (Fig. 2.12) all showed peaks of lower abundance in the 6-AN-treated than in the DMSO-treated control cells. By contrast, purine and pyrimidine precursors (xanthine and uracil) accumulated (Fig. 2.13). Interestingly, adenosine, a nucleoside was also increased in 6-AN-treated cells (Fig. 2.13).

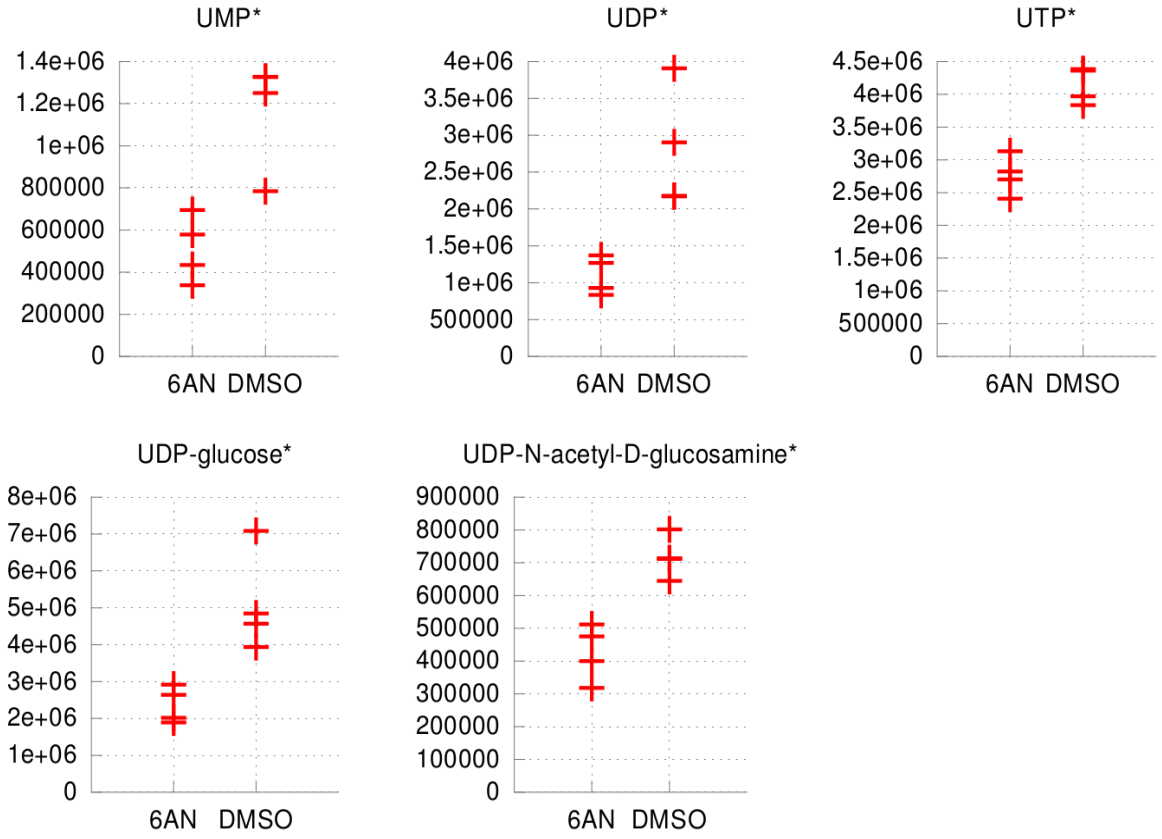


Figure 2.12. Reduced levels of purine and pyrimidine nucleotides, and their carbohydrate derivatives in 6-AN-treated cells, compared to DMSO controls. The Y-axis denotes the signal intensity. *: FDR < 0.05.

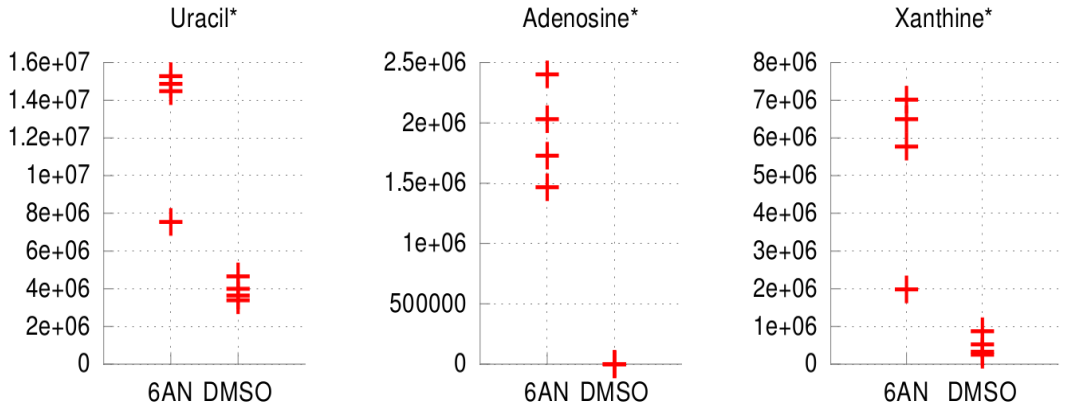


Figure 2.13. Increased pyrimidine precursors and salvaged purine precursors moieties in 6-AN-treated cells compared to DMSO controls. The Y-axis denotes the signal intensity. *: FDR < 0.05.

The salvaged purines are synthesized by the combination of hypoxanthine or xanthine and PRPP to form the first precursor, inosine monophosphate (IMP) or xanthine monophosphate (XMP). The *De novo* synthesis of the pyrimidines implies the formation of orotate first, from simple components and then it binds PRPP to give orotidine monophosphate (OMP) while the salvage pathway involves phosphorylation of uracil (Carter *et al*., 2008). Thus purine salvage pathway and both *De novo* and salvage pyrimidine syntheses are defective in these 6-AN-treated parasites. Interestingly, ATP was found increased in 6-AN treated parasites compared to DMSO-treated controls (Fig. 2.14).

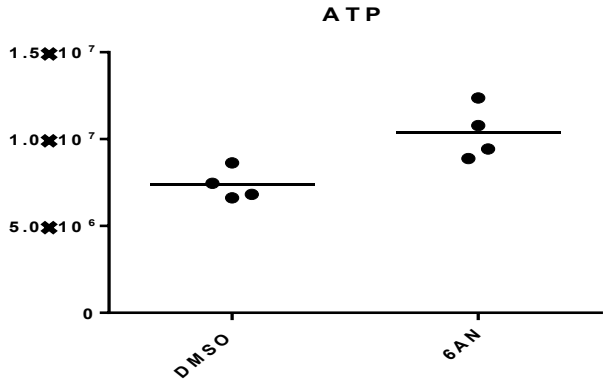


Figure 2.14. Increased ATP level in 6-AN-treated cells compared to DMSO controls.

Other significant changes caused by drug perturbation are in amino acids including L-tryptophan, L-valine and L-arginine which are decreased in 6-AN-treated parasites, and metabolites related to arginine like L- ornithine, which is decreased, while argininic acid, a compound produced from arginine, increases (Fig. 2.15).

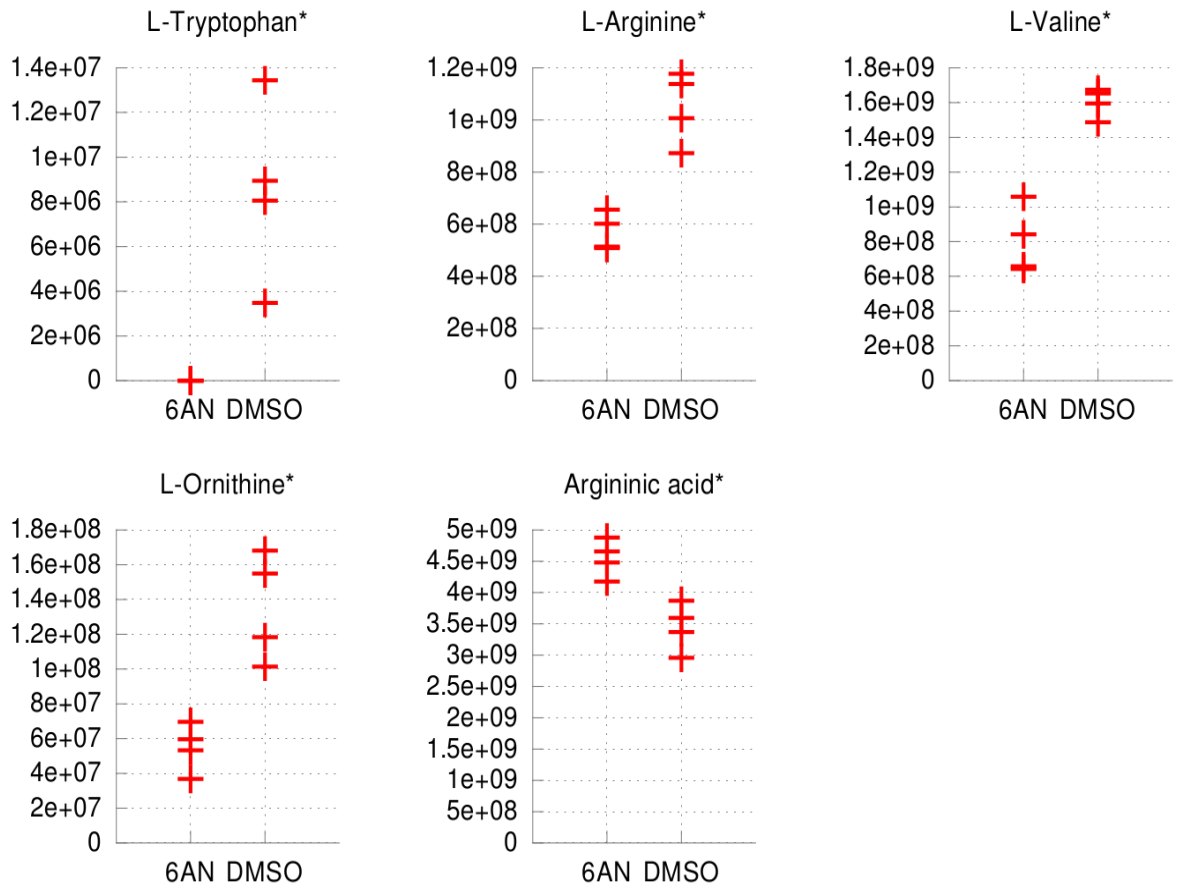


Figure 2.15. Reduced levels of L-tryptophan, L-arginine, L-valine, L-ornithine and increased level of argininic acid in *L. mexicana* promastigotes treated with 6-AN. *: FDR < 0.05.

L. mexicana promastigotes treated with 15.8mM 6-AN in HOMEM medium prepared with ribose and glucose showed improved growth compared to the parasites cultured in HOMEM medium contained glucose alone (Fig. 2.16). This explains that 6-AN-treated parasites utilized the ribose to compensate for the consumed PRPP.

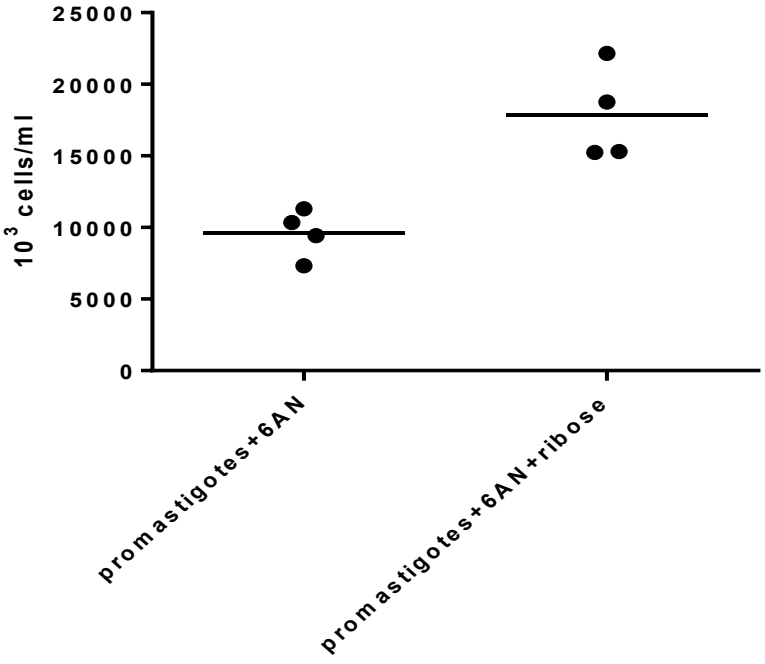


Figure 2.16. Improvement in the growth of 6-AN-treated *L. mexicana* promastigotes by the addition of 6.6 mM ribose. This experiment was done in quadriplicate.

2.3.5.2 *L. infantum*

The metabolomics picture of 6-AN-treated *L. infantum* promastigotes also showed decreases in PRPP and nicotinate, the last even higher than in *L. mexicana*. For *L. infantum*, it was possible to analyse the dinucleotides, NAD^+ and NADPH, which were found significantly decreased together with the PPP intermediate, D-sedoheptulose 7-phosphate and adenosine (Fig. 2.17).

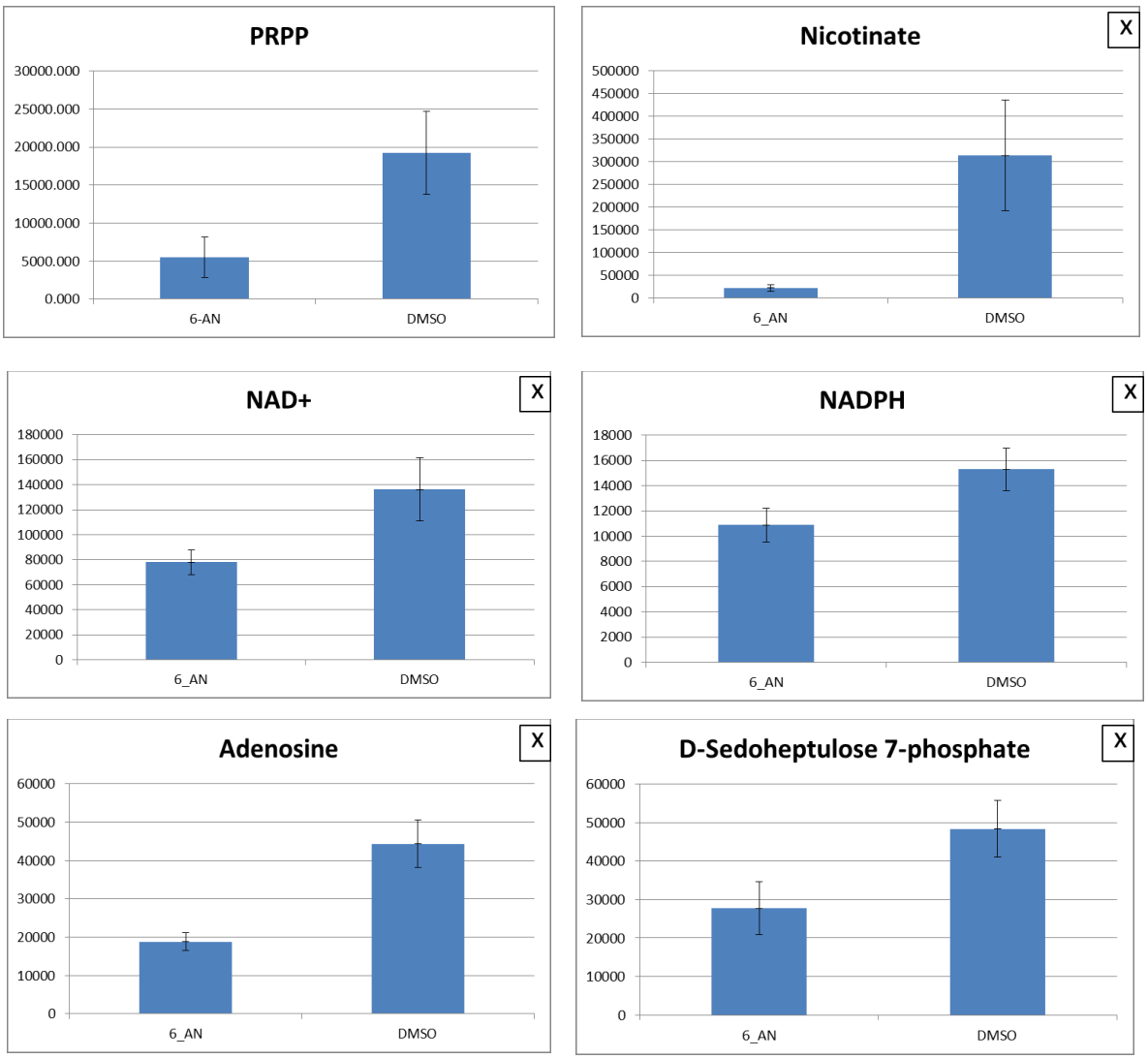


Figure 2.17. Levels of significantly different metabolites of 6-AN-treated *L. infantum* promastigotes compared to DMSO controls. The Y-axis denotes the signal intensity.

2.4 Discussion

Understanding *Leishmania* metabolism and finding promising, specific drug targets is sought. In the present work, we treated *L. mexicana* promastigotes and amastigotes and *L. infantum* promastigotes with 6-AN, and investigated its effect on the parasite growth, infectivity, and metabolism using metabolomics technology.

6-AN showed a leishmaniostatic effect with IC_{50} values in millimolar range and with *L. infantum* more sensitive than *L. mexicana,* and the amastigotes of the latter species were more resistant to 6-AN than its promastigotes. As a comparison, 6-AN inhibits human embryonic palatal mesenchymal cells with an IC_{50} value of 27 μM (Tsuchiya *et al*., 1988), and *Plasmodium falciparum* at an IC_{50} value of 10 μM (Geary *et al*., 1985), indicating that 6-AN is acting differently against *Leishmania* and these other cell types. The so large difference in the 6-AN IC_{50} values between *Plasmodium* and *Leishmania* mirrors their different host environment combatting apparatus, for the first one in the mammal host the environment is erythrocyte, while for the second macrophage, which just uses oxidative stress to fight engulfed microbes. That difference in sensitivity to this compound reflects target protein diversity.

Although *L. mexicana* amastigotes were not sensitive to 6-AN and this compound does not seem to be efficacious as a potential drug, we went ahead to probe changes in the parasite metabolome due to 6-AN treatment. This can highlight important differences between the parasite and mammalian host biochemistry that might be addressed in the pharmacological research. The very higher sensitivity of mammalian cells to 6-AN, whose target is mainly the PPP enzyme, 6PGD (Dietrich *et al*., 1958) points to the *Leishmania* different biochemistry. Striking differences were shown between *T. brucei* and the mammalian 6PGD (Hanau *et al*., 2007), which allowed developing very potent and selective inhibitors (Dardonville *et al*., 2004; Ruda *et al*., 2010). *T. brucei* 6PGD has a strong bent to tetramerization, which is not present in the mammal enzyme (Hanau *et al*., 2013), and *L. mexicana* 6PGD is very similar to *T. brucei* one (González *et al*., 2010). The most peculiar metabolic pattern of trypanosomatids is the glycosome compartmentalization, mainly of most of the glycolytic pathway (Opperdoes and Borst, 1977; Hannaert *et al*., 2003) and is extensively studied as target for new drugs (Barros-Alvarez *et al*., 2014). PPP instead, is mainly in the cytosol and the first enzyme of the pathway G6PDH was

shown very important in decreasing reactive oxygen species in *Leishmania* (Maugeri *et al*., 2003; Ghosh *et al*., 2015) while the second enzyme 6PGL is supposed to be important in removing the highly reactive lactone, produced by G6PDH (Miclet *et al*., 2001). The third enzyme, 6PGDH, whose absence is lethal in the mammalian stage of *T. brucei* (Kerkhoven *et al*., 2013), has been used to type *Leishmania* isolates, as it is a relatively polymorphic marker (Schlein *et al*., 1984). Regarding the nonoxidative branch of PPP, present only in the insect stages of trypanosomatid parasites, the enzyme transaldolase seems essential to *T. brucei* (Creek *et al*., 2015) while transketolase is shown to be present in high percentage in the glycosome too (Veitch *et al*., 2004).

The most evident effect of a 24-hour 6-AN treatment on *Leishmania* promastigotes was a very significant decrease in PRPP, the phosphorylated sugar required for purine and pyrimidine nucleotides, and NAD^+ biosyntheses in these parasites. Therefore, nucleotides (UTP, UDP, and UMP) decreased levels with contemporary increases in purine (xanthine) and pyrimidine (uracil) salvaged nucleobases compared to DMSO-treated controls were noted.

Since the high 6-AN concentration used to inhibit *Leishmania* growth, it can be thought that the profound decrease in *L. mexicana* cellular PRPP is due to interaction of 6-AN with this molecule (although the resultant 6AN-PRPP was not visible in the mass spectrometry platform used).

Strikingly, adenosine increased in 6-AN-treated *L. mexicana* parasites, indicating that this metabolite might have a separate provenance e.g. enhanced transport from the medium in response to falling levels of internal nucleoside in these parasites. In fact, in *L. mexicana*, salvaged adenosine is either directly metabolized to AMP by the action of adenosine kinase (AK), or converted to inosine then to hypoxanthine, whereas in *L. donovani*, adenosine is preferentially transformed to adenine (Glew *et al*., 1988; Boitz and Ullman, 2013). *L. infantum* adenosine metabolism is probably similar to that of *L. donovani* given the phylogenetic proximity and genetic similarity of these species, the possibility to bypass PRPP requirement in adenosine metabolism might explain partially why *L. mexicana* is slightly less sensitive to 6-AN than *L. infantum*.

Addition of ribose sugar to the culture medium improved the growth of the 6-AN-treated parasites by 50% compared to those cultured in a medium containing glucose

alone (Fig. 2.14) and that explains the dire need of the parasites to synthesize PRPP. Indeed, purine and pyrimidine nucleotide synthesis consumes 60-80% of the cell PRPP (Jensen, 1983).

Though purine and pyrimidine nucleotides showed significantly reduced levels, ATP instead increased (Fig. 2.13) which could be due to a shift of glucose metabolism towards glycolysis to compensate for ATP and preserve the parasite viability and integrity. An increase in glycolysis was observed in purine-starved *L. donovani* promastigotes (Martin *et al*., 2014).

Nicotinate and NAD^+ decrease in *L. infantum* indicate inhibition of both nicotinamidase and the general Preiss-Handler pathway. Depletion of NAD^+ in turn affects NAD^+-dependent dehydrogenases, like IMP dehydrogenase involved in purine nucleotide synthesis.

The NAD and NADP derivative of 6AN could not be detected and since also 6PG accumulation has not been found, it can be deduced that the glycohydrolases producing 6ANAD/P from 6AN are not so active in *Leishmania*. Macrophages are more sensitive to 6-AN even at less than 100 μM concentrations again indicating that the effect of this compound on *Leishmania* is different from that on mammalian cells. However, reduced levels of NADP and D-sedoheptulose 7-phosphate in *L. infantum* point to PPP also affected, causing ribose-5-phosphate insufficiency for the production of the needed PRPP.

On the other hand, NAm is a known inhibitor of NAD^+-dependent deacetylase activities (the silent information regulator protein, sirtuin, SIR2). The *Leishmania* SIR2 was shown to have an important role in amastigote stage survival and virulence (Vergnes *et al*., 2002), as well as affecting the survival of promastigotes under starvation conditions using glucose as a unique source of energy (Sereno *et al*., 2006; Ouaissi *et al*., 2006). However since in our case amastigotes were not affected by 6-AN, this enzyme seems unlikely a target.

Other significant changes caused by drug perturbation are in amino acids including L-tryptophan, L-arginine, which are decreased in 6-AN-treated parasites, and metabolites related to arginine like L-ornithine, which is decreased, while argininic acid increased (Fig. 2.14). The latter is formed via deamination of arginine (the substrate for inducible nitric oxide synthase, NOS, in macrophages) followed by reduction, and it is supposed to be produced by *Leishmania* parasites as a

mechanism of evasion of host cell immunity. Therefore, the increase in argininic acid could be a response against drug perturbation.

A significant cellular effect of the oxidizing agents, methylene blue and malachite green (brilliant green) is an increased oxidative stress (Oz *et al*., 2011) and inhibition of 6PGD by malachite green was also reported (Bertelli *et al*., 2001). Methylene blue was found to be an inhibitor of *Plasmodium falciparum* glutathione (GSH) reductase (GR) (Färber *et al*., 1998) however, protective effects from superoxide increase have also been described in other cell types (Oz *et al*., 2011; Tretter *et al*., 2014; Poteet *et al*., 2012). We did not find synergistic interactions between methylene blue and 6-AN, which might indicate that 6-AN does not affect the cell redox balance, anyway we also did not find synergy between methylene blue and malachite green, since the complex way of action by these compounds, involving multiple targets, such as, both are able to bind DNA and many other biomolecules (Oz *et al*., 2011; Zhao *et al*., 1999; Hu *et al*., 2006; Ding *et al*., 2012).

Morphologically, 6-AN-treated promastigotes showed an unusual elongation in the parasite body and flagellum. They assume a morphology similar to that of the nectomonads, the typical stationary phase forms that normally appear after 3 days in the insect gut or the culture medium when the available nutrients are consumed (Gossage *et al*., 2003). Moreover, they cease division, remain viable for about a month, and resume their growth when fresh 6-AN-free medium is added. A significant protraction of the cell body has been reported in *L. donovani* promastigotes cultured in purine-free medium (Carter *et al*., 2010). Therefore, 6-AN treatment seems to induce a form that is typical of starvation and reduced nucleotide metabolism. However, 6-AN treatment of promastigotes for different time lengths from 8 hours to 7 days did not show any consequences on the infectivity of treated parasites to mouse bone marrow macrophages, indicating that the morphological changes does not affect the parasite virulence.

Chapter 3

Detection and identification of a transglutaminase (TGase) from *Leishmania* parasites

3.1 Introduction

3.1.1 General introduction

The transglutaminases (TGases) (protein-glutaminyl-peptide: amine γ-glutaminyltransferase, E.C. 2.3.2.13) are a family of enzymes belonging to the superfamily of papain-like cysteine proteases (Makarov *et al*., 1999; Fesus and Piacentini, 2002). Members of this superfamily have in common a catalytic triad consisting of Cys-His-Asp/Asn (Martin *et al*., 2006).

These enzymes catalyze irreversible post-translational modifications of proteins by creating isopeptide bonds within or between proteins/polypeptides (Lorand and Conrad, 1984). First, TGase attacks a protein-or polypeptide-bound glutamine residue with the formation of thioacyl-enzyme intermediate and release of ammonia as a by-product, then the enzyme crosslinks a protein/polypeptide-bound lysine residue, or a low-molecular mass polyamine generating N^{ε}(γ-glutamyl)lysine isopeptide, or γ-glutamylamine bonds respectively (Folk, 1983; Lorand and Conrad, 1984) (Fig. 3.1). In addition, TGases deamidate glutamine to glutamate, by hydrolyzing the γ–carboxamide group of glutamine and releasing ammonia (Mycek and Waelsch, 1960), hydrolyze GTP (or ATP) into GDP (or ADP) and inorganic phosphate (Pi) and can also use these nucleotides to phosphorylate (Folk, 1983; Lorand and Conrad); another catalyzed activity is isomerization of disulfide bridges (Hasegawa *et al*., 2003). The isopeptide bond they form is highly resistant to chemical, enzymatic and physical degradation (Griffin *et al*., 2002).

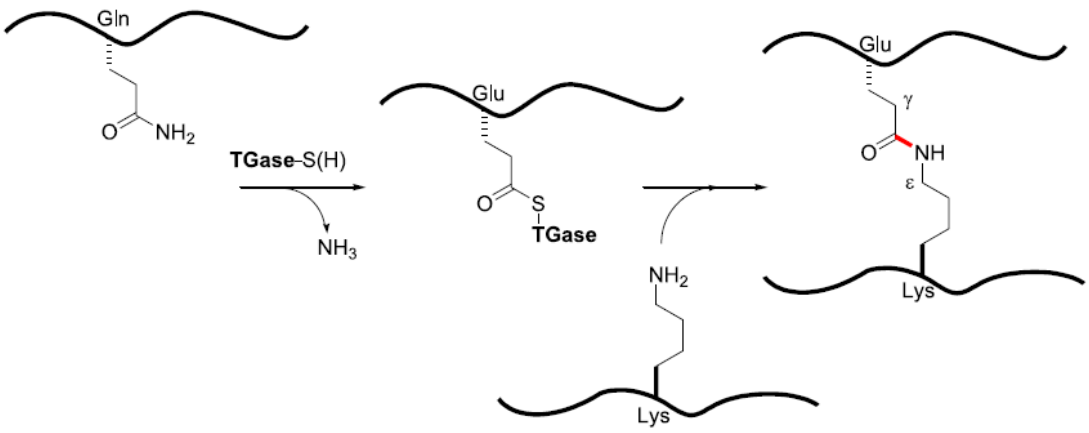


Figure 3.1. The formation of the transglutaminase-catalyzed isopeptide bond (red) between a primary α- amino group of lysine and a γ–carboxamide group of glutamine residues of two proteins or polypeptides.

3.1.2 Mammalian TGases

Clark *et al*., (1957) introduced the term, transglutaminase to depict transamidation catalyzed by guinea pig liver homogenate. Latter, the enzyme TGase was identified by Heinrich Waelsch group in a guinea pig liver tissue (Lorand, 2002). Thereafter, Pisano *et al*., (1968) elucidated the biochemical basis of the TGase reactions. Since then, several proteins from organisms, mammals, invertebrates, fish, and plants with similar crosslinking activities have been identified (Eckert *et al*., 2014). Nine human TGase proteins are identified however, 8 of them are active enzymes and one is inactive (band 4.2) (Grenard *et al*., 2001). They differ in their protein substrates and encoding genes however; they show high sequence homology (Rao and Mehta, 2004). Keratinocytes TGase (type 1) is expressed in the stratified squamous epithelium of the skin, in the upper digestive tract, and in the genital tract of females and it is involved in the differentiation of keratinocytes (Eckert *et al*., 2014). Tissue TGase (type 2) (Fig. 3.2) is abundant in cells and tissues. It is primarily cytosolic but it is also present in the nucleus and the plasma membrane. It is engaged in apoptosis, cell adhesion, stabilization of the extracellular matrix and signal transduction (Eckert *et al*., 2014). The epidermal/hair follicle TGase (type 3) is involved in the terminal differentiation of keratinocytes (Martin *et al*., 2006). Type 4 TGase is found in the

prostate gland, prostatic fluid and seminal plasma and found to be important for fertility in rodents (Dubbink *et al*., 1999). Type 5 TGase is expressed in keratinocytes of the foreskin, in the lining of the epithelial barrier and skeletal muscles (Cassidy *et al*., 2005). Type 6 TGase is expressed in human testes, lungs, and cancer cells with neuronal characteristics (Thomas *et al*., 2013). It is also present in the brain of mice. Type 7 TGase expression is limited to the testes, lungs and brain and it is suggested that this transglutaminase increases in breast cancer cells (Jiang *et al*., 2003). Plasma TGase (FXIIIa) is important in the stabilization of fibrin during blood clotting (Pisano *et al*., 1968). It is present in the platelets, plasma, astrocytes, macrophages, placenta, chondrocytes, synovial fluid, heart, eyes and osteoblastic cells (Nahrendorf *et al*., 2006; Koseki-Kuno *et al*., 2003). It is also involved in bone formation and inflammation related atherogenesis (Dardik *et al*., 2006). Erythrocyte membrane protein band 4.2 (Band 4.2) is the only transglutaminase that lacks the catalytic activity because of a Cys-Ala substitution in the active site of the enzyme. It is important for membrane integrity as well as signal transduction and cell adhesion (Satchwell *et al*., 2009).

The transamidation activity of the mammalian TGases is allosterically regulated. It is activated by calcium (Ca^{2+}) and inhibited by micromolar concentrations of GTP, GDP, or GMP. Since the intracellular concentration of the calcium is very low, the enzyme is mainly exposed to the GTP / GDP and it is thought that TGase is present in an inactive form within cells and that is activated in response to an influx of the calcium (Eckert *et al*., 2014). Even the redox state affects its activities, being inactive in an oxidizing environment, where the critical cysteine forms a disulfide bond with another cysteine. Moreover, phosphorylation, interaction with nitric oxide and various proteins regulate its function (Eckert *et al*., 2014).

Regarding the TGase structure, FXIII consists of four subunits, two of which are plasma protein carriers and the other two the catalytic subunits, after thrombin proteolytic processing the active dimeric enzyme is generated. The single catalytic subunit has the same structure of the monomeric TGases like type 2 tTGase and other animal TGases with 4 domains, an N-terminal β-sandwich, a core domain (containing the catalytic and the regulatory sites) and C-terminal β-barrels 1 and 2 (Griffin *et al*., 2002).

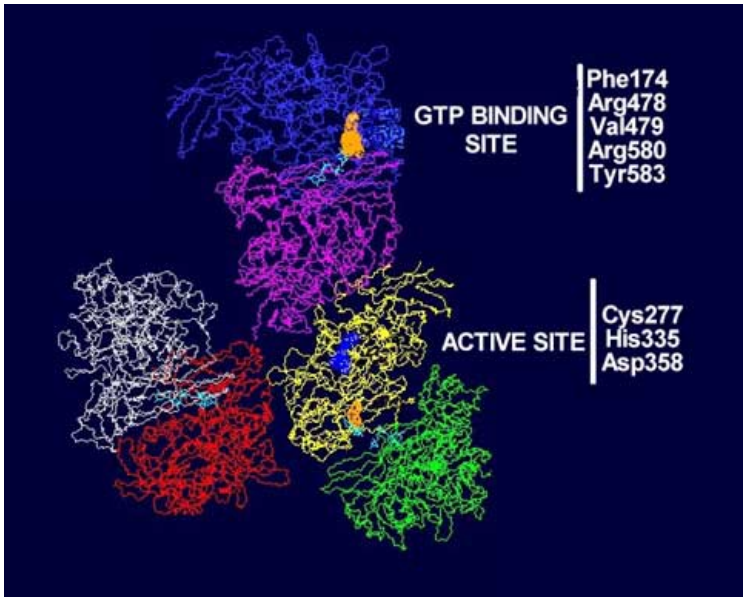


Figure 3.2. The tertiary structure of the mammalian tissue TGase (Type 2) depicting the active site (Cys277) and the GTP binding site. Adapted from Martin *et al*., (2006).

3.1.3 Parasite and microbial TGases

Some TGases of lower organisms have been identified and their properties are described. Singh and Mehta, (1994) purified and characterized a Ca^{2+}-dependent *Brugia malayi* TGase with a molecular mass of 56 kD and the enzyme is inhibited by GTP at concentrations in the millimolar range. This enzyme was found important for eggshell, sheath, and microfilaria production. Dog filarial worm, *Dirofilaria immitis* calcium requiring TGase that is important for molting was identified (Chandrashekar *et al*., 2002). A TGase activity dependent on Ca^{2+} was detected in *Onchocerca volvulus* and the enzyme was suggested to be pivotal for molting and development of the 4^{th} stage larva (L4) (Lustigman *et al*., 1995). In *Plasmodium falciparum*, Nicoll *et al*., (2011) detected a TGase substrate *in vivo* using monoclonal antibodies against N^{ϵ}-(-L-glutamyl)lysine isopeptide formed by the sporozoites in the hepatocytes as well as *in vitro* using tissue TGase and sporozoite-derived Liver Stage Antigen-1 (LSA-1). They suggested that protein cross-linking is important for the protection of the hepatic stages. A weak Ca^{2+}-independent TGase activity in ring-forms and strong in

trophozoites and schizonts of *Plasmodium falciparum* was detected (Adini et al., 2001). It was proposed that protein cross-linking might be important for the modification of the infected red blood cell as well as the building of oocyst capsule in the mosquito gut. Three forms (26, 50, and 13 kDa) of protein disulphide isomerase (PDI) with Ca^{2+}-dependent transamidation activities have been detected in *Giardia lamblia* (Knodler *et al*., 2000). The first microbial TGase (Fig. 3.3) with a molecular weight of 38 kDa was identified in *Streptomyces mobaraensis* (Washizu *et al*., 1994). It is monomeric and its activity does not require Ca^{2+}. Although, some microrganism TGases have a certain homology to mammalian ones, some bacterial enzymes do not have homology to known mammal TGases, representing potential drug targets (Rao and Mehta, 2004). For instance the structure of the *S. mobaraensis* Tgase consists of a single compact $\alpha-\beta$ domain (Kashiwagi *et al*. 2002).

In *Leishmania* parasites, a TGase-like activity has been detected by Brobey and Soong, (2006) in homogenates of promastigotes and amastigotes of *L. amazonensis*, *L. major* and *L. chagasi*. The enzyme activity was found Ca^{2+}-independent, optimum at basic pH (8.5-9.5) and 2-4 times higher in Old World species than in New World ones. In addition, inhibition of *Leishmania* TGase activity inhibited the parasite growth. TGase from leishmanial parasites has not been identified yet however, by searching leishmanial genome with the only known coding sequence for unicellular eukaryote TGase, some sequence identity can be found with other types of proteins, both cytosolic and membrane-bound.

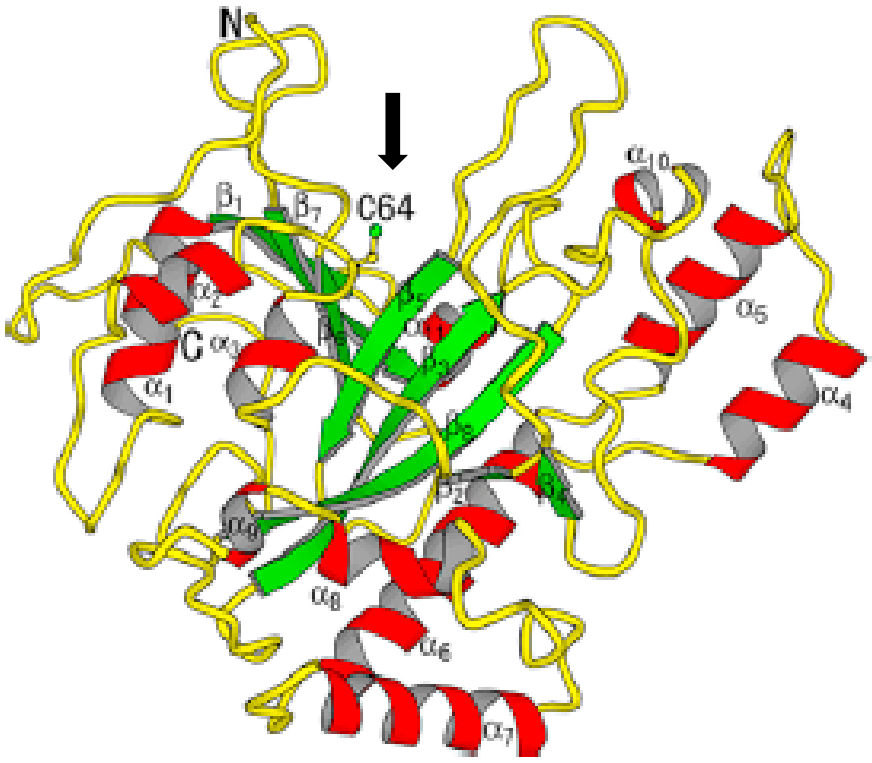


Figure 3.3. The crystal structure of the microbial TGase: The stripe design viewed from above the plate face. The active site Cys64 is shown by ball-and-stick model (arrow). Adapted and modified from Kashiwagi *et al*. (2002).

3.2 Materials and methods

3.2.1 Cultivation of *L. infantum* promastigotes

The promastigotes of canine MON-1 and human MHOM / TN80 / IPT1 *L. infantum* species (provided by IZSLER in Modena, Italy) were routinely cultured in RPMI 1640 medium (Sigma, Germany), pH 7.2, supplemented with 15% heat inactivated and 0.2 micron-filtered fetal calf serum (HIFCS) (Gibco, Paisley, UK), 100U/ml Penicillin, and 100U/ml Streptomycin sulphate in $25cm^2$ non-vented flasks (Corning, USA) (McCarthy-Burke *et al*, 1991), referred here after as complete RPMI (cRPMI). Parasites were incubated at 25°C in a humidified incubator under air as a gas phase with medium replacement every 3-4 days.

3.2.2 Preparation of promastigote lysates and protein estimation

Promastigotes were cultured in cRPMI in large flasks and incubated for 7 days. About $4x10^9$ promastigotes of each canine and human *L. infantum* were first centrifuged at 8,000 rpm, 4°C for 10 minutes to remove the culture medium then washed 4 times in cold, sterile PBS (pH 7.4) and the parasite pellets were frozen at -80 °C till use. The pellet was lysed in five volumes of a buffer consisting of 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM DTT and 1X protease inhibitor cocktail. The sample was incubated in ice for 30 minutes with regular vortexing after every 10 minutes then rapidly freeze/thawed three times, sonicated in ice using Sonicator® Ultrasonic Processor *XL* (Farmingdale, NY, USA) (5 pulse cycles of 20 seconds at an intensity of 50% with 5 cooling pauses of 20 seconds. Lysed parasites were then centrifuged at 14,000 rpm, at 4 °C for 30 min.

The protein content was estimated by Bradford method (Bradford, 1976) using Coomassie Brilliant Blue G-250 in an acid solution, which binds the basic amino acids residues of the proteins forming a colored complex with absorption maximum at 595 nm. Standards of known concentrations of bovine serum albumin (BSA) were prepared and tested together with the serially diluted lysate. In a microtiter plate, 5µl from each sample dilution, and standard were added to respective wells in duplicate. Sterile PBS, pH 7.4, and protein-free lysis buffer were used as blanks. Two hundred microliters Bradford reagent were added to each well and the plate was incubated at

room temperature with continuous mixing. Absorbance was read, standard curve was generated, and the unknown protein concentration was calculated.

The clear supernatant was transferred to a clean Eppendorf tube, aliquoted and stored at -80 °C.

3.2.3 Detection of *Leishmania* TGase activity

3.2.3.1 *In vivo* detection of TGase activity

Fluorescein isothiocyanate (FITC)-cadaverine (FC) (Fig. 3.4) (Thermo Fisher, USA) was used to probe TGase activity in living *Leishmania* parasites using a modified method of Lajemi *et al*., (1997). A bout 10^7 canine *L. infantum* cultured promastigotes were incubated in brown Eppendorf tubes for 3 hours at 25°C with 0.5 mM FC in cRPMI medium. The parasites were then washed three times in sterile PBS (10mM NaH_2PO_4 and 150mM NaCl), pH 7.4, and the smears were prepared on glass slides, air dried and fixed for 10 minutes at - 20°C with cold methanol to remove free intracellular FC which is soluble in methanol (Lorand *et al*., 1983). After that, the smears were incubated for 20 minutes in the dark with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), washed three times (10 minutes each) in PBS, mounted in glycerol in PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine, and examined using a Nikon Microphot FXA fluorescent microscope equipped with a photo camera.

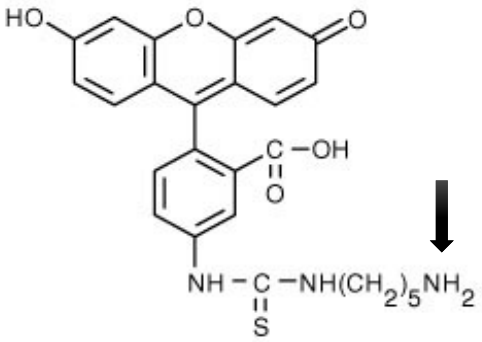


Figure 3.4. The structure of fluorescein-cadaverine. The arrow shows the reactive amine group.

3.2.3.2 *In vitro* detection of TGase activity

Promastigote extracts of canine *L. infantum* promastigotes were incubated for 1 hour at 30 °C with 4 mM FC with or without 50 µl of 20mg/ml dimethyl casein and in absence or presence of 200 µM putrescine (prepared according to the method of Lin *et al*., 1969) under end-to-end roll mixing. After denaturation using Laemmli, (1970) method, samples were loaded onto 15% polyacrylamide mini-gel (PAA) SDS-PAGE. The stacking gel was made of 5% acrylamide/bisacrylamide, 0.1% SDS, 0.125 M Tris-HCl, pH 6.8, 0.026 M TEMED, 0.1% ammonium persulphate and the resolving gel contained 10% acrylamide/bisacrylamide, 0.1% SDS, 0.37 M Tris-HCl, pH 8.8, 0.0165 M TEMED, 0.05% ammonium persulphate. The samples were denatured at 95°C for 5 minutes in the sample buffer that contained 60mM Tris-HCl, pH 6.8, 2% SDS, 1.6% mercaptoethanol, 3.3% glycerol, and 0.01% bromophenol blue as a tracking dye. A fifteen-microliter volume from each denatured sample correspondent to 25µg protein was applied to each well in the gel. Guinea pig liver TGase and the cell lysis buffer were used as positive and negative controls, respectively. ECL Plex Fluorescent Rainbow molecular weight marker conjugated with fluorophores Cyanine 3 and Cyanine 5 was used. Electrophoresis was carried out at 30 mA for 1 hour in a running buffer consisting of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Molecular Imager System PHAROS Bio-Rad FX and software Quantity One 1-D were used for gel analysis. The system includes the green 532 nm, blue 488 nm and red 635 nm lasers and the corresponding “Bandpass filters” BP 605 nm, 530 nm BP and 695 BP.

3.2.3.3 Measurement of TGase activity in micro-well plate

A microplate assay kit (Sigma-Aldrich, USA) was used to quantitate TGase activity. The first substrate is a free amine group of poly-$_L$-lysine that is covalently attached to the plate surface. The other substrate is γ-carboxamide group of biotin-TVQQEL-OH present in the assay buffer. TGase binds the biotinylated peptide to the immobilized amine group and the bound biotin is then detected by streptavidin-peroxidase and 3,3’,5,5’-Tetramethylbenzidine (TMB) liquid substrate. The amount of immobilized biotin is proportional to the amount of the active TGase activity in the sample. The yellow TMB is oxidized to blue and is detected at 450 nm. The assay was performed according to the manufacturer’s instructions. In duplicate, 50µl of each, the positive

control solution (guinea pig liver TGase), and lysate were added to separate wells. Ultrapure water and the lysis buffer were used as negative controls. Assay buffer was prepared and 50µl volume was added to each well. The plate was incubated for 30 minutes at room temperature (RT) followed by 3 washes with ultrapure water. Then 100µl of a fresh 0.1µg streptavidin-peroxidase in PBS-T, pH 7.4 containing 0.05% Tween-20 and 1mM DTT were added to each well and the plate was incubated at RT for 20 minutes. After that, the wells were washed three times (200 µl/well each) in PBS-T. Two hundred microliters TMB were added to each well and 100µl stop solution were added after a maximum of 3 minutes. Absorbance was read at 450 nm using Tecan Infinite M200 (Tecan Trading AG, Switzerland). In some experiments, cell extracts were dialyzed overnight at 4 °C using a buffer consisting of 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1 mM DTT, and protease inhibitor cocktail. A dialysis cellulose membrane with a cutoff of 14 kD was used.

3.2.4 Ammonium sulphate (AS) precipitation of TGase

Salting out using AS precipitation was performed using the method of Duong-Ly and Gabelli, (2014). According to the AS calculator (http://www.encorbio.com/protocols/AM-SO4.htm), grinded, heat-dried AS was gradually added to low-stirring 5 ml canine *L. infantum* promastigote lysate at 4°C to give 15, 30, 45, 60, and 75% saturations. After each AS addition, the lysate was incubated for 20 minutes followed by centrifugation at 15,000 rpm, 4°C and for 20 minutes. The entire supernatant was transferred to a clean eppendorf tube, its volume was measured and AS was added to it to give the subsequent saturation. Each protein pellet was then resuspended in 0.5 ml buffer B consisting of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1X protease inhibitor cocktail. Protein fractions were dialyzed overnight in buffer B containg 0.4 mM phenylmethane sulfonyl fluoride (PMSF), and TGase activity was assayed in all fractions using the mirowell plate kit (Sigma-Aldrich, USA).

3.2.5 Effect of calcium and GTP on TGase activity

Using the microplate assay kit (Sigma-Aldrich, USA), canine *L. infantum* promastigote lysate precipitated at 45% AS saturation was treated in duplicate in two different experiments with 1) 5 and 10 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-

tetraacetic acid (EGTA), 2) 5 mM $CaCl_2$, and 3) 10 mM GTP. After reactions, the absorbance was read at 450 nm using Tecan Infinite M200 (Tecan Trading AG, Switzerland).

3.2.6 Detection of the protein TGase

3.2.6.1 Western blot

Transglutaminase polyclonal antibodies (pAbs, orb2986) (Biorbyt, Cambridgeshire, UK) raised in rabbit against a conserved peptide sequence of human TGase 2 were used to detect the protein TGase in canine and human *L. infantum* promastigote lysates using the method of Towbin *et al*., (1979). About 14µg protein of canine strain whole lysate and its AS fractions (15 and 45%) with TGase activity or human strain total extract were electrophoresed in duplicates in 10% SDS-PAGE under reducing conditions as mentioned earlier. Guinea pig liver TGase was used as a positive control and Precision Plus Protein™ Dual Colour Standards (BIO-RAD, USA) were separated alongside the lysates to facilitate molecular weight determination. The separated proteins were then transferred at 300mA for 90 minutes to 0.45 µm-pore nitrocellulose membranes (Amersham Protran Supported, GE Healthcare) in 1x Tris-glycine-SDS blotting buffer containing 20% methanol. Non-specific sites were then blocked by incubating the membranes for 1 hour at room temperature in a blocking buffer containing 5% Skimmed Milk and 0.5% Tween-20 in PBS (10mM NaH_2PO_4 and 150mM NaCl), pH 7.4. After three washes with PBS/0.5% Tween-20 (PBS/T), blots were incubated overnight with 10µg Tgase pAbs diluted 1:1500 in PBS/T containing 1%BSA, pH 7.4. Blots were then washed three times in PBS/T and incubated with horseradish peroxidase (HRP)-conjugated Goat anti-Rabbit (H+L) secondary antibodies (Novus Biologicals Europe, Abingdon, UK) diluted 1:50000 in the blocking buffer followed by three washes in PBS/T and three washes in PBS. To detect TGase-immunoreactive bands, an enhanced chemiluminescent (ECL) system (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific) was employed according to manufacturer's instructions. This experiment was repeated three times.

3.2.6.2 Immunocytochemical detection of the protein TGase

The immunocytochemistry (Indirect immunofluorescence) was performed using the method of Upchurch *et al*., (1987) with some modifications. On the first day, 10^7 canine *L. infantum* cultured promastigotes were washed twice (5 minutes each) in sterile PBS (10mM NaH_2PO_4 and 150mM NaCl, pH 7.4) at 5000 rpm and 25°C. Parasite smears were prepared on clean glass slides, air-dried and fixed for 20 minutes at room temperature (RT) with 4% w/v paraformaldehyde (PFA) in PBS, pH 7.4 followed by three washes in PBS, 5 minutes each, and a 30-minute wash in PBS/1%BSA buffer with shaking to block non-specific binding sites on glass. Smears were then incubated overnight at 4°C with human TGase 2 rabbit polyclonal antibodies orb2986 (Biorbyt, Cambridge, UK) diluted 1:1500 in PBS containing 0.3% Triton X-100 and 0.1% normal goat serum (Sigma-Aldrich). On the second day, the smears were washed at RT with shaking in PBS (three times, 5 minutes each), PBS/0.1% BSA buffer (three times, 10 minutes each), PBS/0.1% normal goat serum buffer for 30 minutes, and PBS for 10 minutes followed by an incubation for 1 hour in the dark at RT with Fluorescein (FITC)-tagged anti-rabbit antibodies (Chemicon, Temecula, CA, USA) diluted 100 times in PBS containing 0.3% Triton X-100 and 0.1% goat normal serum, with continuous mixing. Smears were then washed three times (10 minutes each) with shaking in PBS/0.2% Triton X-100 buffer then the smears were incubated in the dark with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 20 minutes, washed three times (10 minutes each) in PBS/0.2% Triton X-100 buffer, mounted in glycerol in PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine, and examined using a Nikon Microphot FXA fluorescent microscope equipped with a photo camera.

3.6 Results

3.6.1 *In vivo* detection of TGase activity

A bout 10^7 canine *L. infantum* cultured promastigotes were incubated in the dark for 3 hours at 25°C with 0.5 mM FC in 0.5 ml cRPMI medium. Parasite smears were prepared on slides, fixed and washed for 20 minutes in cold methanol at -20°C. Specific intracellular fluorescence was observed under a Nikon Microphot FXA fluorescent microscope (Fig. 3.5) indicating the presence of TGase activity in canine *L. infantum* promastigotes.

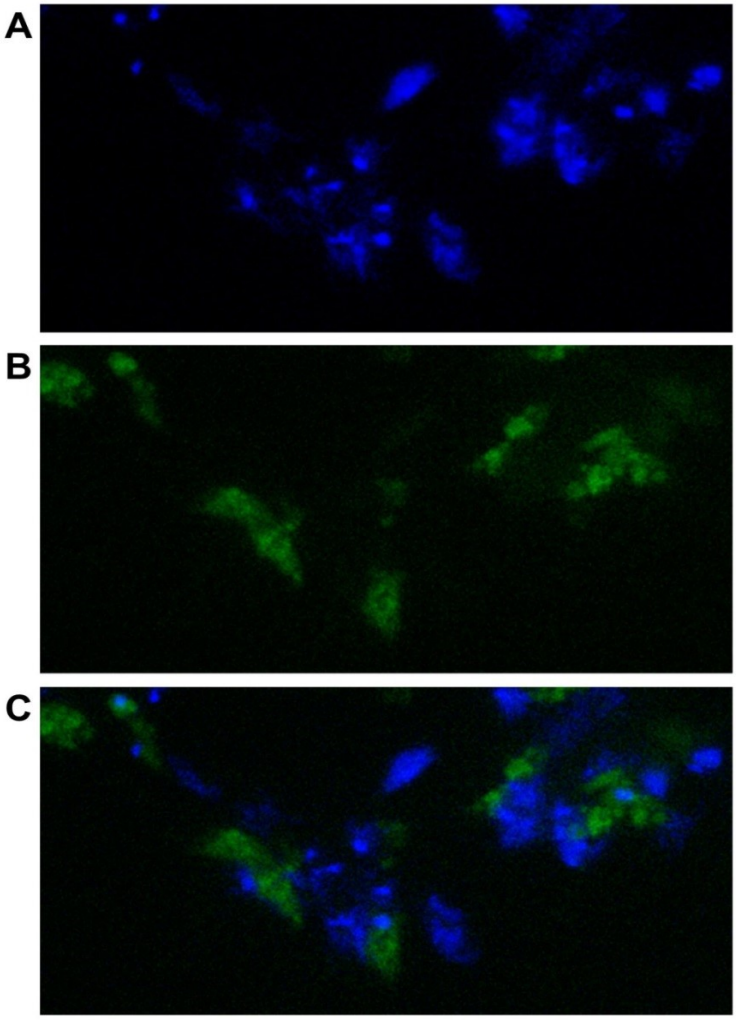


Figure 3.5. In-cell detection of TGase activity in cultured promastigotes of canine *L. infantum*. DAPI-stained nuclei of the parasites (A). FC labelling of indigenous proteins due to TGase activity (B). Merged A and B (C). The green fluorescence was detected by Nikon Microphot FXA fluorescent microscope (X100 magnification).

3.6.2 *In vitro* detection of TGase activity

The debris-free parasite extract prepared from $4x10^9$ canine *L. infantum* promastigotes was incubated with 4 mM FC for 1 hour at 30°C with or without 50 µl of 20mg/ml dimethyl casein and 200 µM putrescine and then separated in 15% SDS-PAGE gel. The analysis of the fluorescence in gel revealed the presence of endogenous substrates under 24 kDa. The activity was weakly inhibited by putrescine at a concentration of 200 µM. The activity of the TGase on dimethyl casein substrate was very low (faint green band above the 38 kDa) (Fig. 3.6).

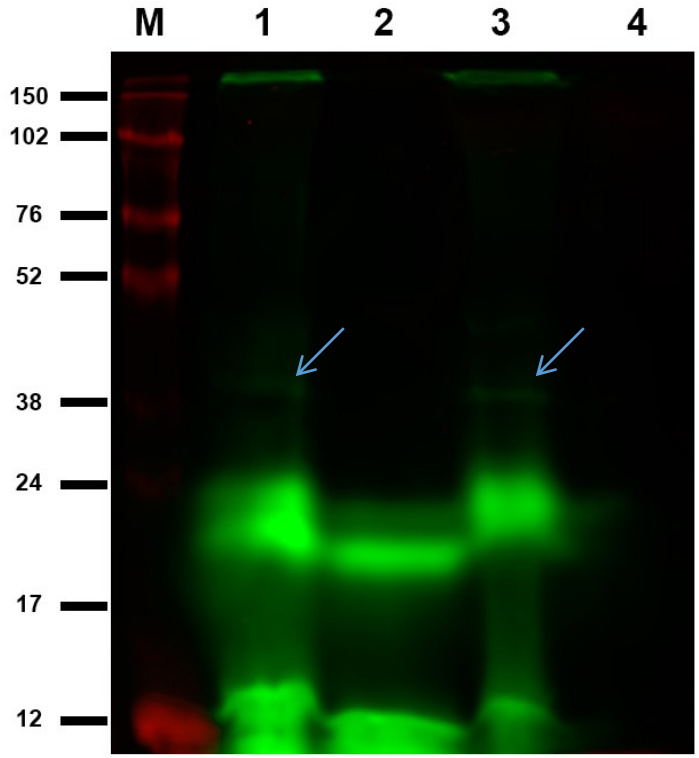


Figure 3.6. SDS-PAGE separation of FC-labelled (TGase activity) canine *L. infantum* promastigote proteins and dimethyl casein. The green bands show excitation of the FITC and the red bands denote excited Cy5, which labels the protein marker. From left, lane M molecular weight marker, lane 1 parasite extract + FC + dimethyl casein (arrows), lane 2 parasite extract + FC, lane 3 parasite extract + FC + dimethyl casein+ putrescine, lane 4 dimethyl casein alone (no fluorescence). Molecular Imager System PHAROS Bio-Rad FX and software Quantity One 1-D were used for gel analysis.

3.6.3 TGase activity assay in micro-well plate

The TGase activity in cell extracts of canine and human *L. infantum* promastigotes extracts prepared from $4x10^9$ parasites each, was measured by micro-plate method using poly-L-lysine immobilized on the plate, and a biotin conjugated to glutamine-containing peptide as substrates. TMB and streptavidin-peroxidase were used to visualize the reactions. Guinea pig liver TGase was used as positive control. The activity was obtained for both canine and human strains. TGase activity in parasite lysate was more in the human strain, approximately 0.053 mU/mg of total protein than in the canine one.

3.6.4 AS precipitation of TGase

AS protein fractionation was performed on extract prepared from $4x10^9$ canine *L. infantum* promastigotes. Fractions precipitated at 15, 30, 45, 60, and 70% AS saturations were collected, dialyzed, and TGase activity was measured. Fractions, 15 and 45% showed the highest total TGase activities of 0.15 and 0.158 mU/ml respectively.

3.6.5 Effects of calcium and guanosine triphosphate (GTP) on TGase activity

To investigate whether Ca^{2+} is required or not for *Leishmania* TGase activity, canine parasite lysate was incubated with or without 5 and 10 mM EGTA and 5 mM $CaCl_2$. The results presented in Fig. 3.7 show that addition of millimolar concentrations of EGTA significantly inhibited the enzyme activity ($p<0.001$) and incubation of the lysate with additional calcium increased the enzyme activity ($p<0.001$) indicating that canine *L. infantum* TGase activity requires Ca^{2+}. On the other hand, treatment with 10 mM GTP markedly reduced the TGase activity ($p<0.001$) showing that the regulation of the activity of this enzyme might be Ca^{2+}/GTP-dependent.

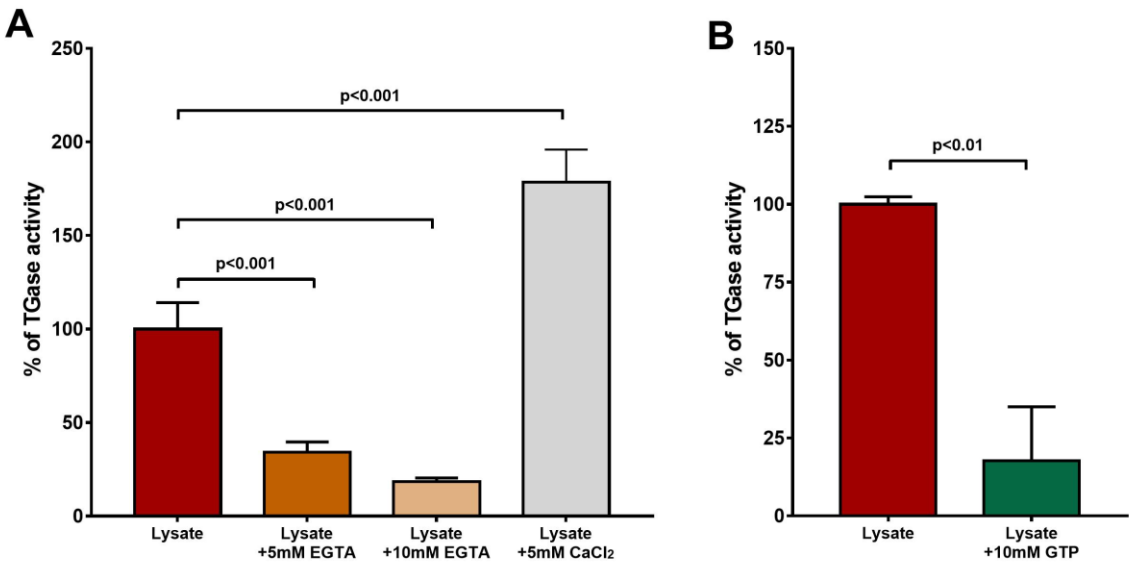


Figure 3.7. Canine *L. infantum* TGase activity is Ca^{2+}-dependent. The percentages of the activities are represented. Addition of EGTA to the reaction mix significantly reduced the enzyme activity ($p<0.001$) and treatment of lysate with additional Ca^{2+} increased the enzyme activity (A). Addition of GTP markedly reduced the TGase activity (B).

3.6.6 Detection of protein TGase by Western blot

Canine *L. infantum* total promastigote extract and 15 and 45% AS precipitated fractions with TGase activity as well as the whole protein extract of human *L. infantum* promastigotes were subjected to 10%SDS-PAGE and western blot analysis using TGase pAbs (orb2986) and ECL system for detection. The immunoblotting of the whole lysates revealed one band of 74.6 KDa for the canine strain and two bands; a major one of 55.34 KDa and a minor one of 65.87 KDa for the human strain (Fig. 3.8). The Canine AS protein fractions showed similar bands to that of its whole extract however, there were some protein degradations due to multiple precipitations and dialysis.

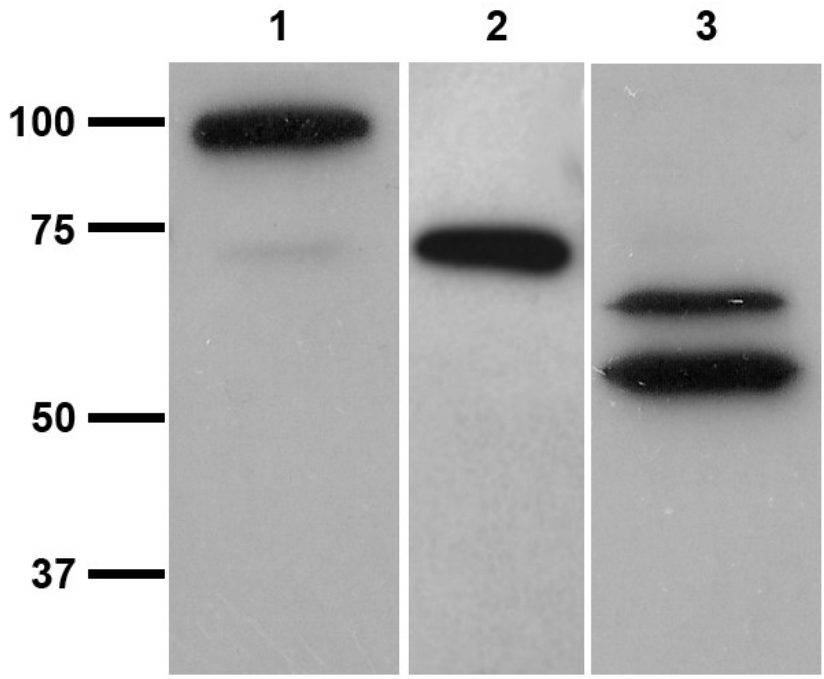


Figure 3.8. Detection of canine and human *L. infantum* putative TGase in promastigotes extracts by employing 10% SDS-PAGE electrophoresis and immunoblotting using transglutaminase polyclonal antibodies (orb2986) and HRP-conjugated Goat anti-Rabbit (H+L) secondary antibodies and ECL detection system. In the WB, lanes 1 purified guinea pig TGase, lane 2 canine *L. infantum* extract, and lane 3 human *L. infantum* lysate.

3.6.7 Immunocytochemical detection of protein TGase

An immunocytochemical analysis was performed to detect the putative protein TGase in canine *L. infantum* promastigotes. Washed 10^7 canine *L. infantum* promastigotes were smeared on glass slides and fixed with 4% w/v paraformaldehyde (PFA) in PBS. The slides were then blocked with 1% BSA/PBS buffer and incubated at 4°C with human TGase2 rabbit polyclonal antibodies (orb2986). Putative protein TGase was revealed (Fig. 3.9) using FITC-tagged anti-rabbit antibodies.

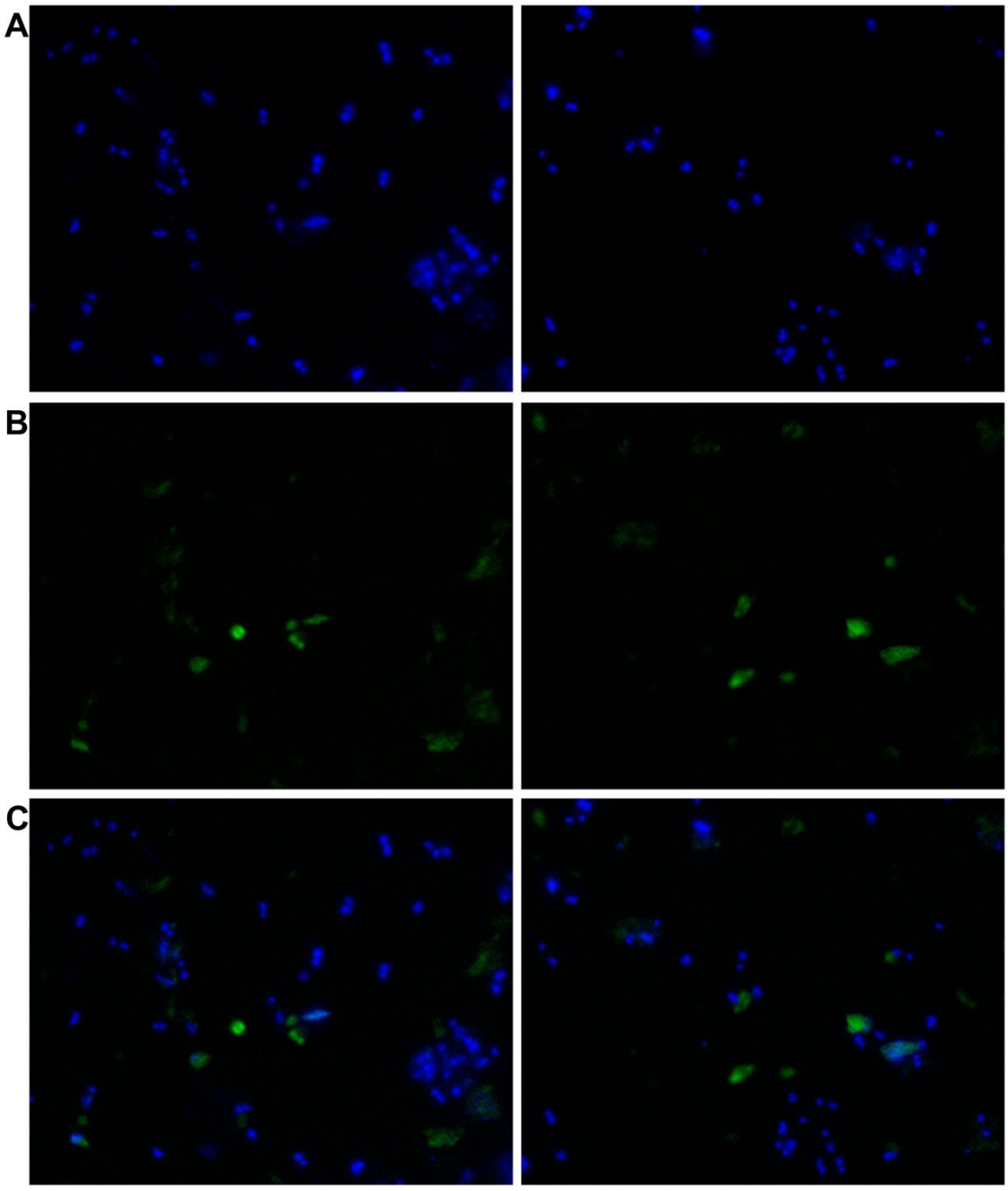


Figure 3.9. Indirect immunofluorescent staining of protein TGase in canine *L. infantum* promastigotes. FITC fluorescence was visualized using a Nikon Microphot FXA fluorescent microscope (X40 magnification). DAPI-stained nuclei of the parasites (A). FITC labelling of protein TGase (B). Merged A and B (C).

3.7 Discussion

Many of the current antileishmanial agents have many serious side effects and the parasites are becoming resistant to them (WHO, 2010) therefore, promising, parasite-specific drug targets are needed to develop safe and effective remedies. On this background, the goal of TGase study in Leishmania was to detect, characterize and understand the function(s) and essentiality of *Leishmania* TGase for the parasite survival and growth. Inhibition of TGase activity markedly reduced the growth of both promastigotes and amastigotes (Brobey and Soong, 2006) and completely inhibited microfilaria production in *Brugia malayi* (Singh and Mehta, 1994). TGases catalyze proteolytic-resistant protein crosslinks (Griffin *et al*., 2002) and the promastigote surface leishanolysin was identified as a substrate for TGase in *L. chagasi* (Brobey and Soong, 2006). We therefore, speculate that the enzyme might be important for attachment and invasion of the immune cells, and for creating protein layers around the intracellular *Leishmania* parasites that can protect them from the lysozymes of the macrophages.

Utilizing the promastigotes of canine and human *L. infantum*, the present study confirms the presence of an active TGase in *Leishmania* through *in vivo* incorporation of FC into parasite proteins and *in vitro* labeling of parasite extract proteins and dimethyl casein with FC, and in quantitative microwell plate assay using biotinylated peptides and streptavidin peroxidase. TGase has weakly utilized dimethyl casein as an acyl donor indicating that this protein is not a suitable substrate for this enzyme.

The partitioning of protein TGase at the 15% AS saturation suggests that this enzyme might be membrane-associated since this lower concentration was used to remove larger cell components. TGase activity in the canine strain promastigote extract was found Ca^{2+}-dependent. Addition of 5 to 10 mM EGTA to our TGase reaction mix has led to a complete loss of enzyme activity, and increased Ca^{2+} concentration in the assay mix increased the TGase activity (Fig. 3.7). Variably, Ca^{2+}-independent Tgase activities were reported in *L. chagasi*. *L. major* and *L. amazonensis* (Brobey and Soong, 2006). Ca^{2+} is essential for mammalian TGase activities however; a TGase-like enzyme with calcium-independent TGase activity was found in rat intestinal mucosa (Tsai *et al*., 1998). Ca^{2+} requirement varies in some parasite and microbial TGases. Ca^{2} is needed for TGase activity in parasitic filarial *Brugia malayi* (Mehta *et*

al., 1992), *Onchocerca volvulus* (Lustigman *et al*., 1995) and PDI transamidation activity in *Giardia lamblia* (Davids *et al*., 2004) whereas *Plasmodium falciparum* (Adini *et al*., 2001) and *Streptoverticillium mobaraense* (Ando *et al*., 1989; Nonaka *et al*., 1989) TGases are Ca^{2+}-independent.

On the other hand, GTP at a concentration of 10 mM in the reaction buffer has completely inhibited canine L. *infantum* TGase activity suggesting that the regulation of the canine strain TGase activity might be Ca^{2+}/GTP ratio dependent. Consistently, GTP at concentrations in the millimolar range inhibits the parasitic *Brugia malayi* TGase (Singh and Mehta, 1994). The mammalian TGase 2 is inhibited by micromolar concentrations of GTP, GDP, or GMP (Eckert *et al*., 2014) and it is Ca^{2+} and GTP controlled (Aeschlimann and Paulsson, 1994) however; the pro-enzymes; coagulation factor XIII, TGase 3 and bacterial TGase which carry activation peptides are controlled by proteolytic cleavage (Takahashi *et al*., 1986; Kim *et al*., 1990; Pasternack *et al*., 1998).

Besides, addition of the polyamine, putrescine at 200 µM concentration to the TGase reaction mix and at 5 mM to the parasites incubated with FC in cRPMI showed a weak inhibition of the enzyme whereas, the polyamine, cystamine at 3 mM reduced the growth of the promastigotes to 50% (Brobey and Soong, 2006) denoting that this enzyme could represent a promising drug target if purified and characterized.

At first, we extensively tried to detect the *Leishmania* protein TGase by Western blot using monoclonal antibodies (mAbs) produced against human TGase 2 however; no protein band was detected. With the pAbs (orb2986) we succeeded in detecting one protein band of a calculated molecular mass of 74.6 KDa for canine *L. infantum* using whole lysates and AS fractions (15 and 45%) with TGase activity, and two bands of 55.34 and 65.87 KDa in the human strain total extract (Fig. 3.8) indicating that the protein TGase can vary between *Leishmania* species, and the reactivity of the pAbs may ease the enzyme purification. In addition, protein TGase detected by pAbs (orb2986) could be used as a marker to differentiate between human and canine *L. infantum*. To confirm this reactivity we performed an immunocytchemical analysis using canine *L. infantum* promastigotes and utilizing FITC-tagged anti-rabbit antibodies and conventional fluorescent microscope. Green fluorescence was seen next to DAPI-stained (blue fluorescence) parasite nuclei (Fig. 3.9). Unfortunately, we do not have an advanced microscope facility in our lab so that we can localize the

enzyme in the parasites. Then we tried to immuno-purify the protein TGase by reacting the enzyme with the pAbs (orb2986) covalently bound to magnetic beads (Invitrogen, Thermo Fisher Scientific, Lithuania) and then eluting the specific protein but we did not succeed. After that we attempted to purify the pAbs (orb2986) utilizing protein G-magnetic beads since their preserving buffer contains albumin and glycerol and these interfere with the binding of these antibodies to the beads. Unfortunatelly, purification of pAbs (orb2986) required a huge starting material however, the pAbs (orb2986) could enormously assist in affinity chromatography purification of this enzyme in order to understand its functions and roles in the parasite survival and growth with a hypothesis that this enzyme might offer a promising drug target.

The biochemical part conclusion

Data of the biochemical study on the 6-AN treatment of *Leishmania* show that this compound in these parasites is not a strong PPP inhibitor as shown in some mammalian cell lines. In mammalian cells, 6-AN in micromolar concentration is converted to 6-ANAD and 6-ANADP by NAD^+ glycohydrolases and the NADP analogue inhibits the dehydrogenases of the PPP however; in *Leishmania* its toxicity is only seen in millimolar range, in which 6-AN consumes the PRPP content of *Leishmania* parasites. The nicotinate decrease indicates that one target could be the nicotinamidase enzyme. However for the PRPP, it is not clear if its drop comes from the ribose 5-phosphate decrease since its level was not changed, although decreased levels of ribose were found as well as low peaks of $NADP^+$ and sedoheptulose7-phosphate in *L. infantum*, which is more sensitive to 6-AN compared to *L. mexicana*. Depletion of PRPP in turn diminishes conversion of nucleobases to their nucleoside products. When given in high doses 6-AN can kill the parasites, probably due to the depletion of nucleotides required for nucleic acid biosynthesis. It seems that in *Leishmania* a NAD^+ glycohydrolase similar to the mammal one does not work however, other approaches like combined ^{13}C-glucose labelling and flux analysis might be useful to ascertain the fate and action mechanism of 6-AN in *Leishmania*. In addition, the parasite growth inhibition due to the depletion of the PRPP highlights that PRPP synthetase should also be a good target for new potential drugs against leishmaniasis.

The biochemical study on TGase in *Leishmania* confirms the presence of TGase activity by detecting a Ca^{2+}-dependent transamidation in canine *L. infantum* promastigotes through *in vivo* and *in vitro* incorporation of FC into the parasite proteins. The protein TGase precipitated at 15 and 45% AS saturations. The inhibition of this TGase by GTP suggests that regulation of the activity of this enzyme might be calcium/GTP regulated. The TGase pAbs (orb2986) detected a calculated major protein band of about 74.6 KDa in the whole extracts as well as 15 and 45% AS fractions with enzyme activity of the canine strain and two bands of 55.34 and 65.87 KDa in the whole lysate of the human strain. The detected protein bands and the *in vivo* immunocytochemical detection of the putative TGase protein suggest that the pAbs (orb2986) could ease the affinity chromatographic purification of this

Leishmania enzyme. Partially purified fractions of the active enzyme should be used for immunoblotting so as to ascertain that the detected protein bands are associated to TGase activity. However, only when having the purified enzyme we could completely validate the use of these antibodies. If purified and studied, this enzyme might offer a good drug target for combating *Leishmania*.

The clinical part

Chapter 4

Subclinical *Leishmania infantum* infections in autoimmune rheumatic patients on biologic drug treatment and living in rural areas of Northern Italy

4.1 Introduction

4.1.1 General introduction

Although environmental aspects such as climate change, growing urbanisation, socio-economic development, etc. are causing changes in the epidemiology of infectious diseases including *Leishmania* (WHO, 2016), host immunity is known to represent a key determinant of the clinical manifestation and outcome of the disease. Immunosuppression in particular, has been identified as a major contributor to disease re-emergence in Europe in patients with autoimmune rheumatic diseases who live in *Leishmania* endemic areas (Ready, 2010; Xynos *et al*., 2009). Autoimmune rheumatic diseases affect the joints and/or connective tissues and worldwide, rheumatoid arthritis (RA) is a common rheumatic autoimmune disease, which causes cartilage and bone damage leading to disability and early death (Firestein, 2003). Psoriatic arthritis (PsA) is a seronegative, major histocompatibility (MHC) class I (HLA-B and HLA-C loci)-associated (Rahman and Elder, 2005; Winchester *et al*., 2008) chronic inflammatory arthritis with the predominance of CD8+ cytotoxic T cells in the synovial tissues and fluid (Costello *et al*., 1999), and develops in patients with chronic skin disease, psoriasis (Amherd-Hoekstra *et al*., 2010). Ankylosing spondylitis (AS) is a type of autoimmune arthritis that affects the joints of the spine (Khan, 1992) in patients positive for HLA-B27, which present unique peptides on MHC class 1 leading to the activation of CD8+ cytotoxic T cells (Kuon and Sieper, 2003) and subsequent damage of synovial tissues. PsA and AS have similarities to RA that make the differential diagnosis more difficult (Cantini *et al*., 2010), and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α),

interleukin-1 (IL-1), and IL-6 that are responsible for synovitis are common to these rheumatic syndromes (Bal *et al*., 2007; van Kuijk *et al*., 2006; McInnes and Schett, 2011) therefore, the immunopathogenesis of RA will be taken as a representative in this study.

The opportunistic behaviour of *Leishmania* has been reported in immunosuppressed and immunocompromized patients (Desjeux and Alvar, 2003). In this setting, a number of published case reports suggest an association between TNF-α blockers used for the treatment of rheumatic diseases, and evident leishmaniasis (Xynos *et al*., 2009; Zanger *et al*., 2012). TNF-α is the key pro-inflammatory cytokine in inflammatory arthritis and is found in high concentrations in patients with RA (McInnes and Schett, 2007).

4.1.2 Leishmaniasis in Italy

Leishmaniasis caused by *Leishmania infantum* is endemic in large parts of southern Europe (Ready, 2010) where there is a focal prevalence of latent infection of up to 53% in the adult population (Moral *et al.*, 2002). However, leishmaniasis is a rare disease in Central Europe and is diagnosed almost exclusively in travellers or migrants coming from tropical or subtropical countries (Poeppl *et al.*, 2013).

Due to increasing population movement, military operations and immigration, the number of imported leishmaniasis cases in countries of Western Europe has increased in past decades. From 1986 to 2012, 105 imported cases were reported, of them 36 were VL (16 co-infected with HIV), and 69 were CL (Di Muccio *et al*., 2015).

VL is an endemic disease in rural and peri-urban areas of continental and insular regions of central-south Italy (Gradoni *et al*., 1993). During the 1990s, most of the imported VL cases caused by *L. infantum* were diagnosed among HIV-positive Italian tourists (Gramiccia, 2003).

Recent epidemiological data have shown a spread of the parasite and sandfly also in some Northern regions, due to temperature increase owing to recent climate changes. In Emilia-Romagna, a Northern region of Italy, 44 cases were diagnosed in the period between 1999 and 2011 of which 26 were VL. The number of cases rose to 30 in 2013 and Bologna was the most affected province (Varani *et al*., www.eurosurveillance.org). In fact, between 1971 and 1972, an outbreak of 60 clinical

cases (with 13 deaths) zoonotic VL occurred in a village near Bologna (Pampiglione et al., 1974).

4.1.3 Asymptomatic *Leishmania* infections

Asymptomatic VL infections represent a challenge in Leishmaniasis control as they act as potential, hidden parasite sources (Stauch *et al*., 2011). The ratio asymptomatic to developed VL are reported to be 50 : 1 in Spain (Moral *et al*., 2002), 18 : 1 in Brazil (Evans *et al*., 1992), 8.9 : 1 in highly-endemic foci in India and Nepal (Ostyn *et al*., 2011), 5.6 : 1 in Ethiopia (Ali and Ashford,1994), 4 : 1 in Kenya (Schaefer *et al*., 1995), and Bangladesh (Bern *et al*., 2007), and 1 : 2.4 in Sudan (Zijlstra *et al*., 1994). Diagnosis of asymptomatic infections alongside the evident VL is therefore important for effective control of *Leishmania*. In the last years, the availability of polymerase chain reaction (PCR) assays such as conventional PCR and real-time PCR that amplify *Leishmania* genetic material of different targets including kDNA, 18S rRNA, ssu rRNA, mini-exon-derived RNA genes, and internal transcribed spacer (ITS) regions (Srividya *et al*., 2012) has been a major step forward in improving the diagnosis of leishmaniasis and the response to treatment compared to serological methods, which may fail to detect asymptomatic *Leishmania* infections especially in endemic areas (Srivastava *et al*., 2013). These techniques have also been successfully used to follow-up VL patients (Sakkas *et al*., 2016) and diagnose subclinical infections (le Fichoux *et al*., 1999; Riera *et al*., 2004; Colomba *et al*., 2009), and were suggested as marker of *L. infantum* infection in asymptomatic individuals in epidemiological studies in Brazil (Srivastava *et al*., 2013). The PCR methods can be done on different biological samples including tissue and lymph node aspirates, peripheral blood, and urine (Motazedian et al., 2008) and qPCR on blood showed 100% sensitivity using kDNA-designed primers for diagnosis of *L. infantum* infections (Mary *et al*., 2004). Although, the molecular methods are satisfactorily sensitive, they require sophisticated equipment, skilled personnel, and are not applicable at field settings (Reithinger and Dujardin, 2007).

4.1.4 Rheumatoid arthritis (RA)

RA is a common inflammatory autoimmune disease that causes pain, swelling, stiffness, and loss of function in the joints particularly, knee joints, wrist, and finger

joints close to the hands. The disease develops when the immune system attacks the synovial membrane lining of the joints causing hyperplasia and progressive damage of articular cartilage and bone (McInnes and Schett, 2011) however, the exact cause of RA remains unknown (Smolen and Steiner, 2003). Systemic complications including cardiovascular, pulmonary, psychological and skeletal disorders can occur (McInnes and Schett, 2011; Hochberg *et al*., 2006). RA ranges from mild to severe however, it is chronic in most cases, and has a symmetrical pattern; if one joint is inflamed the other also is affected (Arnett *et al*., 1988).

4.1.4.1 Immunopathogenesis of RA

The initiation of RA is thought to be a consequence of the appearance of autoantibodies, anticitrullinated protein antibodies (ACPA) (known as anti-cyclic citrullinated peptides antibodies, anti-CCP) and anti-FC portion of the IgG (rheumatoid factor, RF) in the blood, years before the development of the disease (Schellekens *et al*., 1998; Shi *et al*., 2013; Rantapää-Dahlqvist *et al*., 2003). The high-level calcium-dependent enzyme, peptidylarginine-deiminase (PAD) replaces one of the terminal imide groups of peptide-bound arginine by oxygen converting the positively charged arginine to a polar but neutral citrulline (post-translational modifications) (Vossenaar *et al*., 2003) resulting in the appearance of citrullinated proteins with altered structure, antigenicity, and function capable of breaking the immune tolerance (Suzuki *et al*., 2003). Reactions of ACPA with extracellular and/or cell surface citrullinated proteins such as fibrinogen, vimentin, and α-enolase (Klareskog *et al*., 2008), and cartilage collagen II (Snir *et al*., 2010), stress proteins (Shoda *et al*., 2011), and intracellular histone (Pratesi *et al*., 2013), have been reported. In addition, *Porphyromonas gingivalis*, the bacterium that causes periodontitis express PAD14, which is capable of citrullinating mammalian proteins (Wegner et al., 2010), and carbamylated proteins in which lysine is homocitrullinated have also been described (Quirke *et al*., 2013). Although a number of citrullinated proteins maybe present in the RA synovium, citrullinated fibrin, citrullinated vimentin and citrullinated histones are important autoantigens in RA (Vossenaar and van Venrooij, 2004). The antigen presenting cells (APC), dendritic cells, macrophages, and B cells recognize these citrullinated peptides/proteins as non-self thus they uptake, process, and present them in association with class II MHC to naïve CD4+ T

cells in the presence of additional signaling by costimulatory molecules resulting in B cells activation and generation of ACPA (Meulenbroek and Zeijlemaker, 2000). Complex interactions between genetic and environmental factors have been recently suggested to trigger the appearance of ACPA. The human leukocyte antigen HLA-DR4 shared epitope, HLA-DRB1*04 and *0404 allele-expressing patients have elevated risk for recognizing the citrullinated proteins (Gregersen *et al*., 1987; van Gaalen *et al*., 2004; Silman and Pearson, 2002). In fact, 80% of the RA patients have HLA-DRB1*04 cluster (Smolen *et al*., 2007). Other genetic loci which predispose for RA include protein tyrosine phosphatase non-receptor type 22 (PTPN22), PAD14, signal transducer and activator of transcription 4 (STAT4), tumor necrosis factor-associated factor (TRAF1-C5), and tumor necrosis factor alpha-induced protein 3 (TNFAIP3) however, the risk of these alleles represents only 3-5% of the genetic susceptibility to RA (Plenge, 2009). Environmental factors, such as smoking, bronchial stress (e.g., exposure to silica), obesity and infections may also induce citrullination of proteins in the lungs leading to the development of RA (Klareskog *et al*., 2007; Getts and Miller, 2010; Symmons *et al*., 1997; Imboden, 2009). The complex interaction between immune cells, and mediators and immunoglobulins these patients produce is responsible for synovitis and subsequent joint damage (Fig. 4.1) (Smolen and Steiner, 2003). Mechanical trauma or infections of the joints enable circulating ACPA and citrullinated proteins such as fibrinogen to reach the synovial membrane, which contains fibroblast-like synoviocytes and sentinel cells (macrophages, dendritic cells, and mast cells) and as a result a cellular immune response is started (Loewi *et al*., 1974). The IgE ACPAs (Schuerwegh *et al*., 2010) activate mast cells to release histamine and other mediators, which induce vasodilatation, angiogenesis and immune cells recruitment and infiltration including neutrophils (Gaboury *et al*., 1995; Lee *et al*., 2002) whereas macrophages secrete interleukin-1 (IL-1), IL-6, and TNF-α (Smolen and Steiner, 2003). TNF-α plays a fundamental role in RA pathogenesis through the activation of synovial cells, including osteoclasts, and cytokine expression and release whereas IL-1 promotes activation of leukocytes, endothelial cells and chondrocytes, and IL-6 induces autoantibody production (McInnes and Schett, 2011; Smolen *et al*., 2007). In addition, the high-level TNF-α in RA patients inhibits and impairs the functional ability of regulatory T cells thus joint damage continues (Behrens *et al*., 2007). Synoviocytes

produce inflammatory cytokines, prostaglandins (PGs) and matrix metalloproteinases (MMPs) leading to destruction of cartilage (Smolen *et al*., 2007). In addition, APC further present citrullinated antigens in the joint to naïve T cells causing their activation with release of IL-2 and IL-2 receptor alpha (IL-2Rα) and polarization to effective T helper cells (Th1) and occurrence of established local immune reactions in the synovial tissue (Choy, 2012). Moreover, B cells and T cells cause increased production of antibodies and cytokines leading to persistent activation and damage (Smolen and Steiner, 2003). It has been shown recently that macrophage-and dendritic cells-derived transforming growth factor B, IL-6, IL-21, and IL-23 create a milieu that support differentiation of IL-17-secreting CD4+ helper T 17 (Th17) that has a major role in synovitis (Harrington *et al*., 2005). IL-17 synergizes with TNF-α to stimulate a number of cells in inflamed synovium including fibroblasts and chondrocytes to secrete multiple mediators, which create a positive feedback loop leading to a continuous activation of T cells (Chabaud *et al*., 1999; Shahrara *et al*., 2008). However, in the early RA, an elevated transient cytokine profile of Th2 (IL-4- and IL-13), Th17 (IL-17), and IL-15 secreted by microphages dominate (Raza *et al*., 2005). Initially, the serological diagnosis of RA was dependent upon the detection of RF however; it is reported recently that detection of anti-CCP antibodies by enzyme-linked immunosorbent assay (ELISA) using CCPs is of great importance (Schellekens *et al*., 2000). Anti-CCP antibodies are present in about 70% of RA patients and they are more specific than RF (Nishimura *et al*., 2007).

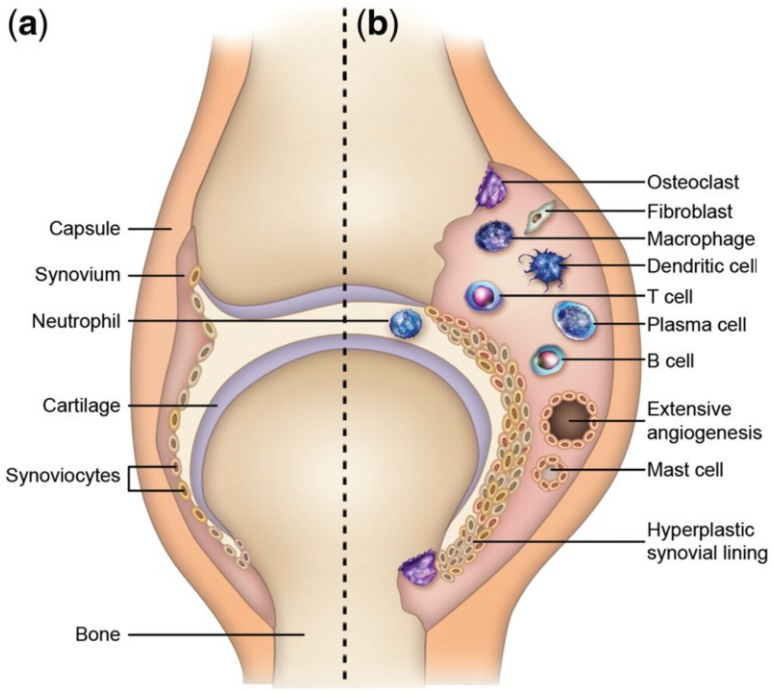


Figure 4.1. A graphic representation shows normal joint (a) and RA-inflamed joint (b). The affected joint has swollen, hyperplastic synovial membrane with infiltration of immune cells. Adapted from Smolen and Steiner, (2003).

4.1.4.2 Treatment of RA:

Elucidation of the role of the pro-inflammatory cytokines in the pathogenesis of RA has led to development of a number of biological agents that target these immune mediators (Table 4.1) particularly, TNF-α, IL-6, IL-1 as well as T and B cells activation signalling. These anti-inflammatory agents are either used alone or in combination with disease-modifying anti-rheumatic drugs such as methotrexate (MTX) (Smolen *et al.*, 2007). TNF-α blockers are the most common and favorable anti-inflammatory agents that have been used for treatment and control of RA (McInnes and Schett, 2011).

4.1.4.2.1 TNF-α blockers

TNF-α is a pro-inflammatory cytokine produced mostly by activated macrophages (Olszewski *et al*., 2007), with an important role in the resistance to several intracellular microorganisms, including *Leishmania* and produced in a host's attempt to control infection (Garcia-Vidal *et al*., 2009), cell recruitment and formation of granuloma (Roach *et al*., 2002). Lloyd J. Old first reported TNF in 1975 (Carswell *et al*., 1975). It is primarily produced as membrane-integrated protein (mTNF-α) from which the soluble form is released (Black *et al*., 1997). TNF-α is encoded by a single-copy gene on human chromosome 6 (Spriggs *et al*., 1992). TNF can bind with high affinity to two receptors, TNFR1, which is expressed in most tissues and activated by both the membrane-bound and soluble forms (sTNF-α) of TNF, and TNFR2 that is found in cells of the immune system and respond to the membrane-bound form (Theiss *et al*., 2005). The mTNF-α is associated with autocrine/paracrine activation where as sTNF-α regulates the endocrine response (Grell, 1995). TNFα and T helper 1 cell-derived gamma interferon (IFNγ) synergize to induce activation of macrophages resulting in up-regulation of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) (Liew *et al*., 1990). In general, the TNF-blocker therapy (Table 4.1) impairs the inflammatory response, increasing susceptibility and reducing the ability to fight infections such as leishmaniasis (Martin-Mola and Balsa, 2009).

Most of leishmaniasis cases in patients on TNF-α blocker therapy were reported from endemic regions of Southern Europe whereas fewer occurred after travel to and migration from endemic areas (Zanger and Gabrysch, 2013). In this regard, opportunistic visceral leishmaniasis (VL) has been shown to occur also under traditional immunosuppressive treatment regimens but with a longer disease onset duration compared with the shorter duration in patients treated with anti-TNF-α therapy (Xynos *et al*., 2009).

4.1.4.2.2 IL-6 signalling blockade

IL-6 is an important mediator that is secreted by macrophages and T cells in response to specific microbial molecules or any tissue damage leading to inflammation (van der Poll *et al*., 1997) however, it acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine as it increases up to 100-fold in the circulation during physical exercise (Pedersen and Febbraio, 2008). IL-6 signals

through a complex cell-surface that consists of membrane-bound IL-6Rα chain (CD126) and the signal-transducing component CD130. When IL-6 binds its receptor, it triggers the CD130 and IL-6R proteins to form a complex thus initiating a signal transduction cascade leading to cell activation (Heinrich *et al*., 1998). The pro-inflammatory effects of IL-6 in RA involve, neutrophil recruitment (Lally *et al*., 2005), B cells proliferation and antibody production, and T cells proliferation and differentiation (McInnes and Schett, 2007), and osteoclast activation (Kudo *et al*., 2003). In addition, IL-6 combines with TGF-β, IL-21, or TNF-α to stimulate differentiation of naïve T cells into Th17 cells (Yang *et al*., 2008; Volpe *et al*., 2008). The focal role of IL-6 in the inflammatory immune response has increased the interest in developing anti-IL-6 agents (Table 4.1) against autoimmune diseases such as RA (Emery *et al*., 2008).

4.1.4.2.3 CD4+ T cell activation modulation

CD4+ T cells play important roles in orchestrating the immune reponses particularly, the adaptive one. These cells help enhance (Th1 and Th17), suppress (Th2) or regulate (Treg) immune responses (Zhu *et al*., 2010; Alberts *et al*., 2002). After development, naïve T cells leave the thymus and migrate throughout the body, including the lymph nodes (Fowlkes *et al*., 1985). During the immune response, the professional APCs such as DCs recognize exogenous or endogenous antigens through their toll-like receptors (TLRs) and phagocytize them (Kumar *et al*., 2011). The activated DC then displays the processed antigenic peptides on MHC class II molecules to naïve CD4+ T cell, which recognizes the antigen through T cell receptors (TCRs), and CD4 molecule that interacts with MHC class II. This interaction represents the primary signal for T cell activation (Gao *et al*., 2002). The interaction of the antigen with TLRs of the DCs stimulates them to the express co-stimulatory molecules, CD80 and CD86, which bind CD28 and CD40 that further binds CD40 ligand (CD40L) on T cells, giving the second signal for full activation of effector T cell (June *et al*., 1987; Mueller *et al*., 1989). Furthermore, DCs direct the polarization of Th1 subset by producing IL-12 and IL-18, as well as IL-21 and IL-23 for formation of Th17 (Murphy *et al*., 2008).

Following the activation, T cells then release IL-2 receptor alpha (IL-2Rα), and the growth factor, IL-2 which acts in an autocrine pattern resulting in activation of T cell proliferation pathways (June *et al*., 1987). A biological drug (Table 4.1) that aborts T

cell activation by preventing APCs from delivering the co-stimulatory signal has been developed (Bristol-Myers Squibb, 2007).

4.1.4.2.4 Depletion of B cells

B lymphocytes are the important cells in the humoral immunity of the adaptive immune response. They present antigens to T cells (Lanzavecchia, 1987), secrete cytokines (Lund, 2008), and produce antibodies after differentiating into plasma cells (Burnet, 1957). Thus, B cells play a crucial role in RA by producing ACPA, as well as in other autoimmune diseases (Edwards and Cambridge, 2001). The B cell membrane CD20 acts as calcium channel however, it has no natural ligand (Cragg *et al*., 2005). Chimeric anti-CD20 monoclonal antibodies have been developed to bind CD20 leading to depletion of CD20+ B cells (Edwards and Cambridge, 2001) through antibody dependent cell-mediated cytotoxicity (ADCC) (Clark and Ledbetter, 2005) leading to improvement in inflamed joint.

4.1.4.2.5 IL-1 receptor neutralization

As mentioned earlier, IL-1 participates to synovitis and cartilage damage by inducing activation of leukocytes, endothelial cells, chondrocytes, and osteoclasts (McInnes and Schett, 2007). IL-1 binds IL-1R leading to activation of downstream inflammation signaling (Weber *et al*., 2010). A biological IL-2R antagonist is used to block IL-1 signaling and reduce the inflammation however; results obtained are humble (McInnes and Schett, 2011).

Table 4.1 The description of the common biological agents used in the treatment of autoimmune rheumatic diseases.

Biological agent	Target	Composition	Mechanism of action	Reference
Adalimumab (ADA)	TNF-α	Synthesized human IgG1 monoclonal antibody	Binds TNF-α and block TNF-α –receptor interaction	(Berkshire, 2010; North Chicago, 2009)
Infliximab (IFX)	TNF-α	Chimeric monoclonal antibody with murine variable region and human IgG1 constant region	Binds soluble and membrane-bound TNF-α and prevent binding of TNF-α to TNF-α receptor	(Malvern, 2009; Scott and Kingsley, 2006; Leiden, 2009)
Golimumab	TNF-α	Human monoclonal antibody	Prevent TNF-α-TNF-α receptor interaction by binding to TNF-α	(Leiden, 2009)
Certolizumab Pegol	TNF-α	Monoclonal antibody with humanized fab fragment	Neutralizes soluble and membrane-bound TNF-α thus prevent binding to TNF-α receptor	(Brussels, 2009; Smyrna, 2009)
Etanercept (ETA)	TNF-α receptor	Construct of human p75 TNF-α receptor fused to an Fc portion of gamma immunoglobulin (IgG1)	Binds to TNF-α and block TNF-α-TNF-α receptor interaction	Berkshire, 2010; Thousand Oaks, 2009)
Tocilizumab (TCZ)	IL-6 receptor	Humanized monoclonal antibody	Binds soluble IL-6 and prevent binding of IL-6 to IL-6 receptor	(Welwyn Garden City, 2009)
Anakinra	IL-1 receptor	IL-1 receptor antagonist	Occupies IL-1 receptor and blocks binding of IL-1 to IL-1 receptor	(Stockholm, 2009)
Rituximab	CD20	Chimeric monoclonal antibody	Depletes B cells by binding to CD20 (B cell differentiation antigen)	(Welwyn Garden City, 2008)
Abatacept (ABT)	CD80 and CD86	Construct of cytotoxic T lymphocyte-associated antigen 4 fused to an Fc portion of IgG (CTLA4-Ig)	Inhibits T cell activation by binding to CD80 and CD86 preventing CD80- and CD86-CD28 interaction	(Uxbridge, 2009)

4.2 Materials and methods

4.2.1 Study patients and design

We performed a retrospective observational study enrolling 50 patients suffering from autoimmune rheumatic diseases (RA, PsA and AS) and treated with BD (either Infliximab, Tocilizumab, or Abatacept) for at least 5 years at the Rheumatology Unit of the University of Ferrara (Group A) and 50 healthy subjects as a control group (Group B).

Demographic and clinical data were collected using standard questionnaires, which included the region, province of residence, location of their home in an urban or rural context, and the ownership of a dog as a family pet.

All the enrolled patients were negative by serologic screening for hepatitis B and C, HIV and Tuberculosis and they did not present any symptoms of leishmaniasis at the moment of the blood sampling. The CD4+/CD8+ ratio was > 2 in all individuals belonging to Group A and B.

4.2.2 Blood collection and peripheral blood mononuclear cell (PBMCs) separation

About 6 ml venous blood was collected from anticubital vein from all participants of the study using disposable syringes, after cleaning the skin with 70% alcohol. The drawn blood was poured into EDTA container. Red blood cells (RBCs), PBMCs, and plasma were fractioned by density gradient centrifugation over Ficoll-paque plus (Amersham Biosciences Europe Gmbh, Milan, Italy), according to the manufacturer's protocol. Plasma and the buffy coat were pipetted into a new eppendorf tube and the RBCs fraction was discarded. Then the plasma and PBMCs were separated by centrifugation at 2000 rpm for 10 minutes, the plasma was pipetted into clean eppendorf tube, and stored at -20°C. The pelleted PBMCs fraction was washed 2 times in 1 ml sterile PBS, pH 7.2 followed by centrifugation at 5,000 rpm for 8 minutes. A volume of 200 µl TE buffer pH 8.8 was added to the cells and they were stored at -80°C till use.

4.2.3 DNA extraction

DNA was extracted from PBMCs of autoimmune rheumatic patients as well as from buffy coat of a patient with confirmed VL (positive biological control) as previously

described by Kellog and Kwok, (1990). The complete lysis buffer was composed of buffer A which contained 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$ and 100 mM KCl, and buffer B which included 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 1% Tween-20, and 1% Triton X-100. About 200µl (100 µl buffer A plus 100 µl buffer B) lysis buffer containing proteinase K (200 mg/ml), were added to each PBMCs sample followed by incubatation for 1 hour at 56 C° and heating for 10 min at 96 C° to inactivate proteinase K. Samples were then cooled to room temperature and stored at -20°C. Reference control DNA was extracted from cultured *L. infantum* promastigotes using a slightly modified protocol of Meredith *et al*., (1993). PBS-washed *L. infantum* promastigotes were re-suspended with gentle mixing in 1 ml sterile lysis buffer composed of 50 mM Tris–HCl, pH 8.3, 10 mM EDTA and 50 mM NaCl followed by addition of sodium dodecyl sulphate (SDS) to a final concentration of 0.5% and gentle shaking until the solution was viscous. The sample was then digested overnight at 60 °C with 100 µg/ml proteinase K. For the extraction of DNA, an equal volume of phenol: chloroform:isoamyl alcohol mix was added to the sample with shaking for at least 3 minutes followed by centrifugation at 16,000 rpm for 10 minutes then the aqueous phase was transferred to a clean tube and an equal volume of chloroform:isoamyl alcohol mixture was added to the aqueous phase with mix gentle mixing and centrifugation as before. Then 100 µl of 3 M sodium acetate and 550 µl ice-cold 96% ethanol were added to the sample with mixing and subsequent incubation at -20°C for 1 hour, then the sample was centrifuged at maximum speed for 30 minutes and the supernatant was discarded. Finally, the DNA pellet was washed in 70% alcohol, dried using a speed vacuum dryer for 10 minutes at 30°C, and dissolved in 100µl TE buffer, pH 8.0, then stored at 4°C until use.

4.2.4 Conventional PCR

Conventional PCR reactions were carried out at the Infectious Diseases Laboratory of the University of Ferrara, Italy, employing strict routine precautions to avoid the risk of contamination by exogenous DNA or PCR product carryover (Kwok and Higuchi, 1989). A conventional PCR assay Kit (Go Taq® G2 Hot Start, Promega, USA) was used to amplify *Leishmania* ssu rRNA gene using the protocol of Schönian *et al*., (2003), with minor modifications. The primers used were R221 (5'-GGTTCCTTTCCTGATTTACG-3') and R332 (5'-GGCCGGTAAAGGCCGAATAG-3')

(van Eys *et al*., 1992) (Sigma, Germany), which amplify 603 bp DNA of all *Leishmania* species. Prepared under a PCR hood and in a total volume of 50 µL, the PCR mixture contained 1X DNA polymerase buffer, 4 mM $MgCl_2$, 250 µM (each) dNTPs (dATP, dCTP, dGTP, and dTTP), 1 U Taq polymerase, 1 µM (each) primer, and 5 µl patient or healthy control DNA sample. Five µl *Leishmania* DNA and *Leishmania*-positive patient DNA, and sterile double distilled water were used as positive and negative controls, respectively. By using a thermocycler (*MasterCycler* gradient, Eppendorf, Germany), 38 amplification cycles were performed with each cycle consisting of: denaturation at 95°C for 5 minutes, annealing at 63°C for 1 minute, and extension at 72°C for 2 minutes. PCR products were electrophoresed at 100V for 1 hour in 1.5% agarose (GellyPhor^{LE}, EuroΘlone, Italy) gel in 40 mM TEA buffer with 1 mM EDTA (Invitrogen, USA) and 250µg/ml ethidium bromide (Sigma, USA). DNA size standards consisting of 12 DNA fractions ranging from 100 to 3000pb (*SHARPMASS*™ 100 PLUS, EuroΘlone, Italy) were separated alongside PCR products to allow sizing of specific DNA bands. DNA was visualized and digitally photographed under UV light using a transilluminator (GENENCO, France). All PCR reactions were repeated at least twice. Samples showing positivity in repeated conventional PCRs were considered positive and further tested by quantitative real-time PCR (qPCR).

4.2.5 Real-time PCR

A real-time quantitative PCR (qPCR) for detection and quantification of *L. infantum* DNA was performed. For accurate sensitivity, kDNA was chosen as the molecular target. External primers were derived from RV1 and RV2, described previously by Lachaud *et al.* (2002). Detection was performed by means of a hybridization probe based on TaqMan chemistry (Applera, Courtaboeuf, France) designed with Primer 3 software. A Stratagene (La Jolla, Calif.) MX 4000 system was used for amplification and detection. Optimization of experiments led us to use the Stratagene qPCR master mix (catalog number 600549-51), followed by addition of 15 pmol of direct primer (CTTTTCTGGTCCTCCGGGTAGG), 15 pmol reverse primer (CCACCCGGCCCTATTTTACACCAA), and 50 pmol TaqMan probe (FAM-TTTTCGCAGAACGCCCCT ACCCGC-TAMRA). Assays were performed in 25 µl final volume with 1 µl sample DNA. The standard curve was generated from *Leishmania* (L) DNA extracted from 5 x 10^6 parasites; 1 µl of serial dilutions, ranging from 50,000

to 0.0001 parasites was pipetted into each reaction tubes. TaqMan chemistry allowed two-step temperature (94 and 55°C) cycling over 45 cycles. All qPCR were performed at Parasitology-Mycology Laboratory of the Archet Hospital, University of Nice in France.

4.2.6 Cytokine analysis

Pro-inflammatory and anti-inflammatory mediators were measured in the sera of LqPCR-positive (n. 18) and LqPCR-negative (n. 20) rheumatic patients and the control subjects (n. 20) using the magnetic bead-based multiplex immunoassays (Bio-Plex, BIO-RAD Laboratories, Milano, Italy). Luminex multiplex technology Kit was used for the measurement of a panel of cytokines including IL-1β, IL-1Ra, IL-2, IL-2Rα, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-15, IFN-α2, IFN-γ, TNF-β and TNF-α.

Following the manufacturer's instructions, 50 µl from each serum sample were diluted 4 times. Diluted sera and reaction standards were added in duplicate to a 96-multi-well plate containing analyte beads followed by incubation at room temperature for 30 minutes. The plate was washed, and the antibody-biotin reporter was added and incubated for 10 minutes with streptavidin phycoerythrin. The cytokine levels were determined using the Bio-Plex array reader (Luminex, Austin, TX). The Bio-Plex Manager software optimized the standard curves automatically and returned the reading data as Median Fluorescence Intensity (MFI) and the cytokine concentration as pg/ml.

4.2.7 Statistical analysis

Data analysis was performed using SPSS Statistics for Windows, version 21.0 (SPSS, Inc., Chicago, IL, USA). Normality of distribution for continuous variable was checked by Shapiro-Wilk test. Since the variables were not normally distributed, group comparisons between controls and patients were performed using Mann-Whitney U test.

Chi-square test was used to compare differences in categorical variables. Binary logistic regression analysis was used to assess the independent effects of living in a rural area, having a dog or taking a steroid in association with a biological drug on the rate of positive PCR results for *Leishmania*. Two-tailed probability values <0.05 were

considered statistically significant. The cytokine data were analyzed by Mann-Whitney non-parametric Student's t-test and represented as graphs using GraphPad Prism 7.0 (GraphPad Software, Inc., USA).

4.2.8 Ethical considerations

The ethics committee of the University of Ferrara in Italy has approved this study. All the study participants knew that they are taking part in a research study and written informed consents were obtained from them before their enrolment.

4.4 Results

Demographical and clinical data of the present study population (n=100) are summarized in Table 4.2. The control subjects (Group B) showed younger ages than Group A ($p<0.001$), whereas no significant difference in sex distribution between the two groups was found.

Group B participants resided mainly in rural areas*[1] rather than in urban context ($p<0.05$), while a statistically significant difference was not found between the two groups regarding the ownership of a dog as a family pet. Finally, Group A showed a statistically significant positivity for circulating *L. infantum* kDNA compared to control group ($p<0.001$). The two groups were then analysed separately.

Table 4.2. Demographic characteristics of autoimmune rheumatic patients (A) and healthy controls (B).

Patient's Characteristics	B (n=50)	A (n=50)
Age (years)	55 (31-60)	61 (49-67) [a]
Gender (females, %)	58	70
Rural area (%)	56	36 [b]
To be owner of a Dog (%)	34	36
Positivity for circulant *Leishmania* DNA (%)	10	36 [c]

Not-normally distributed variables are expressed as median (interquartile range), discrete variables as percentage.

[a] $p<0.001$, Mann-Whitney vs. controls

[b] $p<0.05$ χ^2 vs. controls

[c] $p<0.001$, χ^2 vs. controls

4.4.1 Control subjects (Group B)

Among group B, 1 sample (2%) only resulted positive for *Leishmania* DNA by qualitative PCR, while 4 (8%) were positive by qPCR (10 parasite/ml in 1 patient and

*[1] As defined by the Organization for Cooperation and Economic Development (OECD), the "predominantly rural areas" are areas in which more than 50% of the population resides in rural communes. Rural municipalities are those municipalities with a population density below 150 inhabitants per km^2.

1 parasite/ml in 3 patients). The 4 positive subjects lived in rural areas; 2 lived around Bologna, whereas the remaining 2 around Ferrara and Ravenna (Figure 4.2). Only one subject among those was a dog owner.

Figure 4.2. A geopgraphical map of Emilia-Romagna region, representative of provinces affected by circulating *Leishmania* DNA.

4.4.2 Rheumatic patients (Group A)

Among the 50 analysed rheumatologic samples (one sample from each patient), 18 (36%) resulted positive by qualitative PCR (15 are shown in Fig. 4.3). These were also confirmed by qPCR with the detection of high parasite burdens (1 to 136 parasite/ml in 4 patients, 1.000 to 40.000 in 11 patients and over 1.000.000 in 3 patients). Among the 18 positive patients, 14 (77,7%) were women and 4 men (22.3%), with a median age of 58.7 years. The clinical features of rheumatologic patients divided into negative and positive for *Leishmania* DNA by qPCR analysis (LqPCR), are summarized in Table 4.3. Twelve (77.8%) out of 18 suffered from RA and 3/18 (11.1%) from AS and PA. Every positive patient presented a long history of

an autoimmune rheumatic disease for a period between 8 and 29 years with a mean value of 20.3±5.6. Fourteen (66.6%) out of the 18 patients lived in rural areas, while 4 (22.3%) in urban areas, and 9/18 (50%) owned a dog as a family pet. The province with the highest LqPCR incidence was Ravenna (7 cases), followed by Imola (6 cases), Rovigo (3 cases) and Ferrara (2 cases) (Figure 4.2). To have a dog and being positive for *Leishmania* DNA was not found statistically significant.

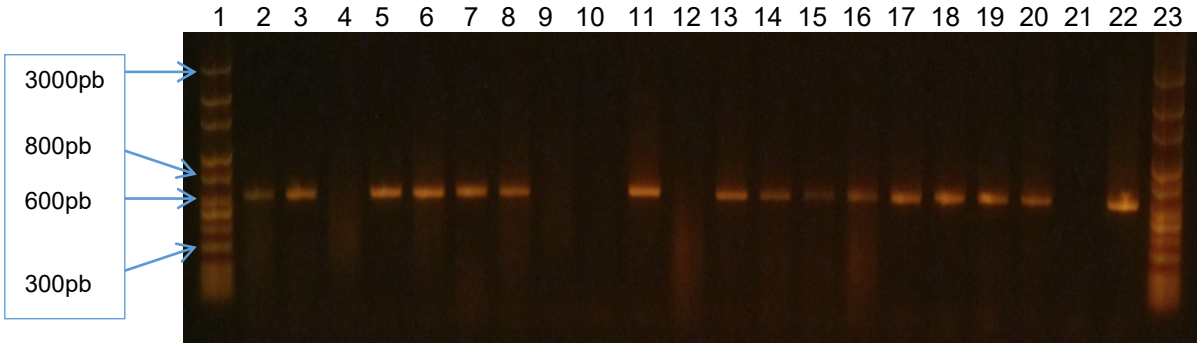


Figure 4.3. 1.5% agarose gel image showing the size of the amplified PCR products of 603 bp of the ssu rRNA gene of *Leishmaia*. Lanes 1 and 23 represent the molecular size (weight) marker (100 bp). Lanes; 2, 3, 5, 6,7,8,11, 13,14,15,16,17,18,19, and 20 are autoimmune rheumatic patient samples positive for *Leishmania* DNA. Lanes; 4, 9,10, and 12 are autoimmune rheumatic patient samples negative for *Leishmania* DNA. Lane 21 is a negative control. Lanes 22 is a positive control.

Table 4.3. Clinical characteristics of autoimmune rheumatic patients divided into negative and positive for *Leishmania* DNA by qPCR analysis.

	***Leishmania* kDNA qPCR**	
Patient's Characteristics	**NEGATIVE (n=32)**	**POSITIVE (n=18)**
Age (years)	58.4±13.2	58.9±9.5
Disease duration (years)	22.0±5.7	20.3±5.6
Therapy duration (years)	10.7±2.7	10.6±3.4
Rural area (%, yes)	21.8	66.6 [a]
To be owner of a Dog (%, yes)	31.3	50.0
Rheumatologic Disease (%)		
RA	61.7	77.8
PsA	6.0	11.1
AS	32.3	11.1
Biological treatment (%)		
anti-TNF-α	81.4	55.6
T lymphocyte modulator	9.3	22.2
anti-IL-6 receptor	9.3	22.2
Steroid drug (%, yes)	25.0	61.1 [b]

Continuous variables are expressed as mean±SD, discrete variables as percentage. RA: rheumatoid arthritis; PA: psoriatic arthritis; AS: ankylosing spondylitis. [a] $p<0.001$, χ^2 vs. negative. [b] $p<0.05$ χ^2 vs. negative.

Positive patients were then grouped according to the pharmacological action of the BD into TNF-alpha antagonists, modulators of T lymphocyte activity and anti-IL-6 receptor antibodies; the possible influence of each type of the drugs on the molecular positivity for *L. infantum* circulant kDNA was assessed using a Chi-square test. This test did not reveal any statistical difference among the different administered treatments ($\chi2$ (2) = 3.37, p=0.185), although the percentage of the positivity for LqPCR seemed higher under therapy with modulators of T lymphocyte activity compared to anti-IL-6 receptor antibodies and TNF-α antagonist treatment (Table 4.3, 55.6%, 22.2% and 22.2%, respectively). Patients were then divided into two groups on the basis of the concurrent, or not, administration of steroids together with the BD. Interestingly, patients that were taking a steroid in association with the BD showed a higher proportion of *Leishmania* positivity than those treated with BD alone (Table 4.3, $\chi2$ (1)=6.18, $p<0.05$; 61.1% vs. 25.0%).

4.4.3 Cytokine profiles

The immune responses were assessed in LqPCR-positive and –negative autoimmune rheumatic patients and the healthy control group by measuring cytokine levels. Pro-inflammatory cytokines of type I immune response including CD4+ Th1 cell cytokine IFN-γ, type II immune response played by CD4+ Th2 cell mediators; IL-4, IL-5 and IL-13, macrophage, DC and other synovial cell mediators; TNF-α, IL-1β, IL-6, IL-7, and IL-15, and the regulatory T cell cytokine; IL-10, were found markedly increased in LqPCR-positive and –negative autoimmune rheumatic groups compared to the control group (Fig. 4.4) confirming the pathologic effects of these mediators during the course of the autoimmune rheumatic diseases characterized by continuous cellular activation, synovitis and cartilage damage. However, IL-8, the neutrophils chemotactic factor was found elevated in LqPCR -negatie autoimmune rheumatic patients compared to LqPCR-positive and controls. On the other hand, LqPCR-positive autoimmune rheumatic patients showed increased levels of Th1 response inflammatory cytokines; IFN-γ, and APC (macrophage and DC)-secreted mediators; IL-12 (p70), TNF-α, IL-1β, IL-6 and IL-15 compared to those of LqPCR-negative autoimmune rheumatic patients. However, Th17-derived IL-17, Th2-secreted cytokines; IL-4 and IL-13, and Treg cell mediator; IL-10 levels (Fig. 4.4) as well as IFN-γ inducible protein-10 (IP-10) and monokine induced by IFN-γ (MIG) were not significantly different in LqPCR-positive and –negative autoimmune rheumatic patients. IL-17 is recently reported to play a principal role in synovitis, cartilage and bone damage as well as in *Leishmania* infections.

The cytokine results indicate that both autoimmune rheumatic diseases and *Leishmania* infections triggered inflammatory responses however, IL-2 levels in LqPCR-negative autoimmune rheumatic patients did not differ from those in the healthy group (Fig. 4.4). On the other hand, analysis of cytokine concentration in LqPCR-positive autoimmune rheumatic patients (Table 4.4 and Fig. 4.5) in relation to the biological drug used and parasite load did not show any statistical significance except for IL-12 and IL-10, which were found increased in patients treated with abatacept compared to infliximab.

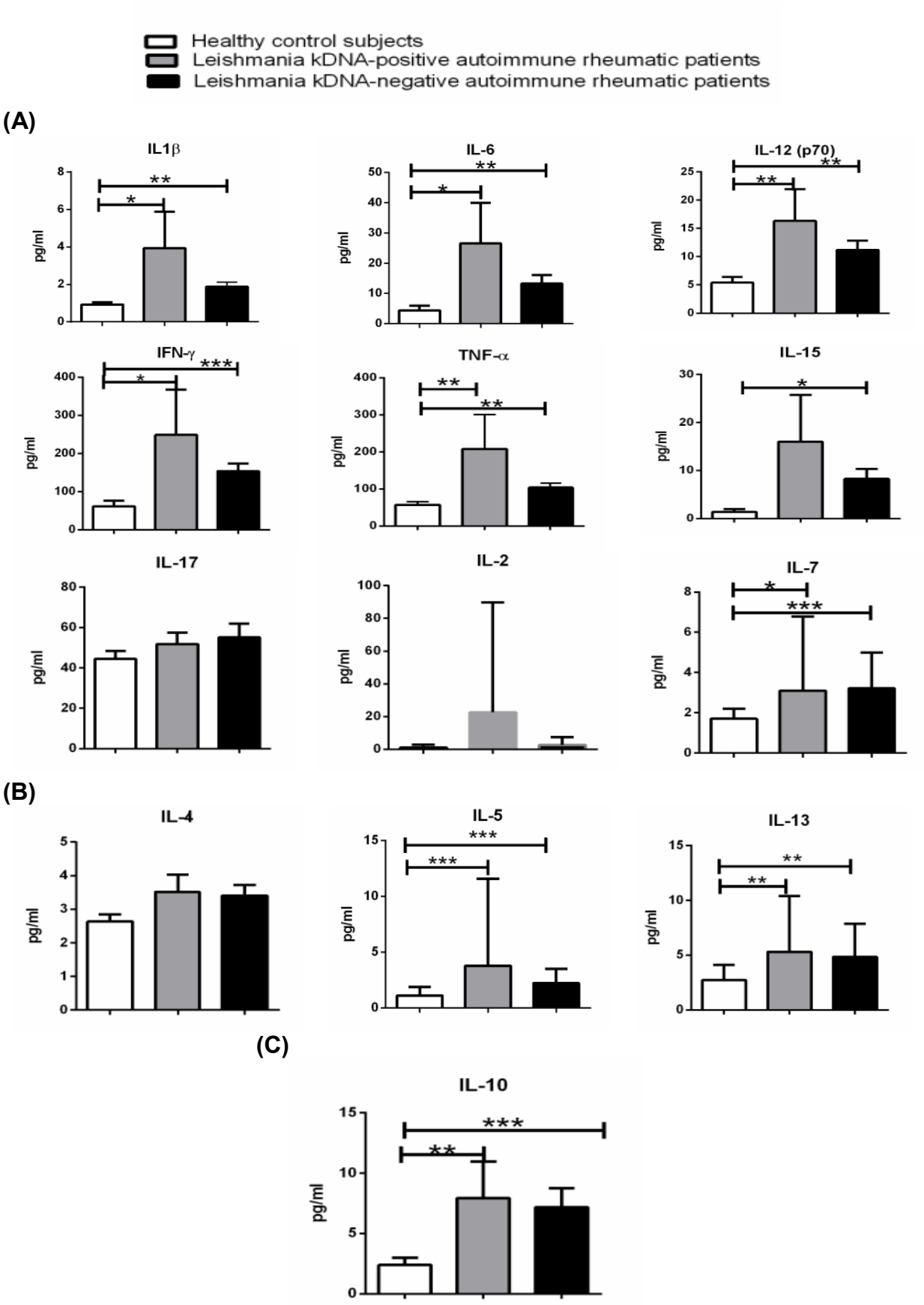

Healthy control subjects
Leishmania kDNA-positive autoimmune rheumatic patients
Leishmania kDNA-negative autoimmune rheumatic patients
(A)
IL1β
IL-6
IL-12 (p70)
IFN-γ
TNF-α
IL-15
IL-17
IL-2
IL-7
(B)
IL-4
IL-5
IL-13
(C)
IL-10
pg/ml

Figure 4.4. Graphic representations of down-regulated or up-regulated pro-inflammatory (A), anti-inflammatory (B) and immune-regulatory (C) cytokines in plasma from autoimmune rheumatic patients positive and negative for *Leishmania* kDNA, and the healthy control group. Mann-Whitney non-parametric Student's t-test was used to compare between the three groups and significant results are marked with asterisks: *P. Value*s, *p <0.05, **p <0.01, ***p <0.001. IL-4 and IL-17 did not show any statistically significant difference between analyzed groups.

Table 4.4. Levels of pro-inflammatory, anti-inflammatory and immune-regulatory cytokines in relation to *Leishmania* parasite density detected by qPCR in autoimmune rheumatic patient treated with biological drugs.

Patient No.	BD	LqPCR Parasite/ml blood	Cytokine level (pg/ml)												
			IL-2	IL-17	IL-1β	IL-6	IL-12 (p70)	IL-15	TNF-α	IFN-γ	IL-4	IL-13	IL-5	IL-7	IL-10
1	ABT	22.67	0.48	67.87	0.85	5.59	9.55	0.25	63.83	50.7	3.33	2.64	0.9	2.17	2.69
2	ABT	2500	0.48	63.23	1.21	9.67	23.49	0.93	96.85	101.6	3.25	11.7	3.77	2.48	9.1
3	ABT	6566.6	181	59.24	15.59	170.8	85.63	122.7	509.89	708.2	4.68	4.35	2.07	3.23	38.51
4	ABT	1268.3	240	133.95	36.14	211.4	83.28	149.5	1830.4	2298	12.0	24.3	35.7	18.1	50.64
5	ABT	<1	6.21	19.9	1.43	32.5	16.45	23.37	95.08	95.89	2.39	13.88	1.56	2.23	18.47
6	IFX	4544.33	0.48	41.64	1.39	0.48	8.12	0.25	67.23	87.92	2.03	3.15	1.9	2.04	3.15
7	IFX	4677.67	0.48	56.81	0.71	0.99	3.27	0.25	50.42	30.33	3.33	1.89	0.53	1.68	0.96
8	IFX	4061	0.48	26.22	1.41	4.36	7.7	0.25	75.82	103.9	1.86	3.64	2.15	2.1	2.95
9	IFX	275	0.48	13.97	0.5	5.69	4.5	0.25	38.99	72.27	1.53	4.94	1.67	1.98	3.7
10	IFX	509.3	0.48	44.12	2.17	6.94	9.55	9.6	89.78	158.9	3.57	5.47	3.23	3	5.22
11	IFX	214444.3	0.48	58.63	1.63	8.01	6.81	0.25	187.35	141.8	3.59	3.71	2.53	2.61	4.12
12	IFX	<1	10.1	79.18	1.18	12.67	16.61	14.01	100.41	131.0	5.56	3.43	0.95	7.32	4.92
13	IFX	1.08	0.48	62.61	1.88	11.26	6.47	1.52	84.52	158.9	4.06	3.01	2.35	2.35	2.5
14	TCZ	992.6	0.48	46.03	1.15	25.78	7.84	0.25	153.37	67.87	2.78	3.29	1.5	1.86	2.82
15	TCZ	326666.6	0.48	55.29	1.84	12.37	10.57	1.81	155.24	151.5	3.46	5.17	2.94	2.58	4.34
16	TCZ	403833.3	0.48	48.1	1.23	2.42	5.53	0.25	122.03	78.92	2.86	3.01	1.28	1.86	2.4
17	ETA	3611	0.48	19.77	0.71	1.7	3.03	0.25	48.76	30.33	1.46	2.54	0.74	1.15	1.36
18	ADA	<1	0.48	31.91	1.28	4.27	5.27	3.02	58.76	83.4	2.39	4.35	1.81	1.92	3.20

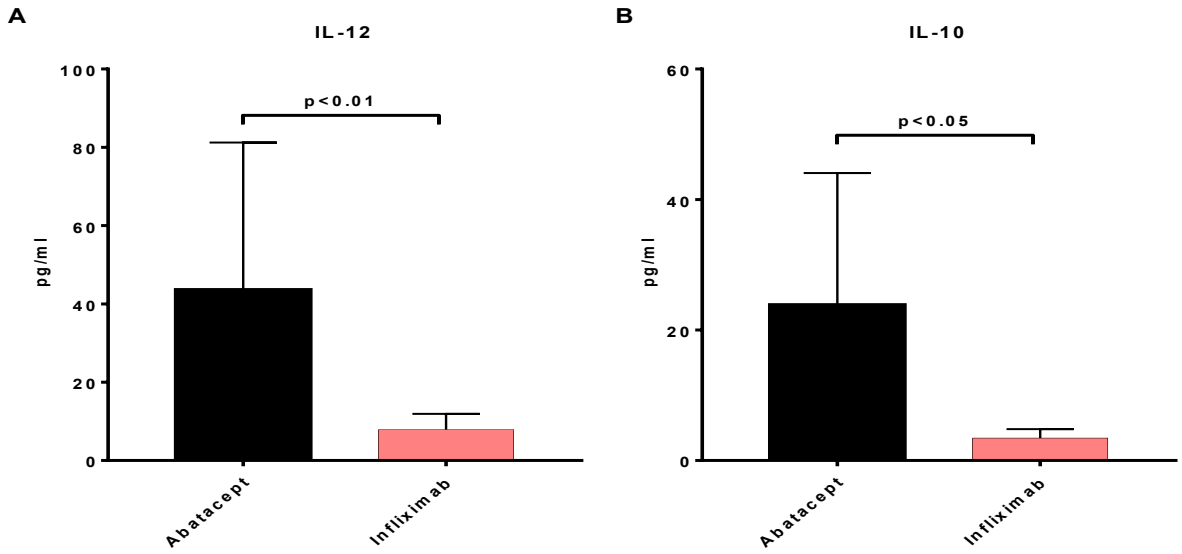


Figure 4.5. Expression of pro-inflammatory (A) and immune-regulatory (B) cytokines that showed statistically significant different concentrations in relation to the biological drug, infliximab or abatacept used to treat autoimmune rheumatic patients. P denotes *P. Value*.

4.5 Discussion

Leishmaniasis is a dynamic disease and different factors such as global warming (affecting the ecology and distribution of the *Phlebotomine* vectors) and man-made risk factors (travels, migration, trade of animals and environmental modifications) are changing the temporal-spatial evolution of leishmaniasis in Europe (Dujardin *et al*., 2008; Di Muccio *et al*., 2015).

In Mediterranean countries, VL is hypoendemic and accounting for 5–6% of the global burden. Most infections remain asymptomatic, but malnutrition and immunosuppression predispose to clinical disease (Okwor and Uzonna, 2013). Indeed, most human *Leishmania* infections are subclinical or asymptomatic, and this can be due to the development of effective cell-mediated immune responses (Ostyn *et al*., 2011)

In Northern Italy, leishmaniasis has spread due to climatic conditions that favour the development of the sandfly, *Phlebotomus perniciosus* (Maroli *et al*., 2013; Signorini *et al*., 2014), and increased mobility of dogs to and from the traditional VL endemic areas (Maroli *et al*., 2008), although the risk for human disease is moderate, as the intensity of transmission seems to be lower than in traditional Mediterranean VL.

Moreover, the use of immunosuppressive drugs, in particular BD, has contributed to expand the number of reported cases in this area. In fact, the commercialization of the BD (such as TNF-α blockers) has become increasingly frequent since the end of the 1990s and has proven effective in the management of several autoimmune diseases, such as RA (van Griensven *et al*., 2014). Their use however, is not free of side effects, especially for the onset of opportunistic infections, such as *Pneumocystis jirovecii* pneumonia, histoplasmosis, cytomegalovirus infection, aspergillosis, cryptococcal meningitis, and granulomatosus diseases such a tuberculosis and leishmaniasis (Marie and Guglielmino, 2010).

Our study represents a survey of asymptomatic human *L. infantum* infections in Emilia-Romagna, an Italian region not traditionally considered endemic for *Leishmania* and characterized by continental climate and a low incidence of officially reported VL cases, but recently switched as “phlebotomus risk region” like some coastal and hilly regions of the middle Adriatic coast and many hilly areas of the pre-Alpine regions of Northern Italy. Canine leishmaniasis, which represents the only

source for human disease, has being increasing in Northern Italy, spreading to rural and periurban areas (Maroli *et al*., 2008).

Traditionally, only southern, central, and insular Italian regions, particularly along the Tyrrhenian littoral, were considered stable, endemic foci for both human and canine leishmaniasis (Pozio *et al*., 1985). Further evidences were found for increasing canine seropositivity and the spread of sandflies in the last decade, as well as by the occurrence of autochthonous human VL cases in the surveyed area (Varani *et al*., 2013).

Our study which included 50 autoimmune rheumatic patients (Group A) treated with BD for at least 5 years and 50 healthy controls (Group B) selected randomly from the apparently health population, has shown a strikingly higher prevalence of *Leishmania* DNA in Group A than in Group B ($p<0.001$, χ^2 vs. controls) (Table 4.1). Among the 18 *Leishmania* kDNA-positive patients of Group A, a large percentage (66.6%) resided in rural areas. The high detection rate of circulating *Leishmania* DNA in a region where the pathogen is not considered endemic suggests that a significant local transmission of *L. infantum* to humans has been going on for years in these areas. In fact, *Leishmania* positivity in both study groups appears to be significantly related to residence in the rural rather than in urban context, highlighting the importance of the suggested repeated and uninterrupted contact with the responsible vector and the animal reservoir (Biglino *et al*., 2010). However, our data showed that dog ownership was not statistically significant and that is consistent with the reported data which show that domestic dogs play only a minor role in the transmission cycle of the parasite (Costa *et al*., 1999; Costa *et al*., 2000) in which the sylvatic hosts (including foxes, wolves and stray dogs) were also found implicated, along with inter-human transmission (Costa *et al*., 2000) however, rates of infection of sandflies fed with VL patients are not dissimilar to those fed with infected domestic dogs (Costa *et al*., 2000) and parasite prevalence in wild canines is often as high as in domestic dogs (Gavgani *et al*., 2002).

The finding of *L. infantum* DNA in 5 out 50 (10%) healthy controls was not surprising as they lived in rural areas, which appears to be a predominant aspect related to the positivity.

In our study, the decision to employ molecular methods for the diagnosis of asymptomatic *Leishmania* infection was supported by the fact that serological

methods are quite specific and sensitive in diagnosing active VL in immunocompetent subjects (Chappuis *et al*., 2006; Kumar *et al*., 2001), although titers decline rapidly in a significant portion of patients after the resolution of the disease (De Almeida Silva *et al*., 2006; Kumar *et al*., 2001). Moreover, the serologic tests frequently give false-negative results in immunocompromised patients even in the presence of positive *Leishmania* qPCR (Garcia-Garcia *et al*., 2006) making their use unsatisfactory for detecting asymptomatic *Leishmania* infection characterized by low or intermittent parasitaemia. In addition, the choice to extract and test DNA from PBMCs, instead of whole blood, has favoured the finding of high parasitic burdens, since PBMCs are target cells for the intracellular parasite (le Fichoux *et al*., 1999).

A possible explanation of the detection of the high parasite load found in Group A could be the immunosuppressive therapies taken by those patients for the treatment of autoimmune rheumatic diseases. Such therapies can impair the ability of host immune system to better control latent infections.

Regarding the type of immunosuppressive treatments, there were no statistically significant differences in relation to the BD administered (TNF-α blockers, modulators of T lymphocyte activity, and anti-IL-6 receptor antibodies) and its possible influence on the molecular positivity for LqPCR ($\chi 2$ (2) = 3.37, p=0.185), although the percentage of the positivity for parasite DNA seemed higher in patients treated with T lymphocyte activity modulators and anti-IL-6 receptor antibodies, compared to TNF-α antagonist treatment (Table 3, 57.1%, 50.0% and 26.3%, respectively). De Kossodo et al., (1994) reported that, in mouse cutaneous leishmaniasis, TNF does not decide the susceptibility versus resistance since neutralization of TNF activity delayed the healing process but did not affect the outcome of the infection. Studies in the literature have shown the important role played by CD4+ T cells in protection against human leishmaniasis. Thus hampering the gamma interferon (IFN-γ)-producing CD4+ T cells response paves the way for the parasite persistence since IFN-γ is important in resolution of VL infection due to its capacity to induce reactive nitrogen species (RNS) by macrophages resulting in the parasite killing (Kaye and Scott, 2011). On the other hand, antigen-encountered T cell exhaustion (gradual loss of function) due to chronic parasite persistence has also been reported (Wherry, 2011).

Several literature data suggest that TNF-α antagonists may increase susceptibility to opportunistic infections, including very rare leishmaniasis (CL, MCL and VL) (Xynos

et al., 2009; Garcia-Gonzalez *et al*., 2012; Fabre et al., 2005; Guarneri *et al*., 2016; Jeziorski *et al*., 2015; Guedes-Barbosa *et al*., 2013). Increased incidence of tuberculosis, histoplasmosis, listeriosis, candidiasis, and aspergillosis after TNF-α blockade treatment has been reported (Wallis *et al*., 2004; Wolfe *et al*., 2004; Gomez-Reino *et al*., 2003; Abbott Laboratories, 2003; Mohan *et al*., 2004). It is therefore, noteworthy that also other categories of BD with different binding affinities and pharmacokinetics can induce immune disorders at multiple levels of adaptive and innate immunity, leading to a macrophage dysfunction and, consequently, to a bad control of the infection itself.

The outcome of *Leishmania* infection depends essentially on the infecting *Leishmania* species, the host genetics and immune response to infection. The early innate immune responses by the host, including DC- and macrophage–parasite interactions, are crucial in determining the persistence or clearance of the parasites (Bosque *et al*., 2000).

Cytokine patterns during autoimmune rheumatic diseases and Leishmaniasis are complex and unpredictable. Our work is the first to study immune responses in *Leishmania* DNA-positive autoimmune rheumatic patients. In this study, cytokine analysis showed that type I immune response mastered by CD+ Th1 cells occurred in both LqPCR-positive and –negative autoimmune rheumatic patients treated with BD with up-regulation of key inflammatory mediators; TNF-α, IL-12p(70), IL-1β, IL-6, and IL-7 secreted by macrophages, DCs, and other cells in the synovium, and IFN-γ produced by differentiated Th1 cells. TNF-α, IL-1β and IL-6 are the predominant cytokines in the synovial tissues and they are responsible for synovitis, cartilage and bone damage (Firestein, 2003). The inflammatory cytokine, IL-17 secreted by a distinct type of T helper cells, Th17, did not show statistically significant difference between the LqPCR-positive and –negative autoimmune rheumatic patients and healthy control group however; Th17 has been reported to play an important role in tissue damage (Annunziato *et al*., 2007) and IL-17 was found elevated in asymptomatic VL patients (Pitta *et al*., 2009). However, in this study, the non-different IL-17 levels could be due to the use of the T cell activity modulator (abatacept) which prevents naïve T cell activation with subsequent production of IL-2 and differentiation into Th17. Th2 (type II immune response) –secreted cytokines; IL-4, IL-13 and IL-5 as well as Treg cell cytokine; IL-10 were also elevated in LqPCR-

positive and –negative autoimmune rheumatic patients implying that Th2 and Treg cells were activated to reduce the immune-inflammatory damage that has been occurring in the affected joints. On the other side, in LqPCR-positive rheumatic patients, increased levels of IFN-γ, and APC (macrophage and DC)-secreted mediators; IL-12 (p70), TNF-α, IL-1β, IL-6 and IL-15 were noted compared to LqPCR-negative autoimmune rheumatic patients which indicates that in qPCR-positive patients a *Leishmania* specific Th1 immune response has happened with the production of inflammatory cytokines capable of at least preventing the occurrence of a full VL course and that is consistent with fact that none of those patients presented a typical clinical picture of VL. In addition, it is likely that those patients did not develop clinical symptoms, despite the high level of parasitaemia, because their normal CD4+ lymphocytes counts were sustained.

Splenomegally and intermittent fever were reported in opportunistic VL in PsA (De Leonardis *et al*., 2009; Tektonidou *et al*., 2007; Romani-Costa *et al*., 2004) and RA (Bagalas *et al*., 2007; Fabre *et al*., 2005) patients treated with TNF-α antagonists however; Bassetti *et al*., (2006) reported a VL/RA case without splenomegaly. De Leonardis *et al*., (2009) suggested that although VL can rarely develop in patients under BD treatment, its occurrence should be linked to fluctuant fever, pancytopenia and splenomegally. However, the high amount of *Leishmania* DNA detected by qPCR in autoimmune rheumatic patients reflects a very high parasitaemia, a finding suggestive of clinically active atypical VL rather than asymptomatic infection. In fact the susceptibility and outcome of *Leishmania* infection depends not only on the immune response but also the host genetics as well as the infecting *Leishmania* species. Of the host genetic factors, it has been reported that the early innate immune responses by the host cells, including DC- and macrophage–parasite interactions with the production of IL-12 or Il-4, are crucial in determining the persistence or clearance of the parasites respectively (Bosque *et al*., 2000).

On the contrary, in the LqPCR-positive healthy subjects, the parasitaemia was lower, indicative of cryptic *Leishmania* infection and that is in line with literature data (le Fichoux *et al*., 1999; Marty *et al*., 1994; Colomba *et al*., 2005).

Th2- and Treg -secreted anti-inflammatory mediator levels in autoimmune rheumatic patients positive for *Leishmania* DNA were similar to those in *Leishmania* DNA-negative but higher than those of the control group. In addition, IFN-γ inducible

protein-10 (IP-10), monokine induced by IFN-γ (MIG), and IL-8 reported to be elevated in VL patients (Kurkjian *et al*., 2006) were also found comparable in LqPCR-positive and –negative autoimmune rheumatic patients with IL-8 higher in the DNA-negative rheumatic group. Variable cytokine profiles have been reported in subclinical VL infections with low IL-10 (Gama *et al*., 2004; Costa *et al*., 2012), IL-12 and IFN-γ (Gama *et al*., 2004). Costa *et al*., (2012) found lower IFN-γ, TNF-α, IL-4 and IL-10 levels in asymptomatic carriers compared to patients with active VL however, IL-2 was higher in asymptomatic than VL patients. The imbalance between Th1 and Th2/Treg cell cytokines i.e., IL-2 and IFN-γ:IL-4 and IL-10 has been described to decide resistance versus susceptibility (Zwingenberger *et al*., 1990; Ghalib *et al*., 1993; Karp *et al*., 1993; Holaday *et al*., 1993; Carvalho *et al*., 1994)

The persistence of *Leishmania* DNA in those autoimmune rheumatic patients could be due to an established Th2 causing inhibition of the production of NO by macrophages and increasing polyamine synthesis in these cells leading to parasite proliferation (Iniesta *et al*., 2005). IFN-γ has been reported to synergize with TNF-α for the activation of macrophage microbial killing machinery (Liew *et al*., 1990). Moreover, we suggest that the significantly increased Treg-released IL-10 during the autoimmune rheumatic diseases was beneficial for the parasite intracellular life establishment as it has been reported to inhibit production of IFN-γ and TNF-α (Ehrenstein *et al*., 2004). However, high level of functional TNF-α in RA patients has been found to impair Treg cells (Behrens *et al*., 2007) denoting that neutralization of TNF-α would induce Treg cells to resume their function in TNF-α blocker-responsive patients and produce IL-10 as reported by Ehrenstein *et al*., (2004), which predisposes autoimmune rheumatic patients to *Leishmania* infections. In theory, patients treated with TNF-α blockers are at high risk in developing fatal Leishmaniasis as reported by Fromm *et al*., (2016) in CL in C57BL/6 mice in spite of exaggerated Th1 response however, in contrary, de Kossodo *et al*., (1994) suggested that in BALB/c and CBA mice, TNF does not decide resistance or susceptibility in *Leishmania* infection. Actually, in animal models either Th1 or Th2 polarization (dichotomy) predominates with clearance of the parasites or development of active *Leishmania* infections respectively (Nieto *et al*., 2011) however, in human infections mixed Th1/Th2 responses is noted (Ghalib *et al*., 1995).

The cytokine level analysis in LqPCR-positive autoimmune rheumatic patients in relation to the BD used and the parasite load detected did not show any statistical significance except for IL-12 and IL-10 which were found increased in patients treated with abatacept (modulation of T cell activity) compared to infliximab (anti-TNF-α antibodies) and this could be ascribed to that APCs worked normally and produced elevated amounts of IL-12 to stimulate the crippled T cells, and IL-10 to stimulate Treg cells to produce more IL-10 to stop tissue damage.

Interestingly, in the total number of the patients positive for *Leishmania* DNA (n. 18), the positivity was more among those receiving the BD in combination with steroids (Table 2, χ^2 (1)=6.18, $p<0.05$; 55.6% vs. 21.2%). This finding is supported by several reports on cases of *Leishmania* reactivation in patients receiving steroid treatment for long periods for different indications, including asthma, sarcoidosis, and myasthenia gravis (van Griensven *et al*., 2014).

To our knowledge, the present study represents a first survey on *Leishmania* subclinical infections in autoimmune rheumatic patients receiving BD in an Italian region not traditionally considered endemic for *Leishmania*. As living in endemic rural areas appears to be a factor closely linked with the risk of developing subclinical *Leishmania* infection and immunosuppression can predispose to overt disease, it would be mandatory to promote molecular screening for *Leishmania* DNA in BD-immunosuppressed patients living in rural areas as well as parasite load follow-up, in order to administer prompt, specific treatment since the first appearance of clinical symptoms.

4.6 The clinical study conclusion

In this study, the high *Leishmania* parasite loads detected in PBMC fractions from autoimmune rheumatic patients suggest that biologic drugs can favor the establishment of opportunistic parasitic infections such as Leishmaniasis through inhibition of the infection-controlling immune responses. In not traditionally considered *Leishmania*-endemic regions as Northern Italy, some autoimmune rheumatic patients under immunosuppressive-BD treatment may have atypical VL or *Leishmania* infection in a latent stage, which might progress to symptomatic disease. This should be taken into account especially for those patients living in rural areas. In this context, molecular screening coupled to cytokine analysis can be used to diagnose and confirm cryptic *Leishmania* infections in order to administer specific treatment since the first appearance of clinical symptoms and before the establishment of the life-threating disease. In addition, these asymptomatic individuals represent potential reservoir host and infection sources. Because PBMCs are target cells for *Leishmania* parasites and non-invasively collected, they could be useful clinical samples to diagnose *Leishmania* asymptomatic infections using the highly sensitive qPCR. Besides, regular parasite load monitoring should be carried out for *Leishmania* DNA-positive, BD-treated autoimmune rheumatic patients.

Thesis conclusion and future perspectives

In this thesis, I have investigated *Leishmania* and leishmaniasis in biochemical and clinical studies searching for new promising drug targets and exploring the occurrence of opportunistic VL during immunosuppression.

In the biochemical part, I considered as potential drug targets, the pentose phosphate pathway and the enzyme transglutaminase.

PPP was studied by a metabolomics approach, after treating *L. mexicana* and *L. infantum* promastigotes with the PPP inhibitor, 6-AN. To cause a significant growth inhibition, I had to use mM concentrations of the compound, which mainly caused depletion of the PRPP content with a marked reduction in nucleotide pool. In addition, nicotinate, the precursor for NAD^+ synthesis was found reduced. 6-AN derivatives of NAD/P and 6-AN/PRPP adduct were not detected. In *Leishmania,* PPP does not seem the major target of the compound contrasting what has been shown in many mammalian cell lines and in agreement with the already demonstrated significant differences between the mammalian and the parasite pathway. However, other approaches like combined ^{13}C-glucose labelling and flux analysis might be useful to ascertain the fate and action mechanism of 6-AN in *Leishmania*.

On the other hand, we confirm the presence of TGase activity in human and canine *Leishmania infantum.* In contrast with the reported Ca^{2+}-independent enzyme activity in some *Leishmania* species, in canine *L. infantum,* transamidation was Ca^{2+}-dependent and further inhibited by GTP. Moreover, pAbs (orb2986) against human TGase 2 succeeded in detecting a calculated protein band of about 74.6 KDa in the whole extract as well as AS fractions with enzyme activity of the canine strain and two bands of 55.34 and 65.87 KDa in the whole lysate of the human strain. These antibodies could assist in the purification of *Leishmania* TGase however, only purified enzyme could confirm the usefulness of these antibodies. If purified and studied, this enzyme might offer a promising drug target pointing to its role in the parasite growth.

In the clinical study, we found that treatment of autoimmune rheumatic patients with biologic drugs predisposes them to VL infections particularly, in the rural areas. Screening for *Leishmania* infections by molecular methods using PBMC fractions, and cytokine analysis should be carried out before treating autoimmune rheumatic patients with biologic drugs. Patients who tested positive for *Leishmania* DNA should

be followed-up with regular parasite load monitoring so as to prevent the occurrence of full courses of VL.

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