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DOTTORATO DI RICERCA IN MOLECULAR MEDICINE AND
FARMACOLOGY
CICLO: XXXIII

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**LEISHMANIASIS: A RE-EMERGING NEGLECTED
DISEASE.
BIOMOLECULAR AND METABOLOMIC STUDIES
AIMED AT IMPLANTING ITS CONTROL IN
EMILIA-ROMAGNA**

Settore Scientifico Disciplinare MED17

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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	Peptidylarginine Deiminase
Treg	Regulatory T Lymphocyte
TLR	Toll-Like Receptor
TCR	T cell Receptor
BD	Biological Drug

Abstract

Background

Leishmaniasis 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. The present thesis presents some results regarding *Leishmania* and leishmaniasis in clinical and biochemical studies.

The first part of the thesis is the clinical one, 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.

According to the biochemical part, the effect of the niacin analogue, 6-AN on *Leishmania* parasite growth and metabolism using the metabolomics technology was the subject of study. The rationale 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.

Methods

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.

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.

Results

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.

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.

Conclusions

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.

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 microorganisms, however, combined ¹³C-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.

1. Overview of *Leishmania*

1.1 Introduction

Leishmaniasis 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 kinetoplast 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 *et 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 transplantation (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 accidentally 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 Glaswegian 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

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 (Santafem 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, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic.

About 90% of mucocutaneous leishmaniasis cases occur in Plurinational State of Bolivia, Brazil and Peru (WHO, 2018). 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, 2018).

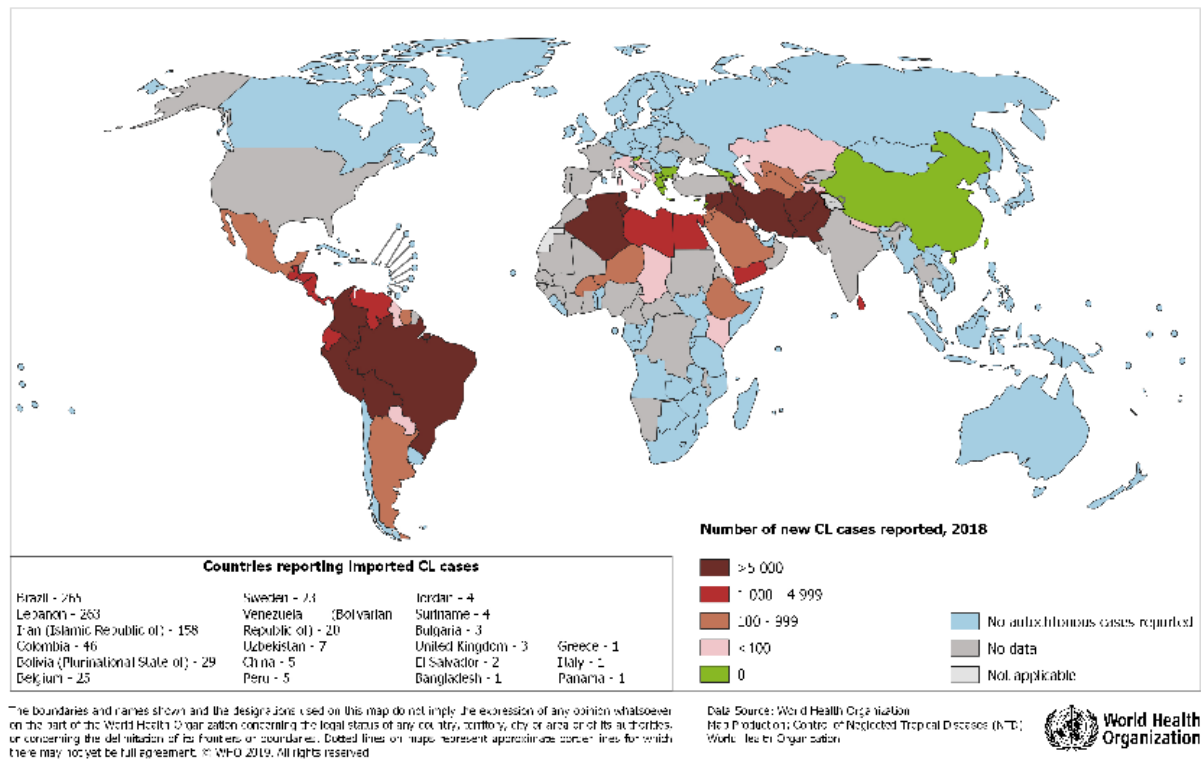
Leishmaniasis ranks second in mortality among tropical diseases (Mathers *et al.*, 2007) however, the mortality data of leishmaniasis are extremely sparse and generally reflect deaths that occur in hospitals only although, 20,000 to 40,000 leishmaniasis deaths occur every year (Alvar *et al.*, 2012).

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 Maltezos, 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, Bangladesh that reported more than 9000 cases in 2006 reported 255 and 192 new cases in 2016 and 2017, respectively (WHO, 2018). 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.

Status of endemicity of cutaneous leishmaniasis worldwide, 2018



B.

Status of endemicity of visceral leishmaniasis worldwide, 2018

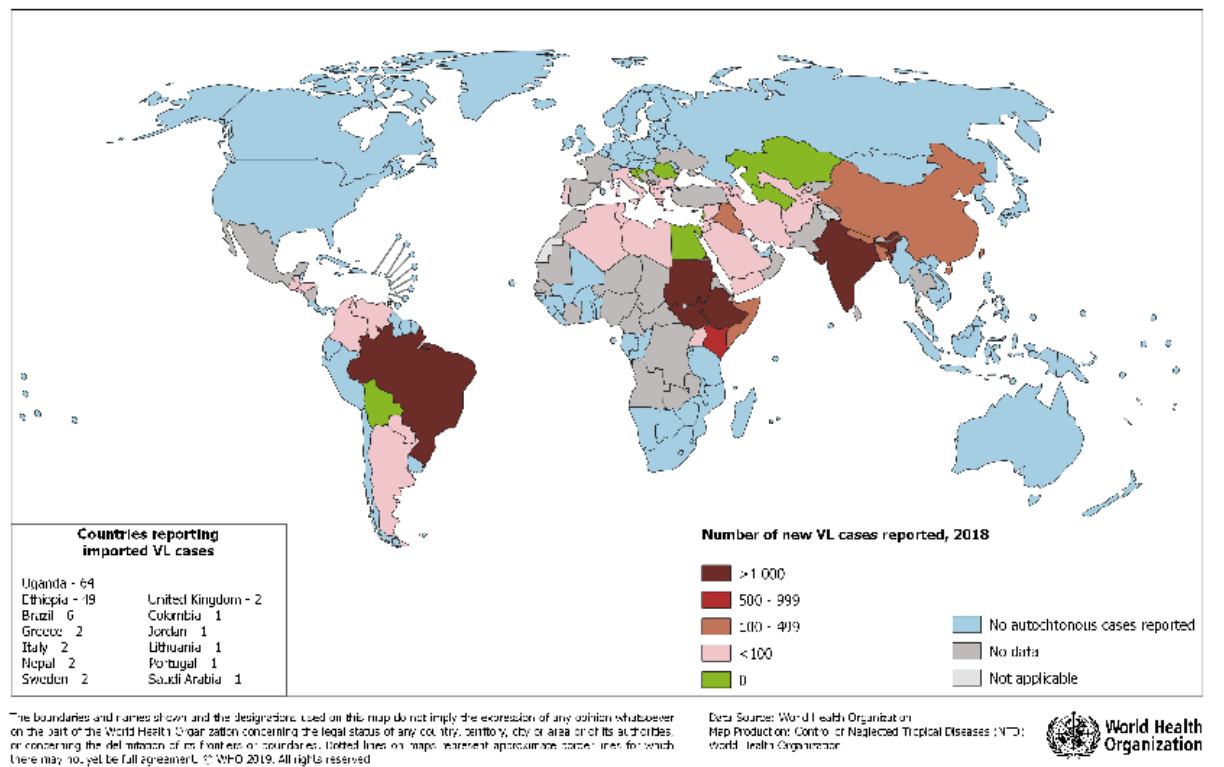


Figure 1.1. The geographic distribution and endemicity map of cutaneous leishmaniasis (A), and visceral leishmaniasis (B) according to WHO 2018 report. (<https://www.who.int/leishmaniasis/burden/en/>).

1.4 Italian epidemiology

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 (Poepl *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).

About 40–70 cases are notified annually, over half of which are from Sicily (data from the Italian Institute of Statistics, ISTAT).

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). A previous outbreak in the same area was registered, between 1971 and 1972, with 60 clinical cases (and 13 deaths) of zoonotic VL occurred in a village near Bologna (Pampiglione *et al.*, 1974).

1.5 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).

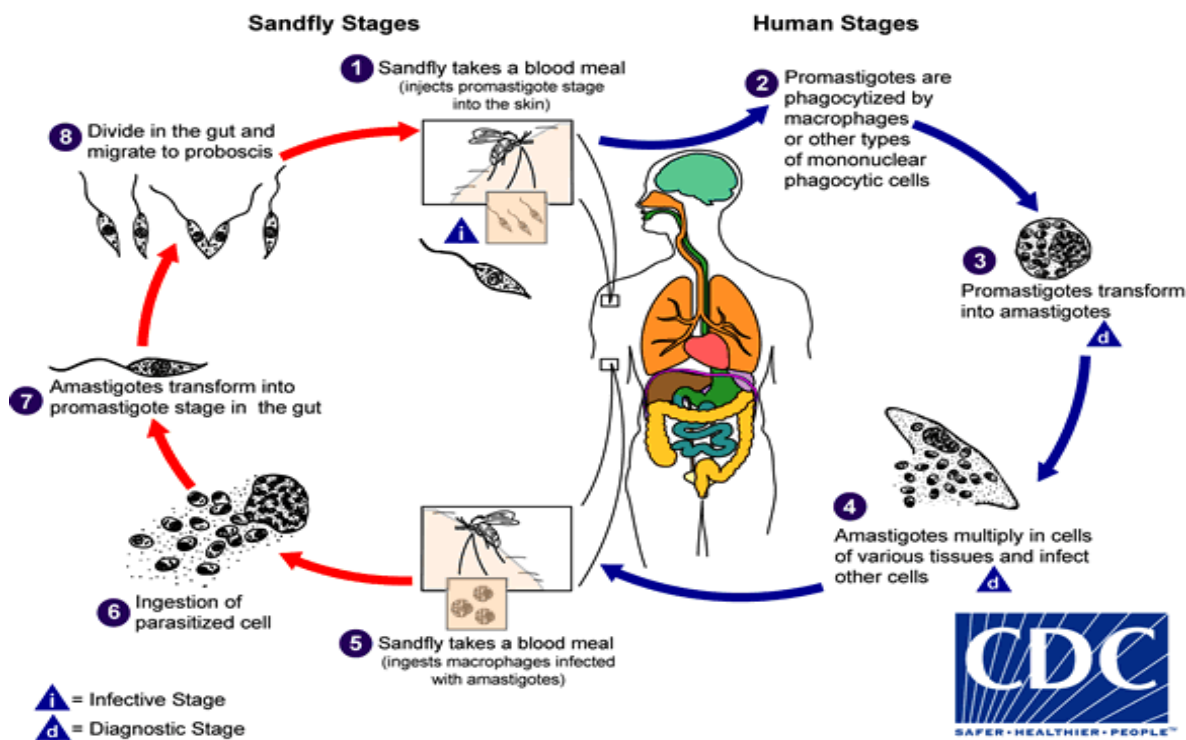


Figure 1.2. The developmental life cycle of *Leishmania* parasites. Adapted from US Centers for Disease Control and Prevention (CDC) (<https://www.cdc.gov/parasites/leishmaniasis/biology.html>).

1.6 Pathogenesis of leishmaniasis

Infection is usually contracted by vector puncture. Regarding the visceral form, however, maternal-fetal transmission as well as infection by transfusion of contaminated blood have also been ascertained. However, these are very rare occurrences. When the phlebotomist stings the man, the promastigotes are immediately phagocytosed by the elements of the monocytic-macrophage system present in the skin, thus transforming into amastigotes. At the point of penetration, a "primary lesion" occurs (evident in the cutaneous forms, of very small dimensions, and therefore inapparent, in VL), which essentially consists of a proliferation of histiocytes, plasma cells and other cells. In them, an active multiplication of the protozoa takes place, until clusters of 50-200 parasites are formed which destroy the cell and release new leishmania parasites, which will be phagocytosed by other elements of the monocyte-macrophage system again; this dynamic allows the dissemination of the pathogen through monocytes and macrophages. The early activation of cell-mediated immunity leads to destruction of leishmania during inoculation (CL), while the very late

intervention of cellular immunity (VL) allows dissemination of the parasite, in particular to the spleen, liver, bone marrow and lymphnodes.

In MCL, on the other hand, the immunological activation occurs in an intermediate period, thus allowing the spread of the infection by contiguity to other skin areas and to those of transition with the mucous membranes.

Pathological phenomena influence the behavior of serum antibodies and the positivization time of the intradermal test with leishmania extracts (reaction to leishmanin or Montenegro reaction). Serum antibodies detectable with the complement fixation reaction, indirect immunofluorescence, immunoenzymatic test, appear only in VL and, inconsistently, in MCL. However, they do not appear to be important for healing purposes.

The intradermal test (expression of the presence of a cell-mediated immunity) results to be positive early in CL, later (after the formation of metastases) in MCL and only 6-8 weeks after clinical healing in the VL. Any clinical form of leishmaniasis renders the patient immune to reinfection with the homologous strain of the parasite.

However, reinfections can occur many years after the first morbid episode, or even relapses following diseases (or treatments) that depress the immune defenses (especially the cell-mediated one). The eradication of the infection and the protection from possible reinfection depend on the proliferation of CD4 + lymphocytes and the development of a TH1-type response with the production of interferon (IFN- γ) and interleukin 2 (IL-2). IFN- γ activates macrophages for the destruction of intracellular amastigotes. Several studies have shown that in patients with progressive disease the TH response is inhibited in favor of a TH2 response, with production of IL-4 and IL-10.

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 interleukin (*IL*)- $2R\beta$ 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-1 β* 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 (Byceson, 1996) as a result of antigen-encountered T cell exhaustion (gradual loss of function) due to chronic parasite persistence (Wherry, 2011).

1.8 Clinical presentations 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, papular, 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).

1.9 *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.*, 1985). 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 coinfection had been reported from 35 endemic countries. Coinfection with HIV intensifies the burden of VL and CL by causing severe forms that are more difficult to manage. VL interaction with HIV infection, as HIV-infected people are particularly vulnerable to VL, while VL accelerates HIV replication and progression to AIDS, poses major challenge in areas where there is high coinfection rate. According to a WHO coordinated monitoring system involving 28 institutions worldwide - the number of new cases has declined in Europe since the end of the 1990s, mainly due to access to highly active antiretroviral therapy. In other parts of the world, however, where there is limited access to such treatment, the prevalence is still high (https://www.who.int/leishmaniasis/epidemic/special_conditions_hiv_coinfection/en/).

1.10 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 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).

1.10.1 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 $24\pm 2^{\circ}\text{C}$ 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).

1.10.2 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).

Serological and antigenic detection methods commonly used in the diagnosis of leishmaniosis are summarized in Table 1.1.

Table 1.1 Serological and antigenic detection methods commonly used in the diagnosis of leishmaniosis.

Assay or method	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages	References
Antibody detection					
IFA test	87c#00	77c#00	Positive in the early stages of infection and undetectable six to nine months after cure.	Requires sophisticated laboratory. No application in the field.	41,42,43
ELISA			Can be used to screen a large scale of epidemiologic studies.	Sensitivity and specificity is greatly influenced by the antigen used. Requires skilled personnel, sophisticated equipment, and electricity.	
CSA as antigen	80c#00	84c#95			45,46,47
with fucose-mannose ligand	95c#00	95			53
rK39 as antigen	75c#98	79c#89			58,59
Western blo	90c#98	98c#00	Provides detailed antibody responses to various leishmanial antigens.	Time consuming, technically cumbersome, and expensive.	69, 70, 71, 72, 73
DAT	85c#00	91c#00	Rapid test, applicable in the field.	Limited to use in regions of endemicity. Long incubation time is needed. Unavailability of commercial source of the positive antigen and fragility of is aqueous form.	74,75,76,77,79
IC test with rK39	90c#00	93c#00	Inexpensive, rapid, simple, and can be performed by untrained person.		84,85,86,88
Antigen detection					
Latex agglutination test in urine (KAtex)	79c#00	60c#00	Simple, easy performing. Useful in diagnosis of disease immunocompromised patients.	Difficult to distinguish weakly positive from negative results and the urine must be boiled to avoid false-positive reactions.	33,34,36,37

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1.10.3 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). Advantages and limitations of the molecular methods conventionally used for the diagnosis of leishmaniasis are reported in Table 1.2.

Table 1.2 Advantages and limitations of the molecular methods conventionally used for the diagnosis of leishmaniasis

Method	Advantage	Limitation
Conventional PCR (cPCR)	High sensitivity, specificity and accurate results. Many applications in molecular analysis. Easy diagnostic interpretation.	Unable to quantify the target DNA. Qualitative test. Time consuming. Limited detection range of some assays.
Quantitative real-time PCR (qPCR)	Higher sensitivity, specificity and security, quantitative capacity and speedy results. Possibility of species differentiation by melting temperature.	High cost due to equipment (thermocycler). Difficulty in interpreting the results, needing thus of a well-trained operator.
Nested-PCR (nPCR)	Higher specificity and sensitivity. Useful technique for studying the molecular epidemiology in the field.	Time consuming and higher cost. Unable to quantify the target DNA. Qualitative test.
Quantitative Nucleic Acid Sequence-Based Assay (QT-NASBA)	High specificity. It is based on an isothermal reaction and thus overcomes the need for a thermocycler; Ideal for lower-tech laboratories. Quantitative capacity. Indicated to detect active diseases; RNA detection.	It uses electrochemiluminescence as tool of detection, which involves more handling steps and procedure time. Assays developed only for RNA detection. . Few studies yet.
NASBA coupled with oligochromatography (NASBA-OC)	High specificity. Speedy results. There is no need of complex laboratorial structure. Simple dipstick format for the detection of amplification products. RNA detection.	Unable to quantify the target RNA. Assays developed only for RNA detection. Few studies yet.
Loop-Mediated Isothermal Amplification (LAMP)	High sensitivity. Low cost. Isothermal reaction, there is no need for a thermocycler. The temperature stability of the reagents enables its use in field conditions.	Unable to quantify the target DNA. Qualitative test. Few studies yet.

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1.11 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). Pentamidine 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 Lindoso, 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 Hariprakash, 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. Leishmaniasis and asymptomatic infection

2.1 Background

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).

The asymptomatic forms characterize infected individuals but in apparently healthy conditions. The subclinical forms, on the other hand, are characterized by at least one clinical manifestation such as lymphadenopathy or mild symptoms (fever, cough, diarrhea, malaise, mild hepatomegaly and possibly splenomegaly) associated with at least one positive diagnostic test, among those routinely used in the diagnosis of visceral leishmaniasis (Badaro *et al.*, 1986).

Diagnosis of asymptomatic infections is therefore important for effective control of *leishmaniasis* and requires the use of direct and indirect methods, such as those used for the diagnosis of VL.

The direct methods are based on the demonstration of parasites by microscopic examination, culture or PCR; indirect methods, summarized in Table 2.1, include the search for LST and antibodies with various techniques: immunofluorescence (IFAT), the direct agglutination test (DAT), single antigen ELISA (rk39), Western blot and immunochromatographic test (rk39) (de Gouvea *et al.*, 2008).

However, the choice of a gold standard method for detecting leishmania asymptomatic carriers is still a matter of debate.

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 lymphnode 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).

Conversely, the reliability of indirect tests in detecting leishmania asymptomatic infection has long been discussed. In fact, the detection of a sporadic positivity to LST or serology may not reflect a chronic infection but simply a recent contact with the parasite. However, in some cases, the finding of a sporadic but confirmed positive serology over time can be the expression, in endemic areas, of a persistent asymptomatic infection rather than of a single contact with leishmania parasite (le Fichoux Y *et al.*, 1999; Biglino *et al.*, 2010).

Furthermore, parasites that nest in non-effector target cells (which are unable to exert antiparasitic activity) could occasionally and for reasons still not fully understood, proliferate leading to repeated antigenic stimulation of the innate and adaptive immune system. This condition would confer, at least in immunocompetent individuals, resistance to development of clinical disease or reinfection. This concept is further supported by the appearance of VL in some patients with asymptomatic infection following severe states of immunosuppression.

All the techniques used to measure the frequency of asymptomatic infection show variable sensitivity and specificity (Bodgan *et al.*, 1993). For example, molecular methods are more sensitive and specific than LST, ELISA or IFAT (Mary *et al.*, 2006). In particular, with regard to serological methods, ELISA tests with crude antigens are generally more sensitive but less specific than those developed with single proteins (D'Oliveira *et al.*, 1997). Finally, the detection of antibodies against *L. infantum* antigens of 14-18 kDa is generally more sensitive and specific than ELISA with crude antigens (Marty P *et al.*, 1995, Biglino *et al.*, 2010).

It should be noted that the weak overlap observed in some studies between direct and indirect tests and also between two serological tests indicates that the use of more than one diagnostic method increases the percentage of asymptomatic infection detected (de Gouvea *et al.*, 2008). Therefore, the use of a single serological method to assess the frequency of

leishmania asymptomatic infection may represent a risk of underestimate the number of cases.

2.2 Asymptomatic carriers in endemic areas

Data regarding the prevalence of asymptomatic *Leishmania* infection are summarized in Table 2.1. Most epidemiological studies reported in literature were carried out in Brazil and southern Europe. Unfortunately, the prevalence of asymptomatic infection, detected by a single test, was highly variable (0.6-71.3%). The most used technique was LST, followed by ELISA, with *Leishmania* crude antigens, and, in more recent studies, PCR. As already mentioned, the use of more than one technique increases the number of positive cases detected (de Gouvea et al., 2008).

Table 2.1 Prevalence of asymptomatic leishmania infection worldwide.

Country/Place	Techniques	Number of tested inhabitants	Results (%)	References
<i>South of Europe</i>				
Sicily, Italy	LST	n/a	16.6	Pampiglione et al. (1975)
Abruzzi, Italy	LST	n/a	27.5	Cited in Marty et al. (1992)
Tuscany, Italy	LST	n/a	15.3	Bettini et al. (1983)
Pyrénées Orientales, France	LST	n/a	15	Cited in Marty et al. (1992)
Sardina, Italy	LST	640	9.7	Gramiccia et al. (1990)
Alpes Maritimes, France	LST	237	30	Marty et al. (1992)
Alpes Maritimes, France	LST/Western blot	50	32/38	Marty et al. (1994)
Alpes Maritimes, France	LST/Western blot	47	46.8/42.5	Marty et al. (1995)
Granada and Malaga province, Spain	LST	1286	44	Accedo Sanchez et al. (1996)
Granada and Malaga province, Spain	LST	1258	42.40	Morillas et al. (1996)
Alacanti region, Spain	LST	184	52.8 of adults and 11.5 of children	Moral et al. (2002)
population of Castilla-Leon, Spain	ELISA (crude antigen)	4825	4.90	Garrote et al. (2004)
Seville, southern Spain	IFAT	95	24.00	Martin-Sanchez et al. (2004)
Epirus region, Greece	IFAT	1200	0.50	Papadopoulou et al. (2005)
Macedonia and Thrace regions, Greece	IFAT and ELISA (crude antigen)	1525	2.80	Diza et al. (2008)
Area of Marseilles	PCR	81	58%	Mary et al. (2006)
Central Piedmont, Italy	WB/PCR in WB+/PCR in WB-	526	7.41/56.4/4.2	Biglino et al. (2010)
<i>South America</i>				
State of Bahia, Brazil	LST/ELISA (crude antigen)	135	45/27	D'Oliveira Junior et al. (1997)
Brotas, Brazil	ELISA (crude antigen)	920	10.30	Jerônimo et al. (2000)
Rio Grande do Norte, Brazil	ELISA (rK39)/ELISA (crude antigen)	168	2.9/8.3	Braz et al. (2002)
Piauí, Brazil	LST/ELISA (crude antigen)	167/168	50/13.9	Werneck et al. (2002)
Piauí, Brazil	PCR/LST	101/87	7.9/71	Costa et al. (2002)
Rio Grande do Norte, Brazil	ELISA (KMP11 antigen)	27	0.05	Passos et al. (2005)
Maranhão, Brazil	LST/ELISA (rK39)/ELISA (crude antigen)	1520	61.1/19.4/19.7	Nascimento Mdo et al. (2005)
General Carneiro district, Brazil	IFAT/ELISA (crude Antigen)/rK39 immunochromatographic	1604	2.4/3.4/5.6	Moreno et al. (2006)
Maranhão, Brazil	Western blot	1100	0.60	Mendes et al. (2007)
Araçatuba, Brazil	rK39 dipstick	A1: 125 A2: 125	Two area: 20 in A1 and 4.8 in A2	Barao et al. (2007)
Minas Gerais, Brazil	LST/IFAT/ELISA (crude antigen)/ELISA (rK39)/PCR	138	2.9/5.1/9.4/5.1/18.1	de Gouvea Viana et al. (2008)
Belém, Brazil	LST/IFAT	946	11.2/3.4	Crescente et al. (2009)
Belém, Brazil	LST/IFAT	946	20.8/6.5	Silveira et al. (2009)
Belo Horizonte, Brazil	PCR/ELISA (crude antigen Amazonensis)/ELISA (crude antigen Infantum)/ELISA (rK39)	136	71.3/60.2/40/28.7	Moreno et al. (2009)
Cametá municipality, Brazil	LST and/or IFAT	174	15.8	Silveira et al. (2009)
Minas Gerais, Brazil	ELISA (crude antigen)/ELISA rK39/ELISA rK26/IFAT	1017	26.6/14.6/12.7/8.5	Romero et al. (2009)
Minas Gerais, Brazil	ELISA (crude antigen)/ELISA rK39/ELISA rK26/IFAT	224	19.2/37.5/21.4/11.2	Romero et al. (2009)
Belém, Brazil	LST and/or IFAT	120	73.4	Silveira et al. (2010a)
<i>China</i>				
Endemic area in China	PCR	n/a	33	Gao et al. (2006)
Gansu Province, China	PCR/ELISA (crude antigen)/rK39-dipstick	n/a	30.9/24.2/0	Wang et al. (2007)
<i>Middle East</i>				
Denizli province, Turkey	Western blot	81	7.41	Sakru et al. (2007)
Eskisehir, Bilecik, Kutahya and Afyon provinces, Turkey	ELISA (commercial ELISA)/ELISA (crude antigen)/ELISA (rK39)/IFAT	572	5.2/4.7/3.6/3.8	Dogan et al. (2008)
Fars province, Iran	DAT/PCR	802	North-western: 1.9/16; South-east: 1.5/8.5	Fakhar et al. (2008)
Ghir Karzin and Sar Mashhad in Iran	LST/IFAT/PCR	388	34/54.4/24.5	Alborzi et al. (2008)

2.3 Immunologic profile of *Leishmania* asymptomatic carriers

Several studies have shown that the clinical forms of VL are under the control of the host's innate and adaptive immune response. In particular, the innate immune response plays a fundamental role during the host's defense resistance reaction to infection, both in controlling the growth of parasites during the initial stages and in guiding the secretion of cytokines by which T cells are activated.

These data were obtained from in vitro stimulation experiments of human peripheral blood mononuclear cells (PBMCs) or T cell clones with *Leishmania* antigens, in order to classify immune responses into type 1 and type 2 based on profiles of secreted cytokines (Carvalho et al, 1989).

Studies in the experimental mouse model show that the type 1 response is characterized by the release of IFN- γ , IL-12 and TNF- α and is associated with resistance to infection, while the predominant type 2 response is accompanied from IL-4, IL-5 and IL-10 and is linked to disease progression.

However, experiments conducted on human *L. chagasi* infection show that VL is accompanied by a mixed cytokine pattern, type Th1 / Th2, including IL-8, IFN- γ , TNF- α , IL-6 and IL-10 (Peruhype-Magalhaes et al., 2006).

According to some authors, the purpose of the mixed response, in fact, could guarantee, on one hand the effective control of parasite proliferation, and on the other, the preservation of immune balance by blocking an excessive cellular response (Machado et al., 2009). In addition, a mixed immune activation pattern (IFN- β produced by CD8 + and CD4+ cells and IL-5 produced by CD4+) was also found for asymptomatic *L. infantum* infection (Mary et al., 1999).

2.4 Asymptomatic infection in immunocompromised

L. infantum asymptomatic infection has been repeatedly demonstrated in immunocompromised people (HIV-positive individuals and patients undergoing immunosuppressive therapy) (Colomba C et al., 2009; Burgeois et al., 2010). In all cases,

there is a real risk that the asymptomatic infection evolves into visceral disease.

For the HIV-positive population, the risk of developing VL generally occurs in the advanced stages of HIV disease, characterized by CD4+ lymphocyte counts below 200×10^6 / ml (83). Furthermore, the proven correlation between HIV viremia and parasitemia highlights a synergism between the two pathogens (Colomba C et al., 2009). HIV increases the risk of developing VL from 100 to 2320 times and leishmania infection, in turn, promotes the clinical progression of HIV and the development of AIDS (Alvar et al., 2008).

Highly active antiretroviral therapy (HAART) has led to a reduction in the incidence of symptoms of VL in HIV patients, but effective treatment of the viral infection is not sufficient to prevent relapse of the parasitic disease (Alvar et al., 2008). In fact, although it provides a good response in over 80 percent of cases, it fails to prevent the relapse of the disease in more than 50 percent of people within the following three years.

Furthermore, recent studies show that patients co-infected with HIV and Leishmania are unable to effectively eliminate the parasite, presenting episodes of clinically manifest or oligosymptomatic disease, interspersed with asymptomatic intervals (Burgeois et al., 2010). Transplant patients, and more generally, those receiving immunosuppressive chemotherapy are also at risk of developing VL.

Transplant recipients who develop VL, manifest the classic clinical form of the disease, which is a syndrome characterized by fever, hepatosplenomegaly, and pancytopenia.

It is worthy of note that in these cases unusual clinical presentations of leishmaniasis are described, with consequent diagnostic delay and sometimes partial responses to specific therapy (Machado et al., 2009). Furthermore, in transplant recipients VL can result from transmission through transplanted tissue, from reactivation of a quiescent infection or from a new infection (Basset et al., 2005).

Many authors recommend screening tests in order to detect any asymptomatic leishmania infection for donors. The geographic origin of the donor is not, in fact, always proven to be a valid criterion, as the habit of travel is becoming more and more widespread and the asymptomatic infection can be contracted even during a short stay in an endemic area. Furthermore, it has been proposed by several authors that transplant patients living or traveling to endemic areas should be tested regularly for leishmaniasis. There is currently no consensus recommendation to identify asymptomatic infection in endemic areas for donors, transplant patients or for those receiving immunosuppressive therapy (Machado et al., 2009). According to the risk presented above, valid screening for asymptomatic infection before starting an immunosuppressive chemotherapy regimen may be a valuable tool to

prevent disease onset (Basset et al., 2005).

3. Rationale and objectives

Detection of the leishmania 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 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.

The present thesis aims to study *Leishmania* and leishmaniasis through clinical and biochemical studies.

The first part of the thesis involves clinical studies that evaluated 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 Northern Italy, using advanced molecular techniques and cytokine analysis;

The second part aimed to study the biochemical aspect, in particular, the effect of the niacin analogue, 6-AN on *Leishmania* parasite growth and metabolism using the metabolomics technology. The rationale 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).

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

4.1 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 determinant key 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 immunocompromised 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).

4.2 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 (Poepl *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).

4.3 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).

4.4 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).

4.5 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-2R α) 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).

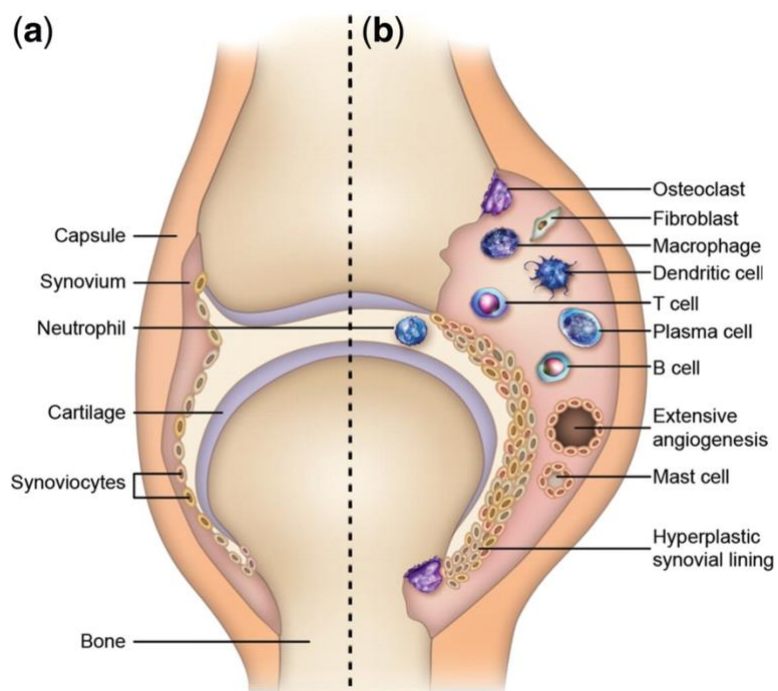


Figure 4.1. A graphic representation shows normal joint (a) and RA-inflamed joint (b). The affected joint has swollen, hyperplastic synovial membrane with infiltration of immune cells. Adapted from Smolen and Steiner, (2003).

4.6 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).

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 whereas sTNF- α regulates the endocrine response (Grell, 1995). TNF α and T helper 1 cell-derived gamma interferon (IFN γ) 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).

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-6R α 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).

CD4+ T cell activation modulation

CD4+ T cells play important roles in orchestrating the immune responses 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 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-2R α), 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).

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.

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-1R 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)

4.7 Materials and methods

4.7.1 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.

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

About 6 ml venous blood was collected from antecubital 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.

4.7.3 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 MgCl₂ and 100 mM KCl, and buffer B which included 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 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 incubation 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.

4.7.4 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 MgCl₂, 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}, Euro@lone, 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*TM 100 PLUS, Euro@lone, 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).

4.7.5 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 (Applied Biosystems, 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-TTTTCGCAGAACGCCCT 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 10⁶ 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.

4.7.6 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-1 β , IL-1Ra, IL-2, IL-2R α , IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-15, IFN- α 2, IFN- γ , TNF- β and TNF- α .

Following the manufacturer's instructions, 50 μ l 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.

4.7.7 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).

4.7.8 Ethical considerations

The present study was approved by the ethics committee of the University of Ferrara, (**ANNO E N. DI PROTOCOLLO**). All the study participants knew that they were taking part of a research study and they gave a written informed consent before their enrolment.

4.8 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 <i>Leishmania</i> 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

*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 km².

4.8.1 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 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 geographical map of Emilia-Romagna region, representative of provinces affected by circulating *Leishmania* DNA.

4.8.2 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.

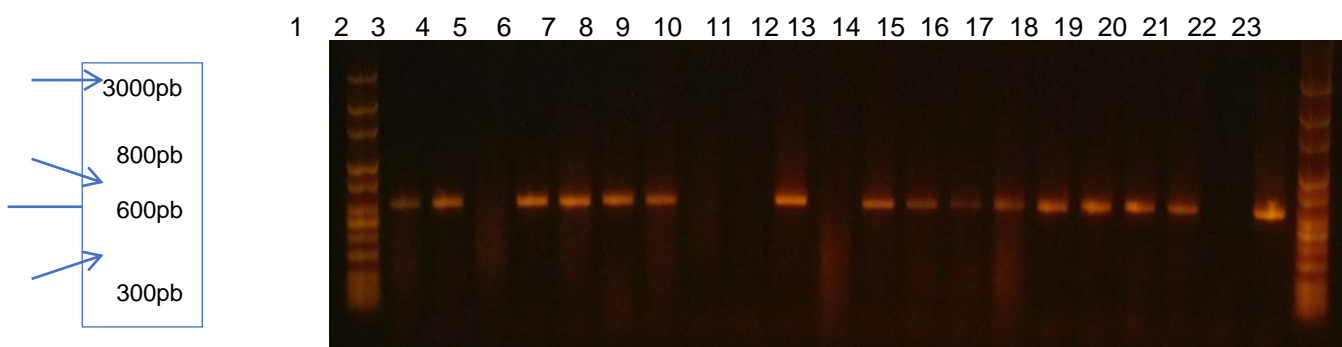


Figure 4.3. 1.5% agarose gel image showing the size of the amplified PCR products of 603 bp of the ssu rRNA gene of *Leishmania*. Lanes 1 and 23 represent the molecular size (weight) marker (100 bp). Lanes; 2, 3, 5, 6, 7, 8, 11, 13, 14, 15, 16, 17, 18, 19, and 20 are autoimmune rheumatic patient samples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


Patient's Characteristics	<i>Leishmania</i> kDNA qPCR	
	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.

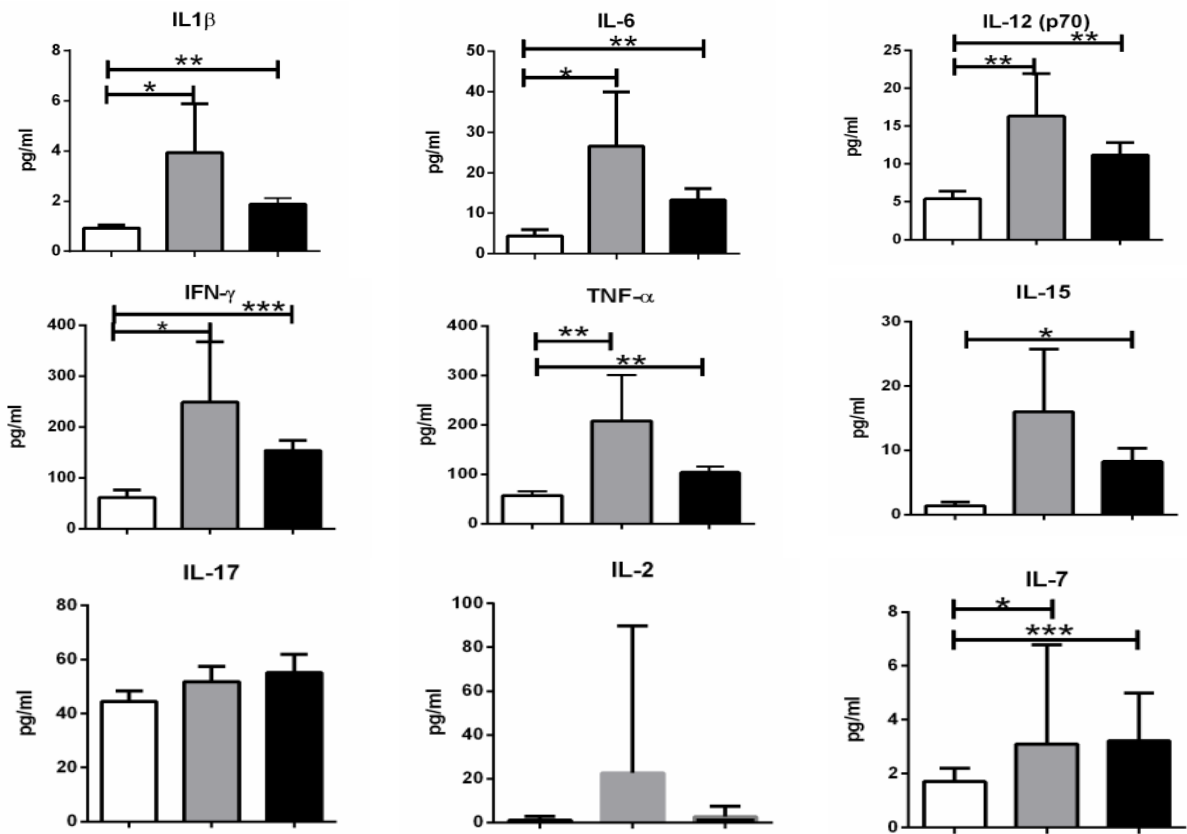
Positive patients were then grouped according to the pharmacological action of the BD into TNF-alpha antagonists, modulators of T lymphocyte activity and anti-IL-6 receptor antibodies; the possible influence of each type of the drugs on the molecular positivity for *L. infantum* circulant kDNA was assessed using a Chi-square test. This test did not reveal any statistical difference among the different administered treatments (χ^2 (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, χ^2 (1)=6.18, p<0.05; 61.1% vs. 25.0%).

4.8.3 Cytokine profiles

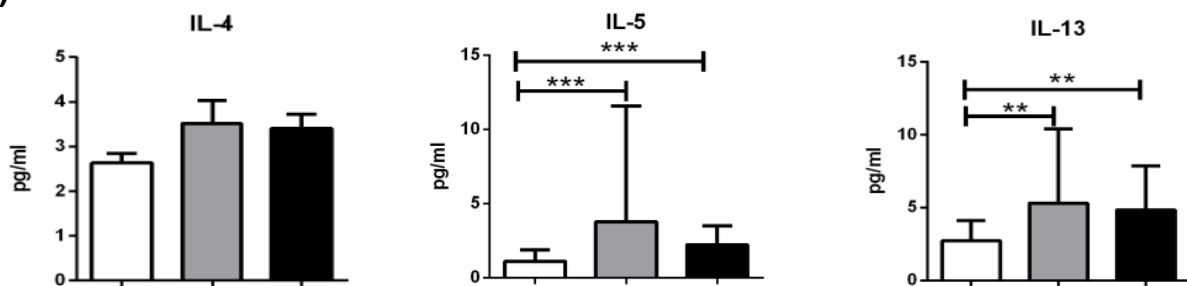
The immune responses were assessed in LqPCR-positive and –negative autoimmune rheumatic patients and the healthy control group by measuring cytokine levels. Pro-inflammatory cytokines of type I immune response including CD4+ Th1 cell cytokine IFN- γ , type II immune response played by CD4+ Th2 cell mediators; IL-4, IL-5 and IL-13, macrophage, DC and other synovial cell mediators; TNF- α , IL-1 β , 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 -negative 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-1 β , 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) and parasite load did not show any statistical significance in relation to the biological drug used, 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)



(B)



(C)

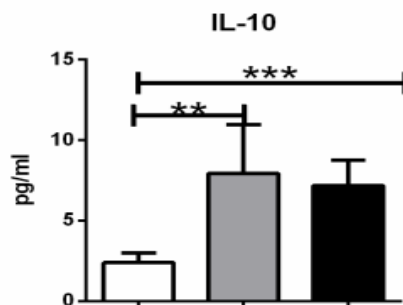


Figure 4.4. Graphic representation of down-regulated or up-regulated pro-inflammatory (A), anti-inflammatory (B) and immune-regulatory (C) cytokines in plasma from autoimmune rheumatic patients positive and negative for *Leishmania* kDNA, and the healthy control group. Mann-Whitney non-parametric Student's t-test was used to compare between the

three groups and significant results are marked with asterisks: *P. Values*, **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-1 β	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

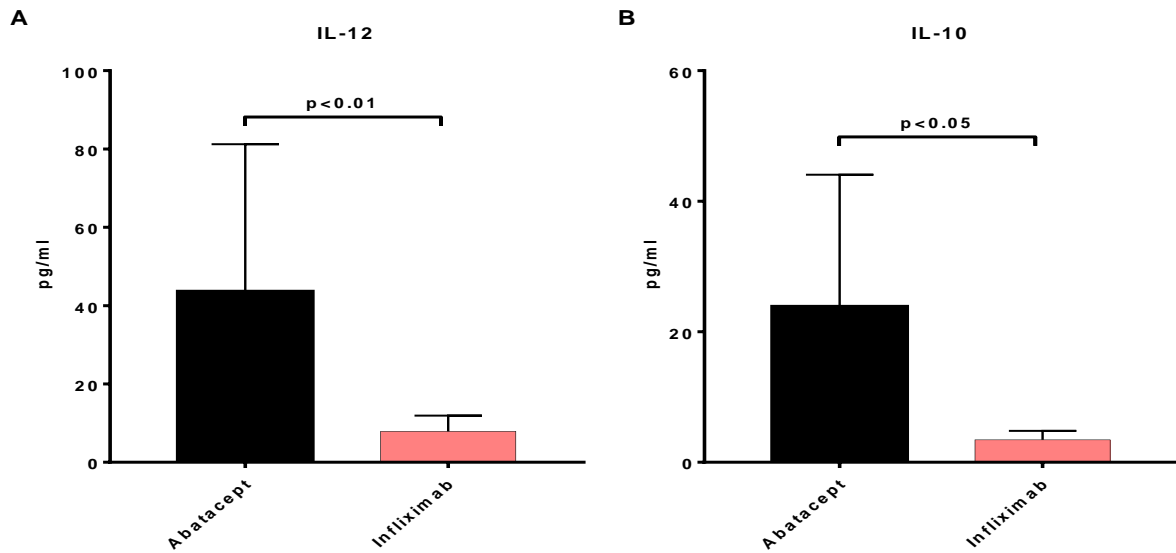


Figure 4.5. Expression of pro-inflammatory (A) and immune-regulatory (B) cytokines that showed statistically significant different concentrations in relation to the biological drug, infliximab or abatacept used to treat autoimmune rheumatic patients. P denotes *P. Value*.

4.9 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 granulomatous diseases such as tuberculosis and leishmaniasis (Marie and Guglielmino, 2010).

Our study is an investigation of asymptomatic human *L. infantum* infections in Emilia-Romagna, an Italian region not traditionally considered endemic for *Leishmania* and characterized by a continental climate and a low incidence of officially reported VL cases, but recently passed as a “phlebotomus risk region” like some coastal and hilly regions of the middle Adriatic Sea and many hilly areas of the pre-alpine regions of Northern Italy. Canine leishmaniasis, which is the only source of disease for humans, is increasing in Northern Italy, spreading in rural and peri-urban 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 involved, 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 (Gavvani *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 (χ^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 4.3, 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, hindering the response of interferon-gamma (IFN- γ)-producing CD4+ T-cells opens the way for parasite persistence as IFN- γ is important in the resolution of VL infection due to its ability to induce reactive nitrogen species (RNS) by macrophages resulting in parasite killing (Kaye and Scott, 2011). On the other hand, T-cell depletion (gradual loss of function) due to chronic persistence of the parasite 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 has been 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-1 β , 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-1 β 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-1 β , 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. Splenomegaly and intermittent fever were reported in opportunistic VL in PsA (De Leonadis *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 Leonadis *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 splenomegaly. 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 patients positive for *Leishmania* DNA (n. 18), positivity was higher among those receiving BD in combination with steroids (Table 4.3, $\chi^2(1)=6.18$, $p<0.05$; 55.6% vs. 21.2%). This finding is supported by several case reports of *Leishmania* reactivation in patients receiving steroid treatment for long time 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.

4.10 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-threatening 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.

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

5.1 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. 5.1) 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.

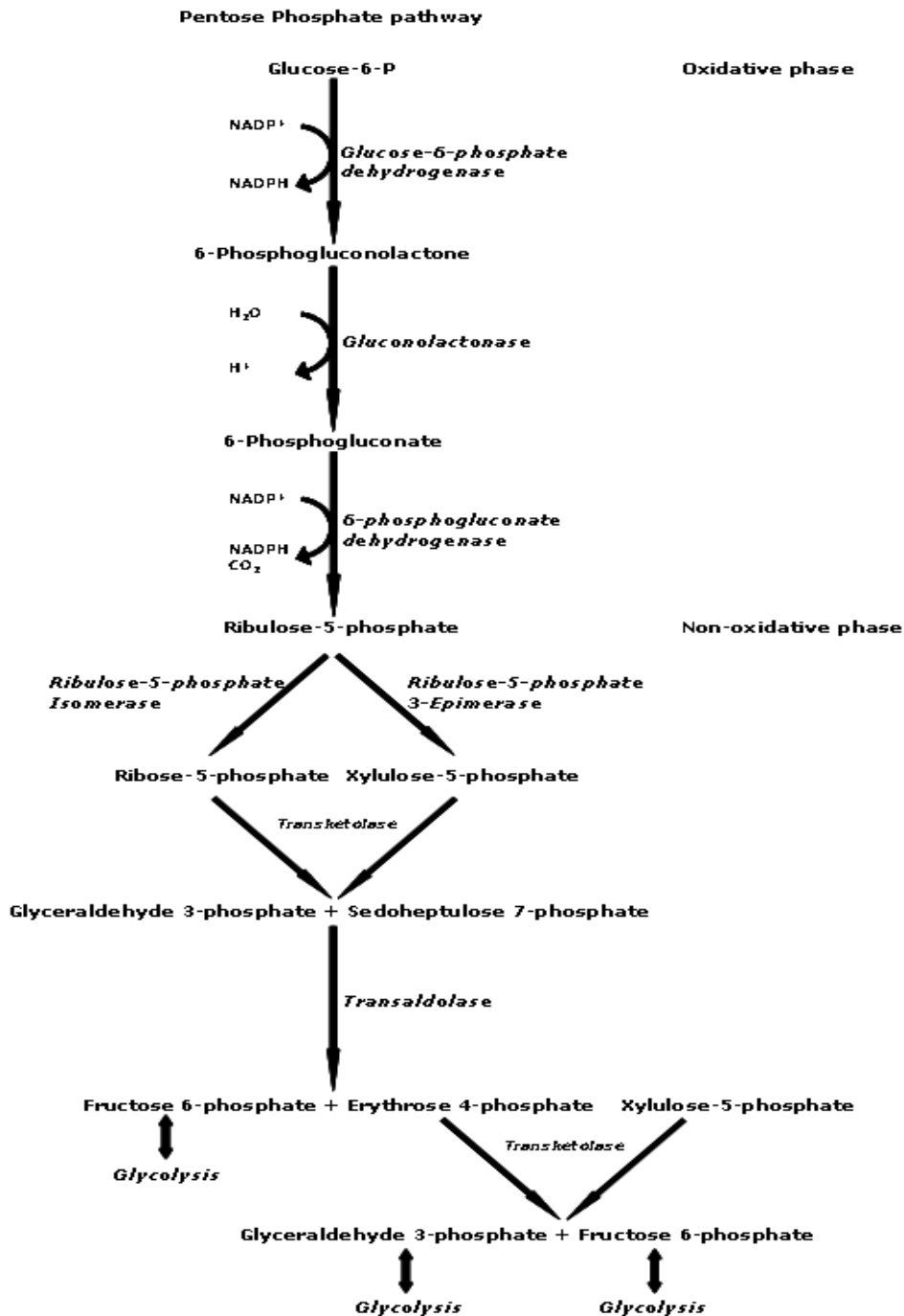


Figure 5.1. A graphical depiction of the pentose phosphate pathway. The oxidative part utilizes glucose 6-phosphate and NADP^+ as a cofactor to produce NADPH and ribulose 5-phosphate while the non-oxidative part proceeds to generate ribose 5-phosphate and other sugar intermediates with some of them are 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).

5.1.2 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 *et 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).

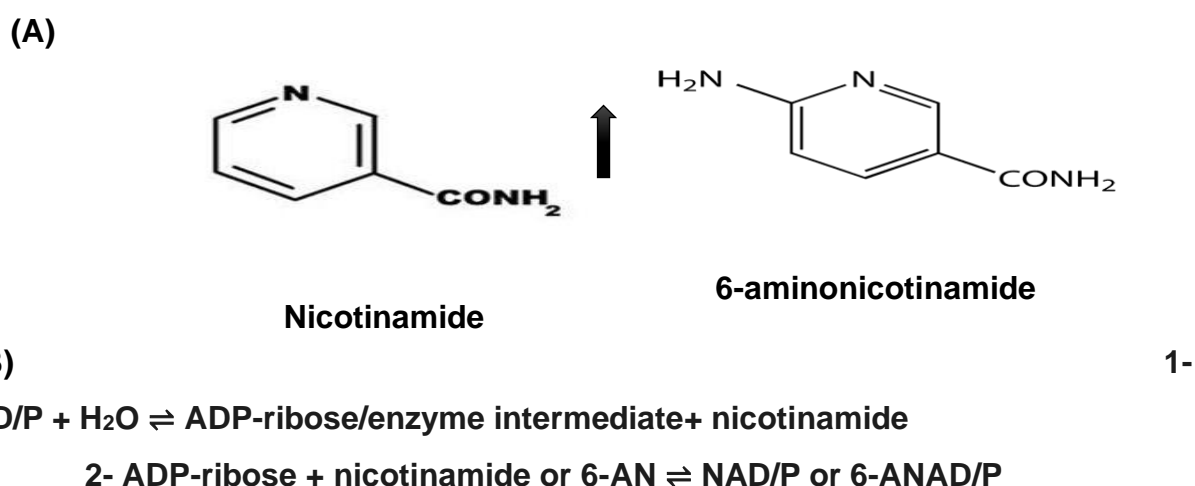


Figure 5.2. A. The chemical structure of nicotinamine and 6-AN. The arrow denotes the additional amine group at carbon 6 of 6-AN. B. The exchange reactions (transglycosidation) catalysed by NAD⁺ glycohydrolases, which hydrolyze NAD/P (B.1) but if nicotinamide or 6-AN is at an inhibitory concentration the reaction will go back generating either NAD/P or 6-ANAD/P (B. 2) (Zatman *et al.*, 1953).

5.1.3 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.

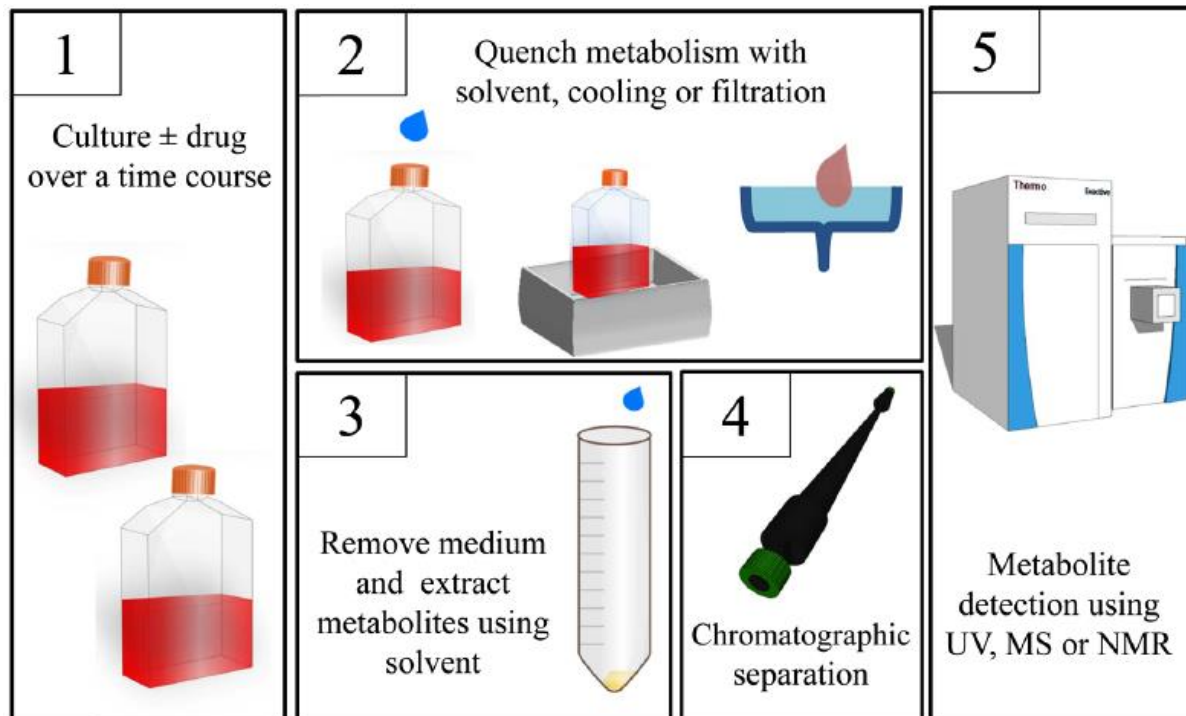


Figure 5.3. Metabolomics work flow (from 1 to 5) for disclosing drug effects and mode of action. Cells are cultured in the presence and absence of the drug. Cells are washed to remove the medium, their metabolism is quenched and small metabolites are extracted.

Metabolites are chromatographically resolved and detected. Adapted from Vincent and Barrett, (2015).

5.2 Materials and methods

5.2.1 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.

5.2.2 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 25cm² 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 25cm² vented tissue culture flasks (Corning, USA) at a density of 10⁶ cells/ml and incubated at 32°C in a humid incubator with 5% CO₂ 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.

5.2.3 Antileishmanial activity

To find IC₅₀ 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 10⁵cells/ml in some experiments or 10⁶ 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 (1/10th 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 IC₅₀ values were calculated using Prism 6.0 software nonlinear dose-response curve mode.

5.2.4 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 IC₅₀ values of either 6-AN, MB or MG were determined first. The starting concentration of both MB and MG were 32 times their IC₅₀ values. To avoid the effect of the DMSO (solvent) on the parasites, 6-AN was prepared at a concentration 4 times its IC₅₀. 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	Combo	Ratio	Volume required
1	5:0	500µl 6-AN	1	5:0	500µl MB
2	4:1	400µl 6-AN+100µl MB	2	4:1	400µl MB+100µl MG
3	3:2	350µl 6-AN+150µl MB	3	3:2	350µl MB+150µl MG
4	2:3	150µl 6-AN+350µl MB	4	2:3	150µl MB+350µl MG
5	1:4	100µl 6-AN+400µl MB	5	1:4	100µl MB+400µl MG
6	0:5	500µl MB	6	0:5	500µl MG

For each ratio, two-fold serial dilutions were done 11 times, and the IC₅₀ values were calculated for each compound, then fractional inhibitory concentrations (FIC) were obtained by dividing the IC₅₀ values by the IC₅₀ 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.

5.2.5 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 10⁵ 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 Sørensen staining buffer. Slides were then washed with tap water, air dried, and examined under a light microscope using 100X magnification.

5.2.6 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 10⁶ 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% CO₂. 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 10⁶ 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% CO₂ (Marim *et al.*, 2010).

Procyclic *L. mexicana* promastigotes were seeded at 10⁵ 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% CO₂. 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.

5.2.7 Metabolomics analysis

5.2.7.1 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 mm² 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.

5.2.7.2 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.

5.2.7.3 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](https://doi.org/10.1093/nar/gks1004). PubMed PMID: [23109552](https://pubmed.ncbi.nlm.nih.gov/23109552/)) with the identifier MTBLS443. The complete dataset can be accessed here (<http://www.ebi.ac.uk/metabolights/MTBLS443>).

5.3 Results

5.3.1 Antileishmanial activity

The effect of 6-AN is leishmaniostatic at high concentrations as seen in growth pattern of *L. mexicana* promastigotes (Fig. 5.4) 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 5.1). 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.

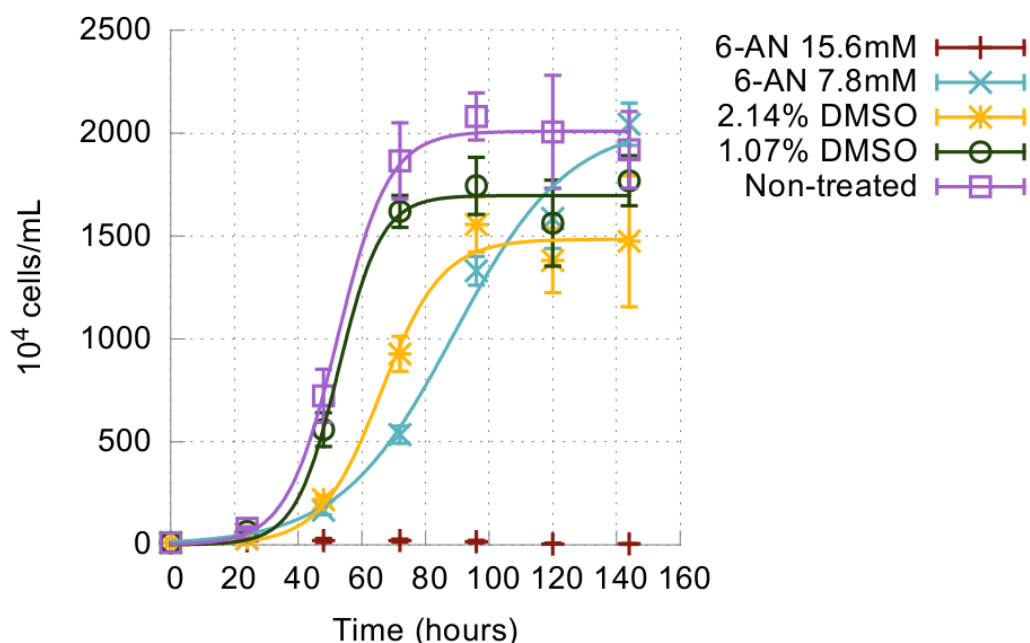


Figure 5.4. 6-AN-treated *L. mexicana* promastigotes growth curve. The graphs are representative of three independent experiments.

Table 5.1. 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 \pm SD of three independent experiments with each performed in triplicate.

Cell type	Compound	IC ₅₀
<i>L. mexicana</i> M379	6-AN	7.8 \pm 1.4 mM
	MB	2.8 \pm 1.6 μ M
	MG	23.5 \pm 11.9 μ M
	AmB	27 \pm 2.3 nM
<i>L. infantum</i> PCM5	6-AN	4.3 \pm 1.34 mM
	AmB	24 \pm 5.35 nM
<i>L. infantum</i> MO1	6-AN	1.8 \pm 0.36 mM

5.3.2 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. 5.5).

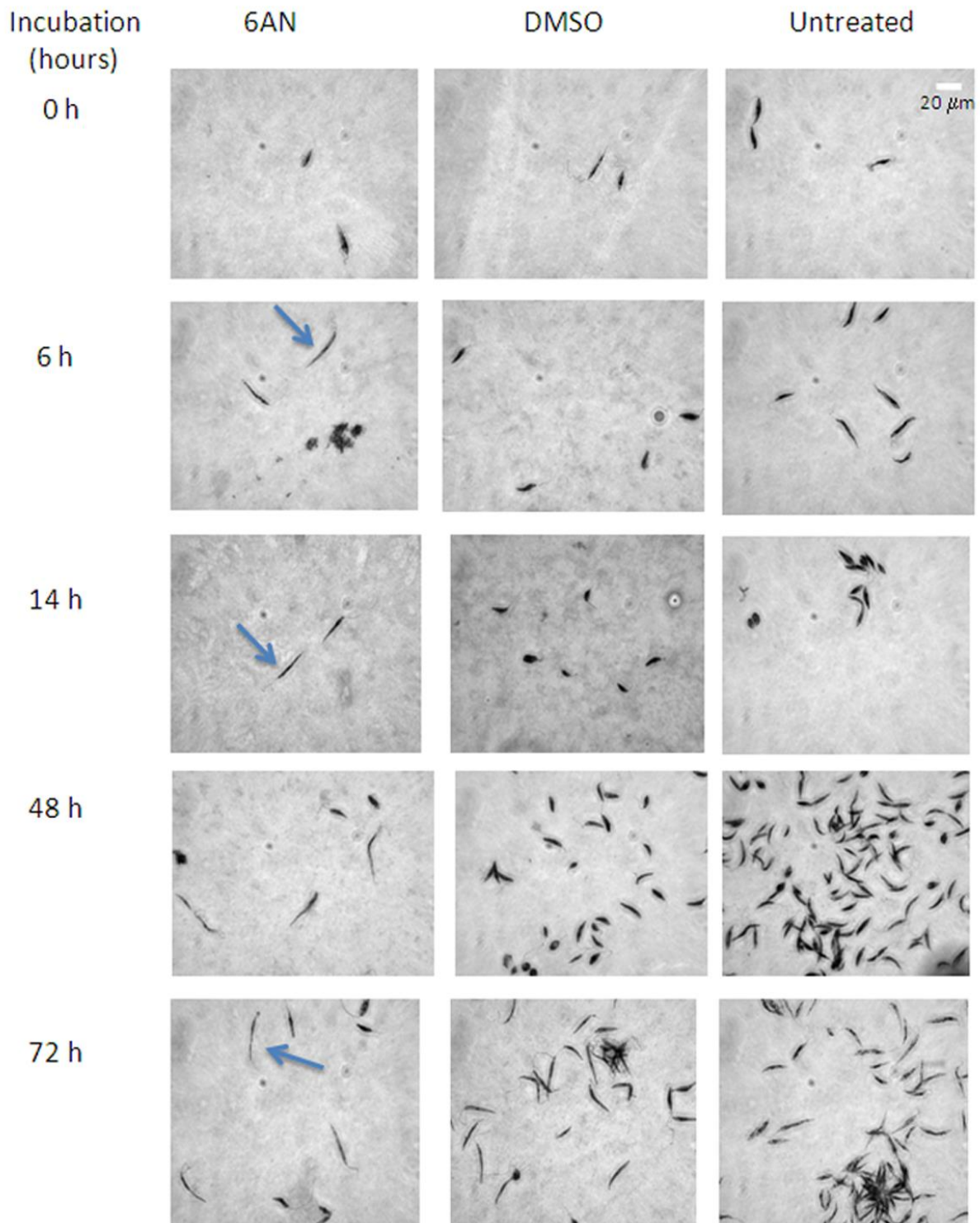


Figure 5.5. General morphology of *L. mexicana* promastigotes treated with 15.6 mM 6-AN, 2.14% DMSO, and untreated controls. Arrows show nectomonad-like elongated parasites seen under a light microscope using 100X magnification. This experiment was replicated three times.

5.3.3 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. 5.6).

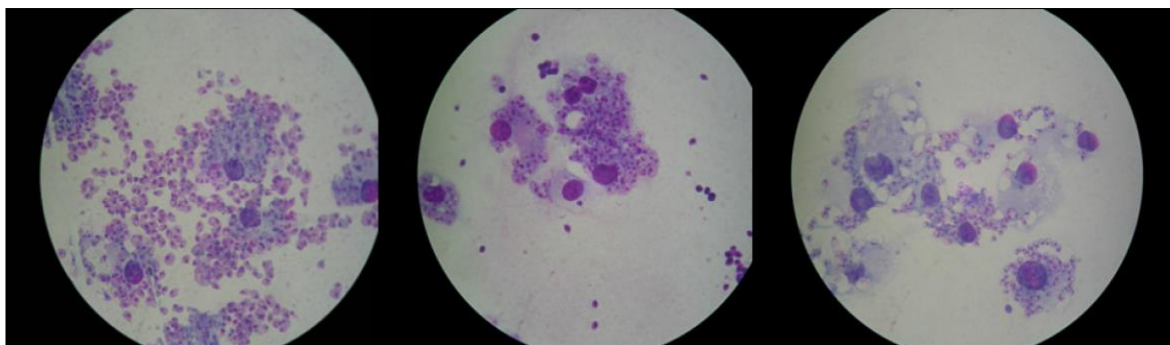


Figure 5.6. *L. mexicana*-infected mouse macrophages. Promastigotes used for infection were untreated (left), treated with 2.14% DMSO (center) and treated with 15.8 mM 6-AN in 2.14% DMSO for 24 hours. Visualization was done under a light microscope using 100X magnification.

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

Although 6AN has relatively little effect against *Leishmania* promastigotes, its proposed activity against PPP, with concomitant disruption of NADPH production and increased sensitivity to oxidative stress, prompted us to determine whether its effect was synergistic with the 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. 5.7, 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.

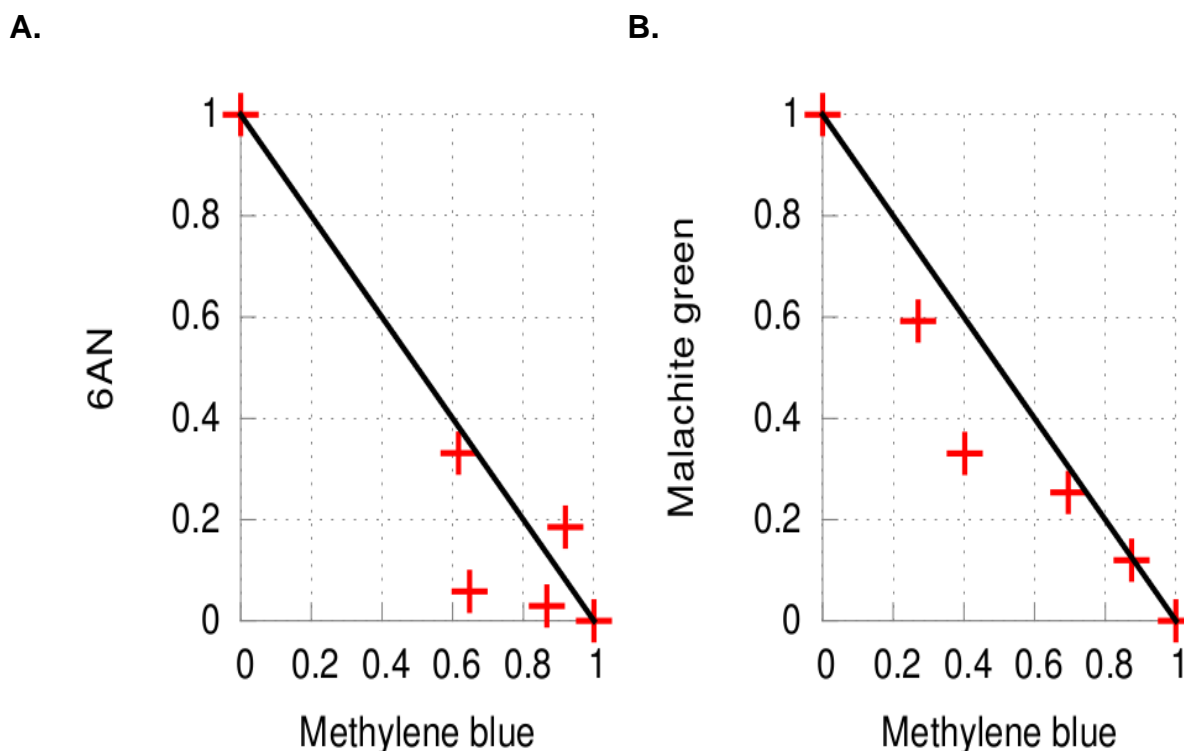


Figure 5.7. Isobolograms of the interaction between 6-AN/MB (A), and MB/MG (B) on *L. mexicana* promastigotes. In the axes the fractional inhibitory concentration (FIC) of 6-AN, methylene blue and malachite green are reported.

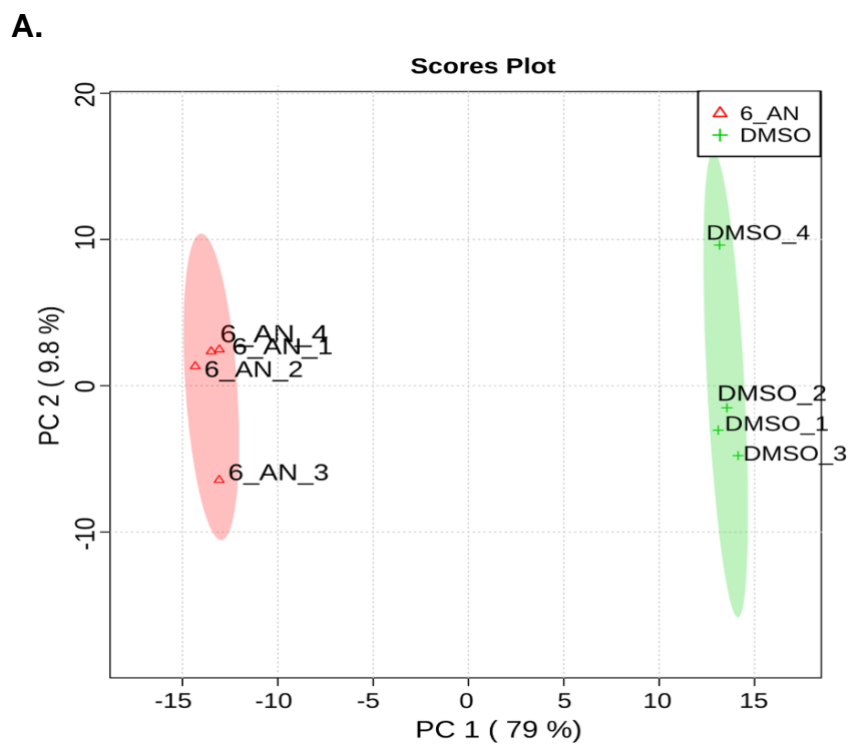
5.3.5 Metabolomics profile

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).

5.3.5.1 *L. mexicana*

The 6-AN and DMSO groups were clearly separated according to principal component analysis (PCA) (Fig. 5.8A); 6AN itself is present only in samples of promastigotes treated with 6AN (Fig. 5.8B), 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. 5.9).



B.

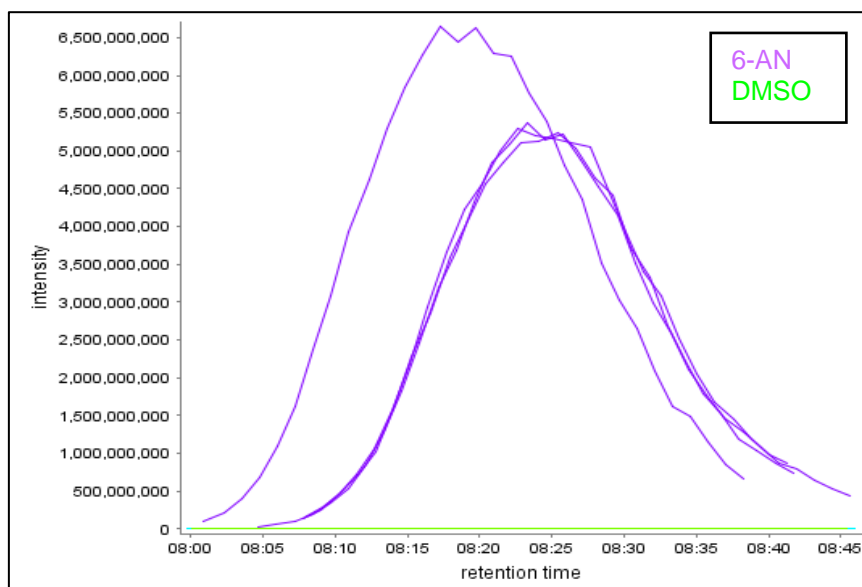


Figure 5.8. A. Principal Component Analysis (PCA) of 6-AN- and DMSO-treated *L. mexicana* samples. 6-AN and DMSO data were uploaded to Metabanalyst; log was transformed prior to PCA (to reduce a biasing effect of high intensity metabolites). The coloured areas denote the 95% confidence interval. B. Absence of 6-AN in DMSO-treated control samples.

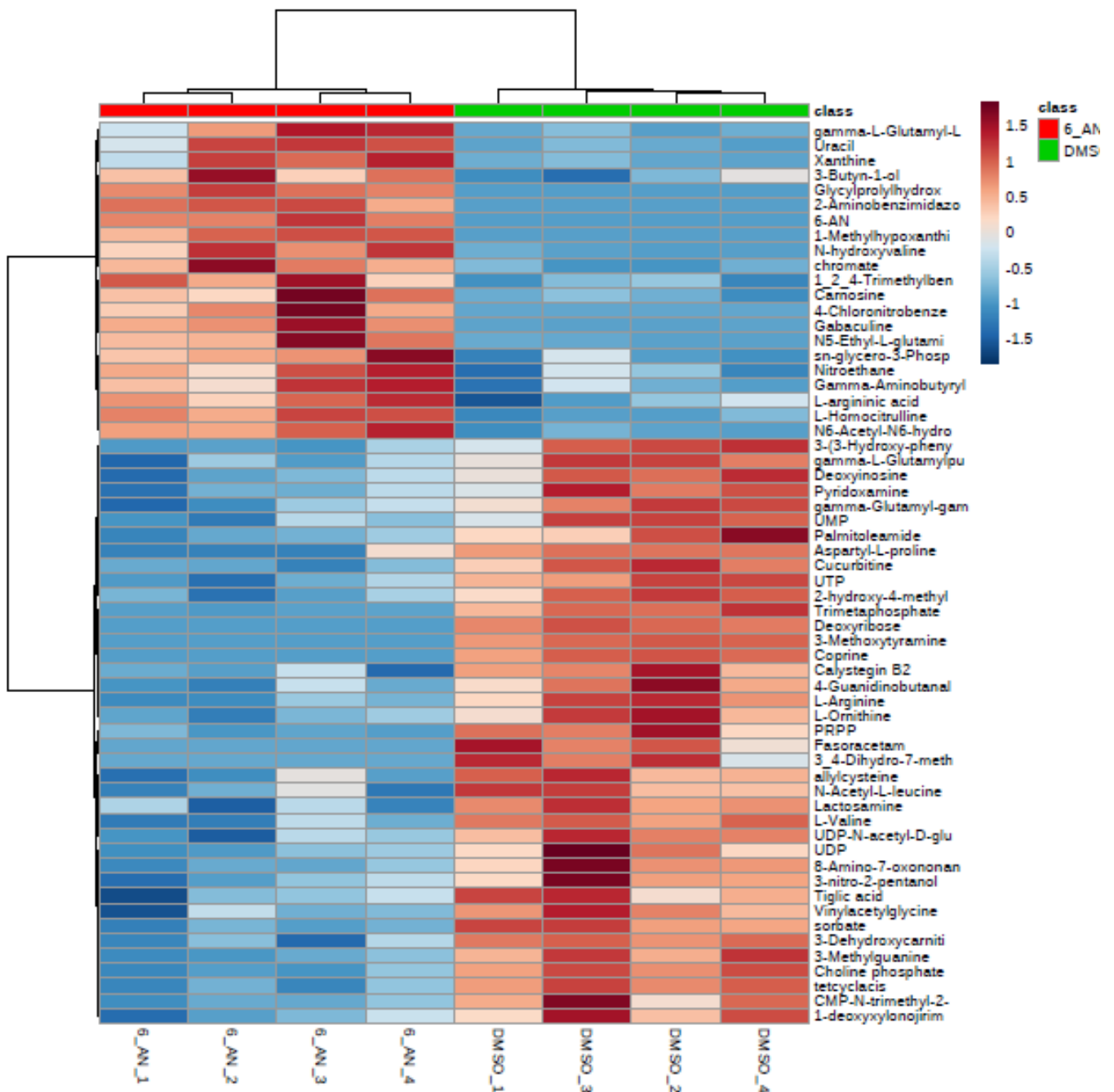


Figure 5.9. The Heat-map of the primary metabolites that differ between 6-AN-treated and control (DMSO-treated) *L. mexicana* promastigotes. The averages of results from four experiments are reported. Red bars depict relative metabolite abundance, while blue bars denote relative metabolite lack.

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).

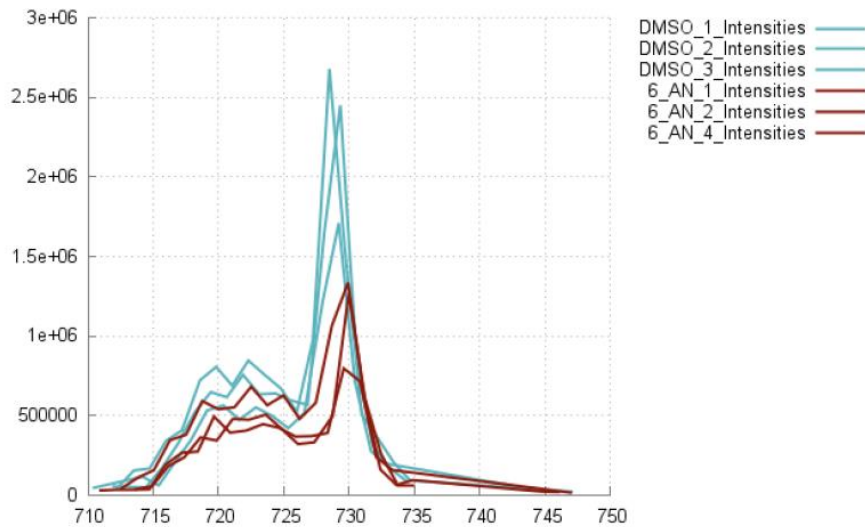
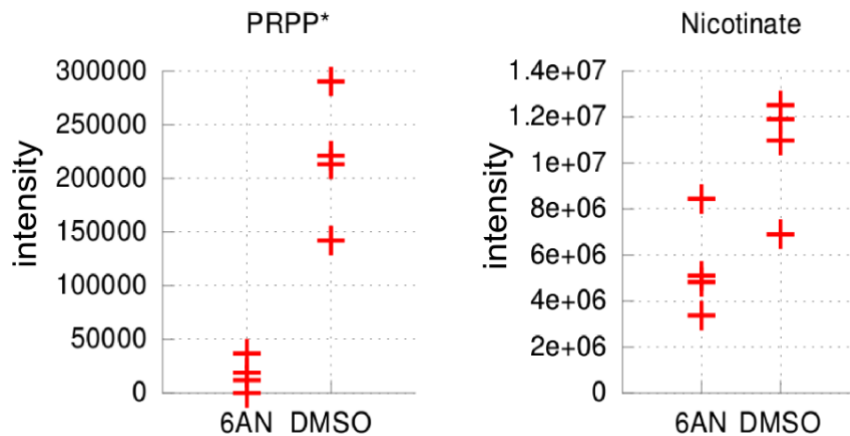


Figure 5.10. Ribose 5-phosphate peaks in 6-AN-treated and -untreated *L. mexicana* promastigotes.

6-AN caused a very marked depletion in the carbohydrate intermediate 5-phospho- α -D-ribose 1-diphosphate (PRPP) and a small (non-significant, FDR=0.09) decrease in nicotinate (Na) (Fig. 5.11A). *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. 5.11B), 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.

A.



B.

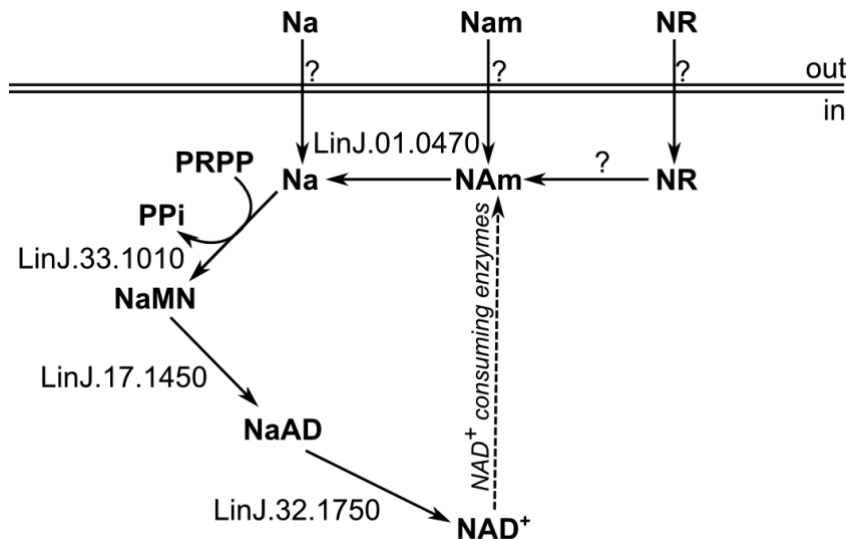


Figure 5.11. A. PRPP and Nicotinate levels in 6-AN-treated parasites and DMSO controls. *: FDR < 0.05. B. The Preiss Handler pathway for NAD⁺ synthesis in *Leishmania*. Abbreviations: NAm, nicotinamide, Na, nicotinate, NaMN, nicotinate mononucleotide, NaAD, Nicotinate adenine dinucleotide, NAD, Nicotinamine 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. 5.12) 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. 5.13). Interestingly, adenosine, a nucleoside was also increased in 6-AN-treated cells (Fig. 5.13).

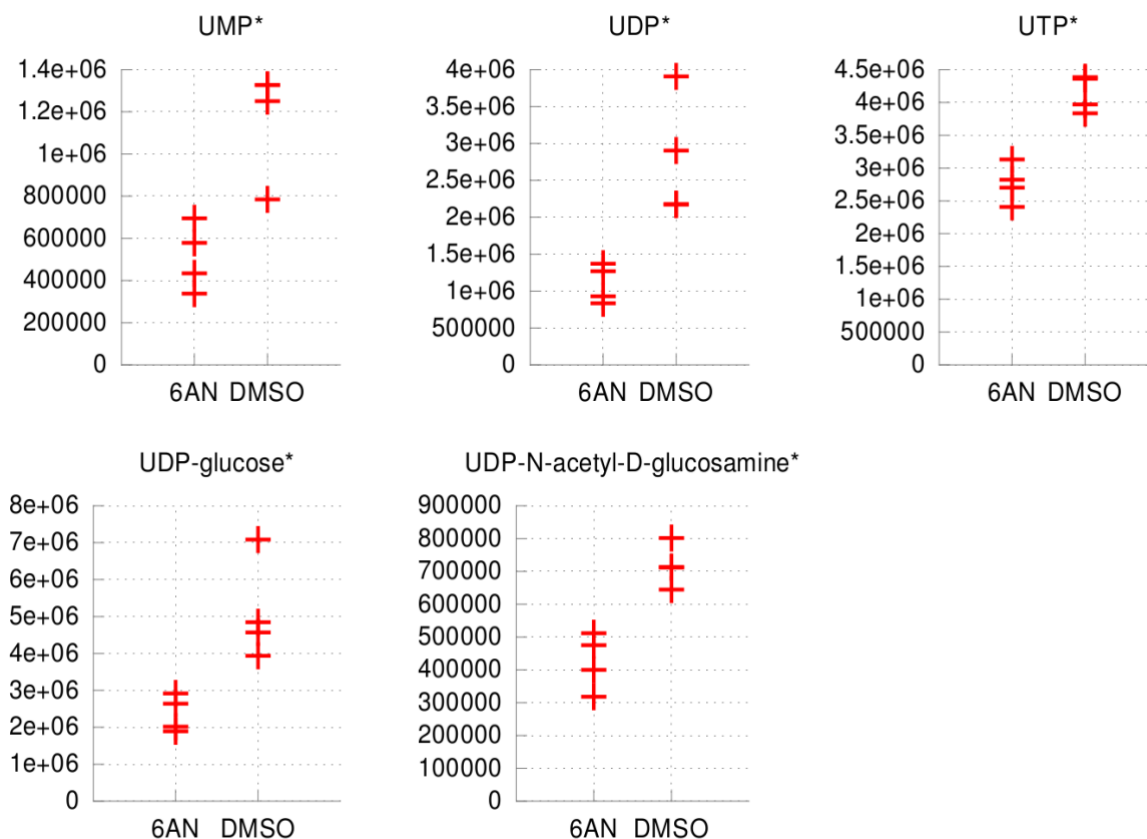


Figure 5.12. Reduced levels of purine and pyrimidine nucleotides, and their carbohydrate derivatives in 6-AN-treated cells, compared to DMSO controls. The Y-axis denotes the signal intensity. *: FDR < 0.05.

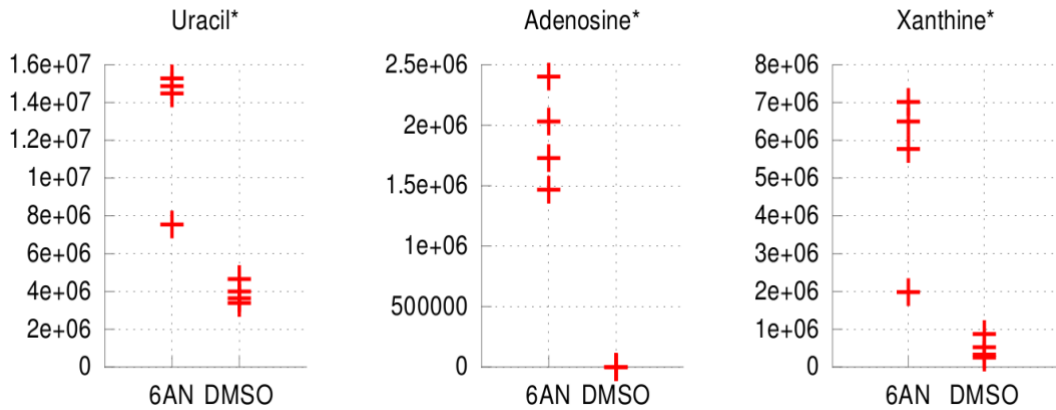


Figure 5.13. Increased pyrimidine precursors and salvaged purine precursors moieties in 6-AN-treated cells compared to DMSO controls. The Y-axis denotes the signal intensity. *: FDR < 0.05.

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. 5.14).

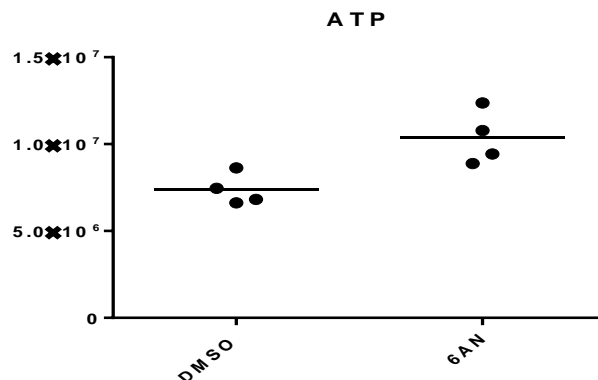


Figure 5.14. Increased ATP level in 6-AN-treated cells compared to DMSO controls. Other significant changes caused by drug perturbation are in amino acids including L-tryptophan, L-valine and L-arginine which are decreased in 6-AN-treated parasites, and

metabolites related to arginine like L- ornithine, which is decreased, while argininic acid, a compound produced from arginine, increases (Fig. 5.15).

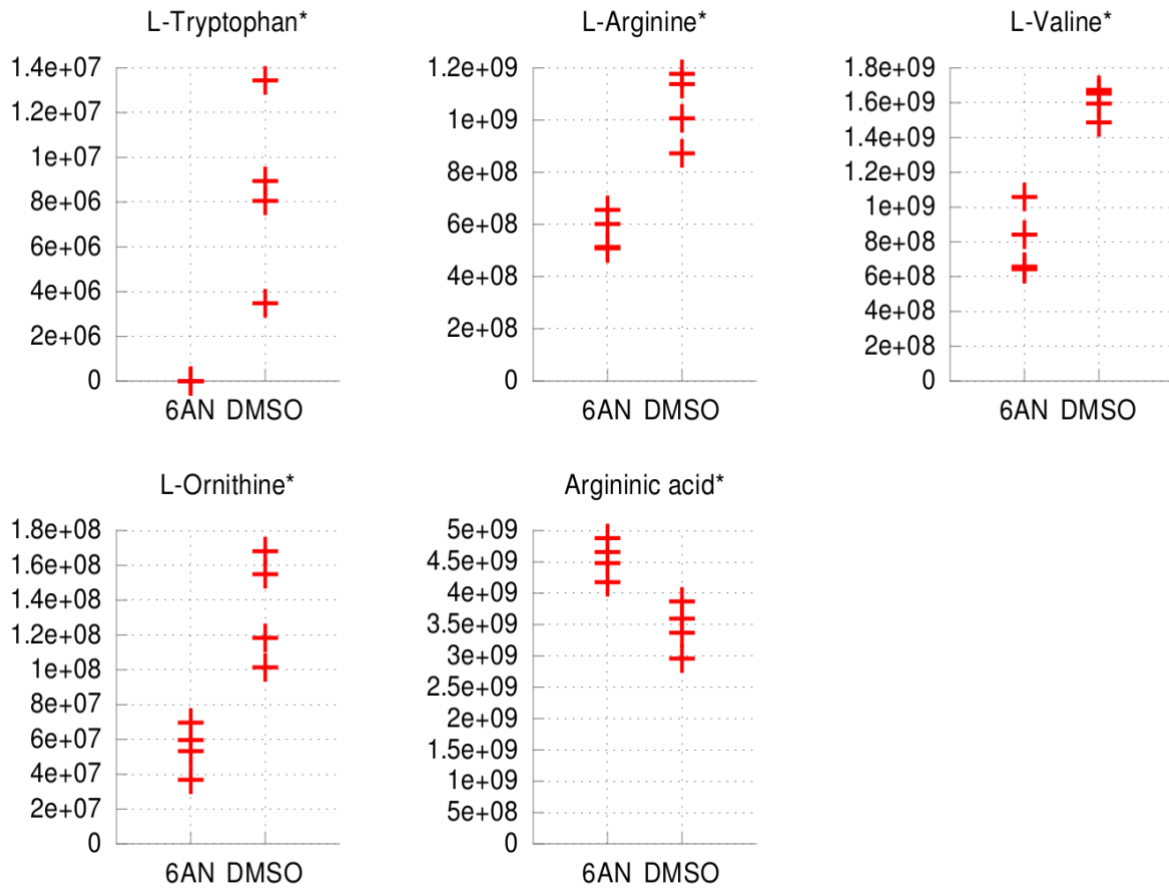


Figure 5.15. Reduced levels of L-tryptophan, L-arginine, L-valine, L-ornithine and increased level of argininic acid in *L. mexicana* promastigotes treated with 6-AN. *: FDR < 0.05.

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. 5.16). This explains that 6-AN-treated parasites utilized the ribose to compensate for the consumed PRPP.

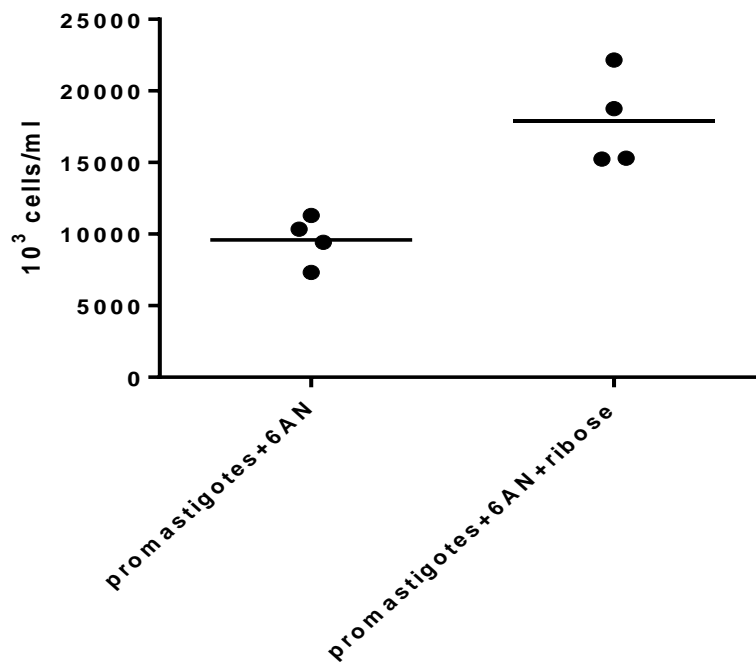
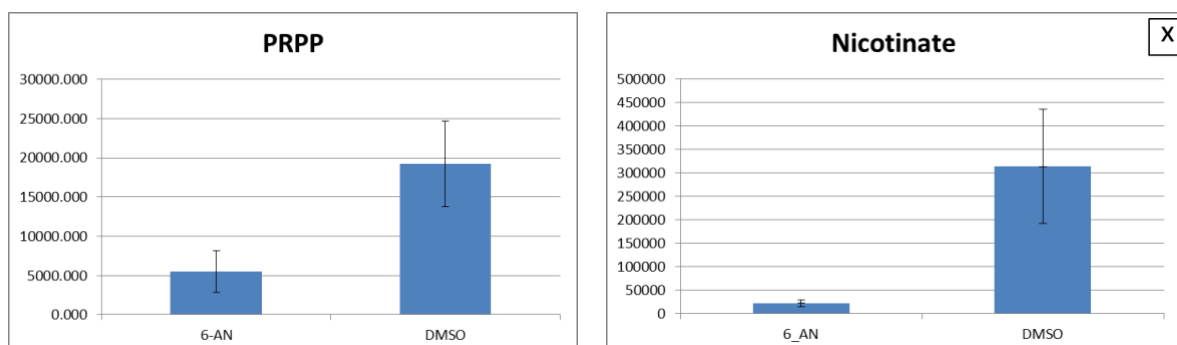


Figure 5.16. Improvement in the growth of 6-AN-treated *L. mexicana* promastigotes by the addition of 6.6 mM ribose. This experiment was done in quadruplicate.

5.3.5.2 *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. 5.17).



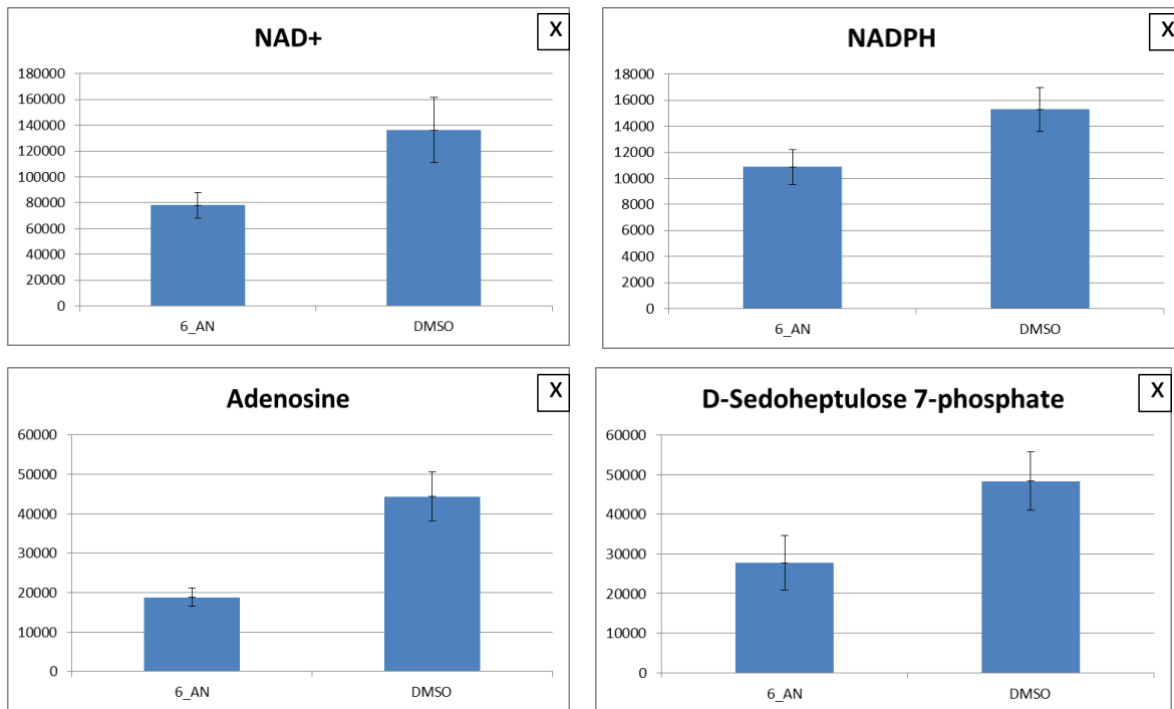


Figure 5.17. Levels of significantly different metabolites of 6-AN-treated *L. infantum* promastigotes compared to DMSO controls. The Y-axis denotes the signal intensity.

5.4 Discussion

Understanding the metabolism of Leishmania and looking for promising drug targets is an interesting goal. In the present work, we treated *L. mexicana* promastigotes and *L. infantum* promastigotes with 6-AN, and studied its effect on parasite growth, infectivity and metabolism using metabolomics technology.

6-AN showed a leishmanistatic 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 IC₅₀ value of 27 μM (Tsuchiya *et al.*, 1988), and *Plasmodium falciparum* at an IC₅₀ 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 IC₅₀ 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. 5.13) 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 μ M 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; Ouaisi *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. 5.14). 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.

5.5 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 ¹³C-glucose labelling and flux analysis might be useful to ascertain the fate and action mechanism of 6-AN in *Leishmania*. In addition, the inhibition of parasite growth due to PRPP depletion shows that PRPP synthetase should also be a good target for potential new drugs against leishmaniasis.

References

- Abbott Laboratories.** Advisory Committee briefing document: Humira (adalimumab). Abbott Park, IL: Abbott Laboratories; 2003.
- Addy, M. & Nandy, A.** Ten years of kala-azar in west Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? *Bull. World Health Organization.* **70**; (1992), P: 341–346.
- Adini A, Krugliak M, Ginsburg H, Li L, Lavie L, Warburg A.** Transglutaminase in *Plasmodium* parasites: activity and putative role in oocysts and blood stages. *Mol Biochem Parasitol.* (2001) Oct; 117(2): 161-8.
- Aeschlimann D, Paulsson M.** Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb Haemost,* Apr. (1994); 71(4):402-15.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts k, Walter P.** Molecular Biology of the Cell. Garland Science: New York, NY pg 1367. "T cells and B cells derive their names from the organs in which they develop. T cells develop in the thymus, and B cells, in mammals, develop in the bone marrow in adults or the liver in fetuses. (2002).
- Ali A, Ashford R. W.** Visceral leishmaniasis in Ethiopia. IV. Prevalence, incidence and relation of infection to disease in an endemic area. *Annals of Tropical Medicine and Parasitology.* (1994); 88(3): 289–293.
- Alvar, J., Aparicio, P., et al.** The relationship between leishmaniasis and AIDS: the second 10 years. *Clin. Microbiol. Rev.* 2008;21, 334–359.
- Alvar J, Canavate C, Molina R, Moreno J, & Nieto J.** Canine leishmaniasis. *Advance Parasitology,* (2004), **57**, P: 1–88.
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al.** Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS ONE,* (2012) 7(5): e35671.
- Amela C, López-Gay D, Alberdi JC, Castilla J.** Injecting drug use as a risk factor for visceral leishmaniasis in AIDS patients. *Eur J Epidemiol.* (1996), 12:91-2.
- Amherd-Hoekstra A, Näher H, Lorenz HM, Enk AH.** "Psoriatic arthritis: a review." *Journal of the German Society of Dermatology.* (2010) May; 8 (5): 332–9.
- Ando H, Adachi M, Umeda K, Matsuura A, Nonaka M, Uchio R, Tanaka H, Motoki M.** Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agric Biol Chem,*(1989); 53:2613-2617.
- Annunziato F, Cosmi L, Santarasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S.** Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) Aug 6; 204(8): 1849-61.
- Antoine J. C., Prina E., Lang T., Courret N.** (1998). The biogenesis and properties of the parasitophorous vacuoles that harbour *Leishmania* in murine macrophages. *Trends*

Microbiol. 6, 392–401.

Arnett FC, Edworthy SM, Bloch DA et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* (1988); 31: 315–24. *Arthritis Rheum.* (2003) Oct; 48(10): 2741-9.

Aruoma OI, Laughton MJ, Halliwell B. "Carnosine, homocarnosine and anserine: could they act as antioxidants in vivo?". *The Biochemical Journal.* (1989); 264 (3): 863–9

Ashford RW, Bates PA. Leishmaniasis in the Old World. In: Cox FEG, Kreier JP, Wakelin D, editors. *Topley & Wilson's microbiology and microbial infections.* Vol.5. *Parasitology.* 9th ed. New York, USA: Oxford University Press; (1998), P: 215-40.

Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, Hommel M. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Trop.* (2001) Jan 15; 78(1): 11-6.

Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, Gollob KJ, Carvalho EM. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infect Immun* (2002) 70:6734–40.

Badaro R, Johnson WD Jr. The role of interferon-gamma in the treatment of visceral and diffuse cutaneous leishmaniasis. *J Infect Dis.* (1993); 167 (Suppl 1): S13–17.

Badaró R, Reed SG, Carvalho EM. Immunofluorescent antibody test in American visceral leishmaniasis: sensitivity and specificity of different morphological forms of two *Leishmania* species. *Am J Trop Med Hyg.* (1983) May; 32 (3): 480-4.

Badaro, R., Jones, T.C., et al., New perspectives on a subclinical form of visceral leishmaniasis. *J. Infect. Dis.* 1986. 154, 1003–1011.

Bagalas V, Kioumis I, Argyropoulou P, Patakas D. Visceral leishmaniasis infection in a patient with rheumatoid arthritis treated with etanercept. *Clin Rheumatol.* (2007); 26: 1344-5.

Bal A, Unlu E, Bahar G, Aydog E, Eksioglu E, Yorgancioglu R. Comparison of serum IL-1 beta, sIL-2R, IL-6, and TNF-alpha levels with disease activity parameters in ankylosing spondylitis. *Clin Rheumatol.* (2007); 26: 211–215,.

Barrett MP, Croft S. Management of trypanosomiasis and leishmaniasis. *Brit Med Bull.* (2012); 104:175-196.

Barrett MP. The pentose phosphate pathway and parasitic protozoa. *Parasitol Today.* (1997) Jan; 13(1):11-6.

Barros-Alvarez X, Gualdrón-López M, Acosta H, Cáceres AJ, Graminha MA, Michels PA, Concepción JL, Quiñones W. Glycosomal targets for anti-trypanosomatid drug discovery. *Curr Med Chem.* (2014); 21(15): 1679-706.

Baserga A, Morsiani M, and Mariuzzi GM. Hematological and Histological Modifications Induced by 6-Aminonicotinamide. *Acta Vitaminologica.* (1956); 6:195-204.

Basset, D., Faraut, F., et al., Visceral leishmaniasis in organ transplant recipients: 11 new cases and a review of the literature. *Microbes Infect.* 2005; 7, 1370–1375.

Bassetti M, Pizzorni C, Gradoni L, Del Bono V, Cutolo M, Viscoli C. Visceral leishmaniasis infection in a rheumatoid arthritis patient treated with adalimumab. *Rheumatology* (Oxford). (2006); 45: 1446-8.

Bates A Paul. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. *Int J Parasitol.* (2007) Aug; 37(10-3): 1097–1106.

Bates PA, Robertson CD, Tetley L, Coombs GH. Axenic cultivation and characterization of *Leishmania mexicana* amastigote-like forms. *Parasitology.* (1992); 105(2): 193–202.

Bates PA, Rogers ME. New insights into the developmental biology and transmission mechanisms of *Leishmania*. *Curr. Mol. Med.* (2004); 4: 601–609.

Behrens F, Himsel A, Rehart S, et al. Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis. *Ann Rheum Dis.* (2007); 66: 1151-6.

Benton HP, Want EJ and Ebbels TMD. Correction of mass calibration gaps in liquid chromatography-mass spectrometry metabolomics data. *BIOINFORMATICS* (2010); 26: 2488.

Berens RL, Brun R, Krassner SM. A simple monophasic medium for axenic culture of hemoflagellates. *J Parasitol.* (1976) Jun; 62 (3): 360-5.

Berger N.A, Catino D.M, and Vietti T.J. *Cancer Res.* (1982); 42, 4382-4386.

Berkshire, UK: Abbott Laboratories Ltd; 2010. Humira 40 mg solution for injection [summary of product characteristics].

Berkshire. UK: Wyeth Europa Ltd; 2010. Enbrel 25 mg powder and solvent for solution for injection [summary of product characteristics].

Berman JD, Gallalee JV, Best JM. Sodium stibogluconate (Pentostam) inhibition of glucose catabolism via the glycolytic pathway, and fatty acid beta-oxidation in *Leishmania mexicana* amastigotes. *Biochem Pharmacol.* (1987) Jan 15; 36(2):197-201.

Bern C, Haque R, Chowdhury R, et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *American Journal of Tropical Medicine and Hygiene.* (2007); 76(5): 909–914.

Bertelli M, El-Bastawissy E, Knaggs MH, Barrett MP, Hanau S, Gilbert IH. Selective inhibition of 6-phosphogluconate dehydrogenase from *Trypanosoma brucei*. *J Comput Aided Mol Des*, (2001); 15: 465-475.

Biglino A, Bolla C, Concialdi E, Trisciuglio A, Romano A, Ferroglio E. Asymptomatic *Leishmania infantum* Infection in an Area of Northwestern Italy (Piedmont Region) Where Such Infections Are Traditionally Nonendemic. *J. Clin Microbiol.* (2010); Vol. 48, No. 1 p. 131–136.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP. "A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells". *Nature*. (1997); 385 (6618): 729–33.

Blackwell J, Goswami T, Evans CW, Sibthorpe D, Papo N, et al. *SLC11A1* (formerly *NRAMP1*) and disease resistance. *Cellular Microbiology*. (2001) 3: 773–784.

Boelaert M. et al. A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*. **70**, (2004), P: 72–77.

Bogdan, C., Gessner, A., et al., Cytokines in leishmaniasis: a complex network of stimulatory and inhibitory interactions. *Immunobiology* 1993; 189, 356–396.

Boitz JM, Ullman B. Adenine and adenosine salvage in *Leishmania donovani*. *Mol. Biochem Parasitol*, (2013); 190: 51-55.

Bosque F, Saravia NG, Valderrama L, Milon G. Distinct innate and acquired immune responses to *Leishmania* in putative susceptible and resistant human populations endemically exposed to *L. Viannia panamensis* infection. *Scand J Immunol*. (2000); 51: 533–541.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. (1976) May 7; 72: 248-54.

Brahmachari U N. *A treatise on kala-azar* (London: John Bale Sons and Daneilson), (1928).

Bristol-Myers Squibb. (March 13, 2007). "ORENCIA label" (PDF). Archived March 7, 2008, at the Wayback Machine. (110 KiB). United States Food and Drug Administration. Retrieved on 2007-05-25.

Brobey RK, Soong L. *Leishmania* species: evidence for transglutaminase activity and its role in parasite proliferation. *Exp Parasitol*. (2006) Oct; 114(2): 94-102.

Brussels, Belgium: UCB, Inc.; 2009. Cimzia® 200 mg solution for injection [summary of prescribing information].

Bryceson ADM. Leishmaniasis. In: Cook GC, editor. *Manson's Tropical Diseases*. 20th edition. (1996). London, United Kingdom: W. B. Saunders. pp. 1213–1245.

Bucheton B, Abel L, El-Safi S, Kheir MM, Pavek S, et al. A major susceptibility locus on chromosome 22q12 plays a critical role in the control of kala-azar. *Am J Hum Genet*. (2003) 73: 1052–1060.

Bourgeois, N., Bastien, P., et al., Active chronic visceral leishmaniasis' in HIV-1- infected patients demonstrated by biological and clinical long-term follow-up of 10 patients. *HIV Med.* 2010; 11, 670–673.

Burnet M. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Aust J Sci.* (1957); 20: 67–69.

Cantini F, Niccoli L, Nannini C, Kaloudi O, Bertoni M, Cassarà E. Psoriatic arthritis: a systematic review. *nt J Rheum Dis.* (2010) Oct; 13(4): 300-17.

Carmona A, Freedland RA. Effect of 6-aminonicotinamide on pentose cycle activity in isolated hepatocytes. *Int. J Biochem,* (1990); 22: 595-599.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. "An endotoxin-induced serum factor that causes necrosis of tumors". *Proc. Natl. Acad. Sci.* (1975); U.S.A. 72 (9): 3666–70.

Carter NS, Yates PA, Arendt CS, Boitz JM, Ullman B. Purine and pyrimidine metabolism in *Leishmania*. *Adv Exp Med Biol* (2008); 625:141-154.

Carter NS, Yates PA, Gessford SK, Galagan SR, Landfear SM, Ullman B. Adaptive responses to purine starvation in *Leishmania donovani*. *Mol Microbiol.* (2010) Oct;78(1): 92-107.

Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL & Reed SG. Restoration of IFN- γ production and lymphocyte proliferation in visceral leishmaniasis. *Journal of Immunology.* (1994); 152: 5949-5956.

Carvalho, E.M., Bacellar, O., et al., Antigen-specific immunosuppression in visceral leishmaniasis is cell mediated. *J. Clin. Invest.* 1989; 83, 860–864.

Cassidy AJ, van Steensel MA, Steijlen PM, van Geel M, van der Velden J, Morley SM, Terrinoni A, Melino G, Candi E, McLean WH. A homozygous missense mutation in TGM5 abolishes epidermal transglutaminase 5 activity and causes acral peeling skin syndrome. *Am J Hum Genet.* (2005); 77: 909–917.

Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, et al. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* (1999); 42: 963-970.

Chakraborty A.K, Majumder H.K. Mode of action of pentavalent antimonials: specific inhibition of type I DNA topoisomerase of *Leishmania donovani*. *Biochem. Biophys. Res. Commun.,* (1988), 152(2), 605-611.

Chandrashekar R, Devarajan E, Mehta K. *Dirofilaria immitis*: further characterization of the transglutaminase enzyme and its role in larval molting. *Parasitol Res.* (2002) Mar; 88(3): 185-91.

Chappuis F, Rijal S, Soto A, Menten J, and Boelaert M. A meta- analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ.* (2006); 333: 723.

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol.* (2007) Nov; 5 (11): 873-82.

Cho YS, Han MK, Kwark OS, Phoe MS, Cha YS, An NH et al. Auto-ADP-ribosylation of NAD glycohydrolase from *Neurospora crassa*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, May (1998); Volume 120, Issue 1, Pages 175–181.

Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology* (Oxford). (2012) Jul; 51 Suppl 5:v3-11.

Clark E A, Ledbetter J A. How does B cell depletion therapy work, and how can it be improved?. *Ann Rheum Dis.* (2005); 64: iv77-iv80.

Clarke DD, Mycek MJ, Neidle A, Waelsch H. The incorporation of amines into protein. *Archives of Biochemistry and Biophysics.* (1959); Volume 79, Pages 338-354

Cobb JR, Percy SC, Gholson RK. Metabolism of 6-aminonicotinic acid in *Escherichia coli*. *J Bacteriol* (1977); 131: 789-794.

Colomba C, Saporito L, Polara VF, Barone T, Corrao A, Titone L. Serological screening for *Leishmania infantum* in asymptomatic blood donors living in an endemic area (Sicily, Italy). *Transfus Apher Sci.* (2005) Nov; 33(3): 311-4.

Colomba C, Saporito L, Vitale F, Reale S, Vitale G, Casuccio A, et al. Cryptic *Leishmania infantum* infection in Italian HIV infected patients. *BMC Infect Dis.* (2009) Dec 10; 9: 199.

Contini C, Grilli A, Badia L, Guardigni V, Govoni M, Seraceni S. Detection of *Chlamydophila pneumoniae* in patients with arthritis: significance and diagnostic value. *Rheumatol Int.* (2011); 31:1307–13.

Costa AS, Costa GC, Aquino DM, Mendonça VR, Barral A, Barral-Netto M, Caldas Ade J. Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis. *Mem Inst Oswaldo Cruz.* (2012) Sep; 107(6): 735-9.

Costa CHN, Gomes RBB, Silva MRB, Garcez LM, Ramos PKS, Santos RS, Shaw JJ, David JR, Maguire JH. Competence of the human host as a reservoir for *Leishmania chagasi*. *J Infect Dis.* (2000); 182: 997–1000.

Costa CHN, Pereira HF, Pereira FCA, Tavares JP, Araújo MV, Gonçalves MJO. Is the household dog a risk factor for American visceral leishmaniasis in Brazil. *Trans R Soc Trop Med Hyg.* (1999); 93: 464.

Costello P, Bresnihan B, O'Farrelly C, FitzGerald O. Predominance of CD8+ T lymphocytes in psoriatic arthritis. *J Rheumatol.* (1999); 26: 1117–1124.

Cragg MS, Walshe CA, Ivanov AO, Glennie MJ. "The biology of CD20 and its potential as a target for mAb therapy". *Current Directions in Autoimmunity*. *Current Directions in Autoimmunity*. (2005); 8: 140–74.

Creek DJ, Jankevics A, Breitling R, et al. Toward Global Metabolomics Analysis with Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry: Improved Metabolite Identification by Retention Time Prediction. *Anal. Chem.* 2011, 83, 8703–8710.

Creek DJ, Jankevics A, Breitling R, Watson DG, Barrett MP, Burgess KEV. Toward global metabolomics analysis with hydrophilic interaction liquid chromatography-mass spectrometry: Improved metabolite identification by retention time prediction. *Anal Chem* (2011); 83: 8703–8710.

Creek DJ, Jankevics A, Burgess KEV, Breitling R, Barrett MP. IDEOM: an Excel interface for analysis of LC–MS-based metabolomics data. *Bioinformatics* (2012); 28: 1048–1049.

Chambers MC, Maclean B, Burke R, Amodei D, Ruderman DL, Neumann S et al. A Cross-platform Toolkit for Mass Spectrometry and Proteomics. *Nat Biotechnol*, (2012); 30(10):918–920.

Creek DJ, Mazet M, Achcar F, Anderson J, Kim DH, Kamour R, Morand P, Millerioux Y, Biran M, Kerkhoven EJ, Chokkathukalam A, Weidt SK, Burgess KE, Breitling R, Watson DG, Bringaud F, Barrett MP. Probing the metabolic network in bloodstream-form *Trypanosoma brucei* using untargeted metabolomics with stable isotope labelled glucose. *PLoS Pathog.* (2015) Mar 16; 11(3):e1004689.

Croft SL, Seifert K, Yardley V. Current scenario of drug development for leishmaniasis. *Indian J. Med. Res.*, (2006), 123(3), 399-410.

Cruz AK, de Toledo JS, Falade M, Terraio MC, Kamchonwongpaisan S, Kyle DE, Uthaipibull C. Current treatment and drug discovery against *Leishmania* spp. and *Plasmodium* spp.: a review. *Curr. Drug Targets*, (2009), 10(3), 178-192.

Cunningham D D. On the presence of peculiar parasitic organisms in the tissue of a specimen of Delhi boil. Scientific memoirs by officers of the Medical and Sanitary Departments of the Government of India; Government printing: Calcutta, India, (1885); 1, 21–31.

Cunningham ML, Fairlam AH. Trypanothione reductase from *Leishmania donovani*. Purification, characterisation and inhibition by trivalent antimonials. *Eur J Biochem* (1995); 230:460-468.

Cupolillo E, Medina-Acosta E, Noyes H, Momen H, Grimaldi G Jr. A revised classification for *Leishmania* and *Endotrypanum*. *Parasitol Today*. (2000); 16: 142–144.

D'Oliveira Junior, A., Costa, S.R., et al., Asymptomatic *Leishmania chagasi* infection in relatives and neighbors of patients with visceral leishmaniasis. *Mem. Inst. Oswaldo Cruz* 1997; 92, 15–20.

Dardik R, Leor J, Skutelsky E, Castel D, Holbova R, Schiby G, Shaish A, Dickneite G, Loscalzo J, Inbal A. Evaluation of the pro-angiogenic effect of factor XIII in heterotopic mouse heart allografts and FXIII-deficient mice. *Thromb Haemost.* (2006); 95: 546–550.

Dardonville C, Rinaldi E, Barrett M P, Brun R, Gilbert IH. and Hanau S. "Selective inhibition of *Trypanosoma brucei* 6-phosphogluconate dehydrogenase by high energy intermediate and transition state analogues" *Journal of Medicinal Chemistry.* (2004); 47: 3427-37.

Das S, Rani M, Pandey K, Sahoo GC, Rabidas VN, Singh D, Das P. Combination of paromomycin and miltefosine promotes TLR4-dependent induction of antileishmanial immune response in vitro. *J Antimicrob Chemother.* (2012) 67: 2373- 2378.

David CV, Craft N. Cutaneous and mucocutaneous leishmaniasis. *Dermatol Ther.* (2009) Nov-Dec; 22 (6): 491-502.

Dauids BJ, Mehta K, Fesus L, McCaffery JM, Gillin FD. Dependence of *Giardia lamblia* encystation on novel transglutaminase activity. *Mol Biochem Parasitol*, Aug. (2004);136(2):173-80.

De Almeida MC, Cardoso SA, Barral-Netto M. *Leishmania (Leishmania) chagasi* infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. *Int J Parasitol.* (2003), 33: 153-162.

de Gouvea Viana, L., de Assis, T.S., et al., Combined diagnostic methods identify a remarkable proportion of asymptomatic *Leishmania (Leishmania) chagasi* carriers who present modulated cytokine profiles. *Trans. R. Soc. Trop. Med. Hyg.* 2008; 102, 548–555.

de Kossodo S, Grau GE, Louis JA, Müller I. Tumor necrosis factor alpha (TNF-alpha) and TNF-beta and their receptors in experimental cutaneous leishmaniasis. *Infect Immun.* (1994) Apr; 62(4): 1414-20.

De la Loma A, Alvar J, Martínez-Galiano E, Blázquez J, Alcalá-Muñoz A, Nájera R. Leishmaniasis or AIDS? *Trans R Soc Trop Med Hyg.* (1985); 79: 421-2.

De Leonardis F, Govoni M, Lo Monaco A, Trotta F. Visceral leishmaniasis and anti-TNF- α therapy: case report and review of the literature. *Clinical and Experimental Rheumatology.* (2009); 27: 503-506.

Deborggraeve S, Claes F, Laurent T, Mertens P, Leclipteux T, Dujardin JC, Herdewijn P, and Buscher P. Molecular dipstick test for diagnosis of sleeping sickness. *Journal of Clinical Microbiology.* 44, (2006), P: 2884-2889.

Dedet JP, Pratlong F, Pradinaud R, Moreau B. Delayed culture of *Leishmania* in skin biopsies. *Transaction of the Royal Society and Tropical Medicine and Hygiene.* 93, (1999), P: 673-674.

Denton H, McGregor J.C, Coombs G.H. Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. *Biochem. J.*, (2004), 381(2), 405-412.

Desjeux P, and Alvar J. *Leishmania/HIV* co-infections: epidemiology in Europe. *Ann. Trop. Med. Parasitol.* (2003); 97(Suppl.1): 3–15.

Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immun Microbiol Infect Dis*, (2004); 27:305-318.

Desjeux P. The increase in risk factors for leishmaniasis worldwide. *Transaction of the Royal Society and Tropical Medicine and Hygiene*, 95, (2001), P: 239-43.

Di Muccio T, Scalone A, Bruno A, Marangi M, Grande R, Armignacco O, Gradoni L, Gramiccia M. Epidemiology of Imported Leishmaniasis in Italy: Implications for a European Endemic Country. *PLOS ONE*. (2015) July 31; 10(7): e0134885.

Dickens F, & Glock G. E. Direct oxidation of glucose-6-phosphate, 6-phosphogluconate and pentose-5-phosphates by enzymes of animal origin. *Biochem J.* (1951) Nov; 50(1): 81-95.

Dietrich LS, Friedland IM, Kaplan LA. Pyridine nucleotide metabolism: mechanism of action of the niacin antagonist, 6-aminonicotinamide. *J Biol Chem* (1958); 33: 964-968.

Dietrich LS, Friedland IM. 6-aminonicotinamide and 6-aminonicotinic acid metabolism in nucleated and non-nucleated erythrocytes. *Arch Biochem Biophys* (1960); 313-317.

Ding F, Li XN, Diao JX, Sun Y, Zhang L, Ma L et al. Potential toxicity and affinity of triphenylmethane dye malachite green to lysozyme. *Ecotox Environ Safe*, (2012); 78: 41-49.

Dubbink HJ, Cleutjens KB, van der Korput HA, Trapman J, Romijn JC. An Sp1 binding site is essential for basal activity of the human prostate-specific transglutaminase gene (TGM4) promoter. *Gene*. (1999); 240: 261–267.

Dujardin JC, Campino L, Cañavate C, Dedet J-P, Gradoni L, Soteriadou K et al. Spread of vector-borne diseases and neglect of Leishmaniasis, *Europe. Emerg Infect Dis*. (2008); 14: 1013–1018.

Dujardin JC, Campino L, Canavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M. Spread of vector-borne diseases and neglect of leishmaniasis, *Europe. Emerg. Infect. Dis.*, (2008), 14(7), 1013-1018.

Duong-Ly KC, Gabelli SB. Salting out of proteins using ammonium sulfate precipitation. *Methods Enzymol*, (2014); 541:85-94.

Eckert RL, Kaartinen MT, Nurminskaya M, Belkin AM, Colak G, Johnson GV, Mehta K. Transglutaminase regulation of cell function. *Physiol Rev.* (2014) Apr; 94(2): 383-417.

Edwards JC, Cambridge G. Prospects for B-cell-targeted therapy in autoimmune disease. *Rheumatology (Oxford)*. (2005); 44: 151–6.

Edwards JC, Cambridge G. Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. *Rheumatology* (Oxford). (2001); 40: 205-11.

Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF α therapy. *J Exp Med.* (2004) Aug; 2; 200(3): 277-85. Epub 2004 Jul 26.

Emery P, Keystone E, Tony HP, Cantagrel A, van Vollenhoven R, Sanchez A, Alecock E, Lee J, Kremer J. "IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial". *Annals of the Rheumatic Diseases.* (2008) Nov; 67 (11): 1516–23.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, E. Vetter A, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill III FR, and Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Reviews.* 19, (2006), P: 165-256.

Evans DA. Handbook on isolation, characterization, cryopreservation of Leishmania. Geneva, *World Health Organization' (UNDP/ World Bank)*, (1989).

Evans TG, Teixeira MJ, McAuliffe IT, et al. Epidemiology of visceral leishmaniasis in Northeast Brazil. *Journal of Infectious Diseases.* (1992); 166(5): 1124–1132.

FABRE S, GIBERT C, LECHICHE C, DEREURE J, JORGENSEN C, SANY J. Visceral leishmaniasis infection in a rheumatoid arthritis patient treated with infliximab. *Clin Exp Rheumatol.* (2005); 23: 891-2.

Färber PM, Arscott LD, Williams Jr CH, Becker K, and Schirmer RH. Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue. *FEBS Lett.* (1998); 422:311-314.

Faria J, Loureiro I, Santarém N, Cecílio P, Macedo-Ribeiro S, Tavares J, Cordeiro-da-Silva A. Disclosing the essentiality of ribose-5-phosphate isomerase B in Trypanosomatids. *Sci Rep.* (2016) May; 27: 6:26937.

Fesus L, and Piacentini M. Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem Sci.* (2002) Oct; 27(10): 534-9.

Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature.* (2003) May 15; 423(6937): 356-61.

Fivelman QL, Adagu IS, Warhurst DC. Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*. *Antimicrob Agents Chemother* (2004); 48(11): 4097-4102.

Folk JE. *Enzymol. Relat. Areas Mol. Biol.* (1983); 54: 1-56.

Fowlkes BJ, Edison L, Mathieson BJ, Chused TM. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J Exp Med.* (1985) Sep; 1;162(3): 802-22.

François C, Shyam S, Asrat H, Hashim G, Suman R, Rosanna WP, Jorge A, Marleen B. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews* 5, 873–882.

Fromm PD, Kling JC, Remke A, Bogdan C, Körner H. Fatal Leishmaniasis in the Absence of TNF Despite a Strong Th1 Response. *Front Microbiol.* (2016) Jan 22; 6:1520.

Fruth U, Solioz N, Louis JA. *Leishmania major* interferes with antigen presentation by infected macrophages. *J Immunol.* (1993) Mar 1;150 (5):1857-64.

Fukutani K, Figueiredo V, Celes F, Cristal J, Barral A, Barral-Neto M, et al. Serological survey of *Leishmania* infection in blood donors in Salvador, Northeastern Brazil. *BMC Infect Dis* (2014); 14: 422.

Gaboury JP, Johnston B, Niu XF, Kubes P. Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. *J Immunol.* (1995); 154: 804–813.

Gao GF, Rao Z, Bell JI. Molecular coordination of $\alpha\beta$ T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. *Trends Immunol.* (2002); 23: 408–413.

Garcia-Garcia A, Martin-Sanchez J, Gallego M, Rivero-Roman A, Camacho A, Riera C, Morillas-Marquez F, Vergara S, Macias J, and Pineda JA. Use of noninvasive markers to detect *Leishmania* infection in asymptomatic human immunodeficiency virus-infected patients. *J. Clin. Microbiol.* (2006); 44: 4455–4458.

Garcia-Gonzalez E, Guidelli GM, Bardelli M, Maggio R. Mucocutaneous leishmaniasis in a patient treated with anti-TNF- α therapy. *Rheumatology* (Oxford). (2012) Aug; 51(8): 1517-8.

Garcia-Vidal C, Rodríguez-Fernández S, Teijón S, Esteve M, Rodríguez-Carballeira M, Lacasa JM, et al. Risk factors for opportunistic infections in infliximab-treated patients: the importance of screening in prevention. *Eur J Clin Microbiol Infect Dis.* (2009); 28(4): 331–7.

Gavvani ASM, Mohite H, Edrissian GH, Mohebbi M, Clive RD. Domestic Dog Ownership In Iran Is A Risk Factor For Human Infection With *Leishmania Infantum*. *Am. J. Trop. Med. Hyg.* (2002); 67(5), pp. 511–515.

Gazanion E, Garcia D, Silvestre R, Gérard C, Guichou JF, Labesse G et al. The *Leishmania* nicotinamidase is essential for NAD⁺ production and parasite proliferation. *Mol. Microbiol* (2011); 82: 21–38.

Gazanion E, Vergnes B, Seveno M, Garcia D, Oury B, Ait-Oudhia K et al. *In vitro* activity of nicotinamide/antileishmanial drug combinations. *Parasitol Int* (2011); 60:19-24.

Geary TG, Divo AA, Jensen JB. Nutritional requirements of *Plasmodium falciparum* in culture. II. Effects of antimetabolites in a semi-defined medium. *J Protozool.* (1985); 32: 65-69.

Getts MT, Miller SD. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: triggering of autoimmune diseases by infections. *Clin Exp Immunol.* (2010); 160: 15-21.

Ghalib HW, Piurezman MR, Skeiky YW, Siddig M, Hashim FA, EL-Hassan AM, Russo DM, Reed SG. Interleukin-10 production relates with pathology in human *Leishmania donovani* infections. *Journal of Clinical Investigation.* (1993); 92, P: 324-329.

Ghalib HW, Piuvezam MR, Sheiky Y, Siddig M, Hashim FA, El Hassan AM, Russo DM & Reed SG. Interleukin-10 production correlates with pathology in human *Leishmania donovani* infections. *Journal of Clinical Investigation.* (1993); 92: 324- 329.

Ghalib HW, Whittle JA, Kubin M, Hashim FA, el-Hassan AM, Grabstein KH, Trinchieri G, Reed SG. IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. *J Immunol.* (1995) May 1; 154 (9):4623-9.

Gholson RK. The pyridine nucleotide cycle. *Nature* (London). (1966); 212: 933-935.

Ghosh AK, Sardar AH, Mandal A, Saini S, Abhishek K, Kumar A, Purkait B, Singh R, Das S, Mukhopadhyay R, Roy S, Das P. Metabolic reconfiguration of the central glucose metabolism: a crucial strategy of *Leishmania donovani* for its survival during oxidative stress. *FASEB J.* (2015) May; 29(5): 2081-98.

Ghosh J, Anderson PJ, Chandrasekaran S., Caparon, MG. Characterization of *Streptococcus pyogenes* β -NAD⁺ Glycohydrolase. *J Biol Chem.* (2010); 285: 5683–5694.

Gidwani K, Rai M, Chakravarty J, Boelaert M, Sundar S. Evaluation of leishmanin skin test in Indian visceral leishmaniasis. *Am J Trop Med Hyg.* (2009) Apr; 80(4): 566-7.

Glaser L, & Brown, D. H. Purification and properties of d-glucose-6-phosphate dehydrogenase. *Journal of Biological Chemistry,* (1955); 216: 67–79.

Glew RH, Saha AK, Das S, Remaley AT. Biochemistry of the *Leishmania* species. *Microbiol Rev.* (1988); 52: 412-432.

Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. *Arthritis Rheum.* (2003); 48: 2122-7.

González D, Pérez JL, Serrano ML, Igoillo-Esteve M, Cazzulo JJ, Barrett MP, Bubis J, Mendoza-León A. The 6-phosphogluconate dehydrogenase of *Leishmania (Leishmania) mexicana*: gene characterization and protein structure prediction. *J Mol Microbiol Biotechnol.* (2010); 19(4): 213-23.

Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* (2003) Jan; 3 (1):23-35.

Gossage SM, Rogers ME, Bates PA. Two separate growth phases during the development of *Leishmania* in sand flies: implications for understanding the life cycle. *Int J Parasitol.* 2003; 33:1027-1034.

Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous

leishmaniasis. *Expert Rev Anti Infect Ther.* (2010) Apr;8(4): 419-33.

Gradoni L, Pizzuti R, di Martino L, Gramiccia M, Pempinello R, Gaeta GB, Ferrara M, Scotti S, Altieri S. The epidemiology and surveillance of visceral leishmaniasis in the Campania region of Italy. The value of zymodeme typing. *Epidemiol Infect* (1993) 111 (2): 297-306.

Gramiccia M. The identification and variability of the parasites causing leishmaniasis in HIV-positive patients in Italy. *Ann Trop Med Parasitol.* (2003); 97(S1): 65–73.

Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* (1987) Nov; 30(11): 1205-13.

Grell M. Tumor necrosis factor (TNF) receptors in cellular signaling of soluble and membrane-expressed TNF. *J Inflamm.* (1995); 47(1–2): 8–17.

Grenard P, Bates MK, Aeschlimann D. Evolution of transglutaminase genes: identification of a transglutaminase gene cluster on human chromosome 15q15. Structure of the gene encoding transglutaminase X and a novel gene family member, transglutaminase Z. *J Biol Chem* 276. (2001); 33066–33078.

Griffin M., Casadio R., Bergamini C.M. Transglutaminases: Nature's biological glues. *Biochem J.* (2002); 368: 377-396.

Guarneri C, Bevelacqua V, Patterson JW, Tchernev G. Cutaneous and visceral leishmaniasis during anti-TNF α therapy. *Wien Med Wochenschr.* (2016) Nov. DOI 10.1007/s10354-016-0527-1

Guedes-Barbosa LS, Pereira da Costa I, Fernandes V, Henrique da Mota LM, de Menezes I, Aaron Scheinberg M. Leishmaniasis during anti-tumor necrosis factor therapy: report of 4 cases and review of the literature (additional 28 cases). *Semin Arthritis Rheum.* (2013) Oct; 43(2): 152-7.

Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, Wasunna MK, Bryceson AD. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect. Dis.* (2002), 2, 494-501.

Gupta S, Igoillo-Esteve M, Michels PA, Cordeiro AT. Glucose-6-Phosphate Dehydrogenase of Trypanosomatids: Characterization, Target Validation, and Drug Discovery. *Mol Biol Int.* (2011); Article ID 135701.

Hanau S, d'Empaire LP, Capone I, Alberighi S, Montioli R, Dallochio F. Evidence for dimer/tetramer equilibrium in *Trypanosoma brucei* 6-phosphogluconate dehydrogenase. *Biochim Biophys Acta.* (2013) Dec; 1834(12): 2647-52.

Hanau S, Montin K, Gilbert I.H, Barrett M.P. and Dallochio F. "Inhibitors of *Trypanosoma brucei* 6-Phosphogluconate Dehydrogenase" *Current Bioactive Compounds.* (2007); 3: 161-169.

Hannaert V, Bringaud F, Opperdoes FR, Michels PA. Evolution of energy metabolism and its compartmentation in *Kinetoplastida*. *Kinetoplastid Biol Dis.* (2003) Oct 28; 2(1):11.

Haralambous C, Antoniou M, Pratlong F, Dedet J, et al. Development of a molecular assay specific for the *Leishmania donovani* complex that discriminates *L. donovani/Leishmania infantum* zymodemes: a useful tool for typing MON-1. *Diagn Microbiol Infect Dis.* (2008); 60:33–42.

Harith AE, Kolk AHJ, Kager PA et al. A simple and economical direct agglutination test for serodiagnosis and seroepidemiological studies of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* (1986) 80, 583– 587.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* (2005); 6: 1123-1132.

Hasegawa G, Suwa M, Ichikawa Y, Ohtsuka T, Kumagai S, Kikuchi M, Sato Y, Saito Y. *Biochem. J.* (2003); 373: 793-803.

Haug K, Salek RM, Conesa P, Hastings J, de Matos P, Rijnbeek M et al. MetaboLights - an open-access general-purpose repository for metabolomics studies and associated meta-data. *Nucleic Acids Res.* (2013); 41:D781-D786.

Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. "Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway". *The Biochemical Journal.* (1998) Sep; 334 (Pt 2): 297–314.

Hendricks L, Wright N. Diagnosis of cutaneous leishmaniasis by in vitro cultivation of saline aspirates in Schneider's Drosophila Medium. *Am J Trop Med Hyg.* (1979) Nov;28(6):962-4.

Hendrickson N, Allen T, Ullman B. Molecular characterization of phosphoribosylpyrophosphate synthetase from *Leishmania donovani*. *Mol Biochem Parasitol.* (1993) May; 59(1): 15-27.

Hernández-Pérez J, Yebra-Bango M, Jiménez-Martínez E, et al. Visceral leishmaniasis (Kala-azar) in solid organ transplantation: report of five cases and review. *Clin Infect Dis.* (1999); 29:918-21.

Herter FP, Weissman SG, Thompson HG, Hyman G, Martin DS. Clinical Experience with 6-aminonicotinamide. *Cancer Res.* (1961); 21: 31-37.

Herwaldt BL. Leishmaniasis. *Lancet.* (1999) Oct 2; 354 (1985): 1191-9.

Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B: recent progress and future directions. *Clin. Infect. Dis.* (1996), 22(2), S133-144.

Hochberg MC, Johnston SS, John AK. The incidence and prevalence of extra-articular and systemic manifestations in a cohort of newly diagnosed patients with rheumatoid arthritis between 1999 and 2006. *Curr Med Res Opin.* (2008); 24: 469-80.

Holaday BJ, Pompeu MM, Jeronimo S, Teixeira MJ & Sousa A de A. Potential role for IL-10 in the immune suppression associated with kala-azar. *Journal of Clinical Investigation.* (1993); 92: 2626- 2632.

Holman JD, Tabb DL, Mallick P. Employing ProteoWizard to Convert Raw Mass SpectrometryData. *Curr Protoc Bioinformatics* (2015); 46: 13.24.1–13.24.9.

Hommel M, Peters W, Ranque J, Quilici M, Lanotte G. The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. *Ann Trop Med Parasitol.* (1978) Jun; 72(3): 213-18.

Hove-Jensen B. Mutation in the phosphoribosylpyrophosphate synthetase gene (prs) that results in simultaneous requirements for purine and pyrimidine nucleosides, nicotinamide nucleotide, histidine, and tryptophan in *Escherichia coli*. *J Bacteriol* (1998); 170:1148-1152.

Hu X, Jiao K, Sun W, You JY. Electrochemical and spectroscopic studies on the interaction of malachite green with DNA and its application. *Electroanal.* (2006); 6:613-620.

Hughes KT, Cookson BT, Ladika D, Olivera BM, Roth JR. 6-aminonicotinamide-resistant mutants of *Salmonella typhimurium*. *J Bacteriol.* (1983); 154:1126-1136.

Hunting D, Gowans B, Henderson JF. Effects of 6-aminonicotinamide on cell growth, poly(ADP-ribose) synthesis and nucleotide metabolism. *Biochem Pharmacol.* (1985) Nov 15; 34(22): 3999-4003.

Imboden JB. The immunopathogenesis of rheumatoid arthritis. *Annu Rev Pathol.* (2009); 4: 417-34.

Iniesta V, Carcelen J, Molano I, Peixoto PM, Redondo E, Parra P, Mangas M, Monroy I, Campo ML, Nieto CG, Corraliza I. Arginase I induction during *Leishmania major* infection mediates the development of disease. *Infect. Immun.* (2005). 73, 6085–6090.

Jacob N, Stohl W. Autoantibody-dependent and autoantibody-independent roles for B cell in systemic lupus erythematosus: past, present, and future. *Autoimmunity.* (2010); 43: 84–97.

Jaffe CL, Bennett E, Grimaldi G Jr, McMahon-Pratt D. Production and characterization of species-specific monoclonal antibodies against *Leishmania donovani* for immunodiagnosis. *Journal of Immunology.* (1984); 133, P: 440-447.

Jensen KF. Metabolism of 5-phosphoribosyl 1-pyrophosphate (PRPP) in *Escherichia coli* and *Salmonella typhimurium*, p. 1-25. In A. Munch-Petersen (ed.) (1983), Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. AcademicPress, Inc., London.

Jeziorski E, Dereure J, Mac Bullen G, Blanchet C, Ludwig C, Costes V, Rodière M. Mucosal relapse of visceral leishmaniasis in a child treated with anti-TNF α . *Int J Infect Dis.* (2015) Apr; 33: 135-6.

Jiang WG, Ablin R, Douglas-Jones A, Mansel RE. Expression of transglutaminases in human breast cancer and their possible clinical significance. *Oncol Rep.* (2003); 10: 2039–2044.

Johnson WJ, and McColl JD. *Science.* (1955); 122, 834.

June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* (1987); 7: 4472–4481.

Kahana SE, Lowry OH, Schulz DW, Passonneau JV, and Crawford EJ. The kinetics of phosphoglucose isomerase. *J. Biol. Chem.* (1960); 235: 2178-2184.

Karp CL, El Safi SH, Wynn TA, Satti MM, Kordofani AM, Hashim FA, Hag-Ali M, Neva FA, Nutman TB & Sacks DL. *In vivo* cytokine profiles in patients with kala-azar. *Journal of Clinical Investigation.* (1993); 91: 1644-1648.

Kashiwagi T, Yokoyama K, Ishikawa K, Ono K, Ejima D, Matui H, Suzuki E Crystal structure of microbial transglutaminase from *Streptovorticillium mobaraense*. *J Biol Chem.* (2002); 277 46:44252–44260.

Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol.* (2011) Jul; 11; 9(8): 604-15.

Kellog DE, Kwok S. 1990. Detection of human immunodeficiency virus, p 339–343 *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. (ed), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

Keniry MA, Hollander C, Benz CC. The effect of gossypol and 6-aminonicotinamide on tumor cell metabolism: a ³¹P-magnetic resonance spectroscopic study. *Biochem Biophys Res Commun.* (1989); 164(2): 947-953.

Kerkhoven EJ, Achar F, Alibu V, Burchmore RJ, Gilber I H, Trybilo M et al. Handling Uncertainty in Dynamic Models: The Pentose Phosphate Pathway in *Trypanosoma brucei*. *PLoS Comput Biol* (2013); 9(12): e1003371.

Kerkhoven JE, Achar F, Alibu VP, Burchmore RJ, Gilbert IH, Trybilo M, et al. Handling uncertainty in dynamic models: the pentose phosphate pathway in *Trypanosoma brucei*. *PLoS Comput Biol.* (2013); 9(12)e1003371.

Khan MA. Spondyloarthropathies. *Rheum Dis Clin North Am.* (1992); 18(1): 1-276.

Killick-Kendrick, R. Biology of *Leishmania* in *Phlebotomine* sandflies. *In Biology of the Kinetoplastida.* (1979); Vol. 2, (ed. Lumsden, W. H. R. & Evans. D. A.), pp. 395-460. Academic Press, London/New York.

Kim HC, Lewis MS, Gorman JJ, Park SC, Girard JE, Folk JE, & Chung SI. Protransglutaminase from guinea pig skin. Isolation and partial characterisation, *J. Biol. Chem.* (1990); 265: 2 1 971221 978.

Klareskog L, Rönnelid J, Lundberg K, et al. Immunity to citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol.* (2008); 26: 651 – 675.

Klareskog L, Padyukov L, Alfredsson L. Smoking as a trigger for inflammatory rheumatic diseases. *Curr Opin Rheumatol.* (2007); 19: 49-54.

Knodler LA, Noiva R, Mehta K, McCaffery JM, Aley SB, Svard SG, Nystul TG, Reiner DS, Silberman JD, Gillin FD. Novel protein-disulfide isomerases from the early-diverging protist *Giardia lamblia*. *J Biol Chem.* (2000) Sep; 8: 275(36): 28339.

Kobets T, Grekov I, and Lipoldová M. Leishmaniasis: Prevention, Parasite Detection and Treatment *Current Medicinal Chemistry*. (2012); 19, 1443-1474 1443.

Kohler E, Barrach HJ, Neubert D. Inhibition of NADP dependent oxidoreductases by the 6-aminonicotinamide analogue of NADP. *FEBS Lett.* (1970); 6: 225-228.

Kolbe H, Keller K, Lange K, and Herken H. *Biochem. Biophys. Res. Commun.* (1969); 36, 93.

Kolbe H, Keller K, Lange K, Herken H. Metabolic consequences of drug-induced inhibition of the pentose phosphate pathway in neuroblastoma and glioma cells. *Biochem Biophys Res Commun.* (1976); 73(2): 378-382.

Koseki-Kuno S, Yamakawa M, Dickneite G, Ichinose A. Factor XIII A subunit-deficient mice developed severe uterine bleeding events and subsequent spontaneous miscarriages. *Blood.* (2003); 102: 4410–4412.

Kovarova J, Barrett MP. The pentose phosphate pathway in parasitic *Trypanosomatids*. *Trends in parasitology.* (2016) August; Volume 32, Issue 8, Pages 622–634.

Kubar, J., Marty, P., et al., Visceral leishmaniasis in HIV-positive patients: primary infection, reactivation and latent infection Impact of the CD4+ Tlymphocyte counts. *AIDS* 1998; 12, 2147–2153.

Kudo O, Sabokbar A, Pocock A, et al. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone.* (2003); 32: 1-7.

Kumar H, Kawai T, and Akira S, “Pathogen recognition by the innate immune system. *International Reviews of Immunology.* (2011); vol. 30, no. 1, pp. 16–34.

Kumar R., K. Pai, K. Pathak, and S. Sundar. Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. *Clin. Diagn. Lab Immunol.* (2001); 8:1220–1224.

Kuon W, Sieper J. Identification of HLA-B27-restricted peptides in reactive arthritis and other spondyloarthropathies: computer algorithms and fluorescent activated cell sorting analysis as tools for hunting of HLA-B27-restricted chlamydial and autologous cross-reactive peptides involved in reactive arthritis and ankylosing spondylitis. *Rheum Dis Clin North Am.* (2003); 29:595–611.

Kurkjian KM, Mahmutovic AJ, Kellar KL, Haque R, Bern C, Secor WE. Multiplex analysis of circulating cytokines in the sera of patients with different clinical forms of visceral leishmaniasis. *Cytometry A.* (2006) May; 69(5): 353-8.

Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature.* (1989) May 18; 339(6221): 237-8.

Lachaud L, Chabbert E, Dubessay P, Dereure J, Lamothe J, Dedet JP, and Bastien P. Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. *Parasitology.* (2002); 125:197–207.

- Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP & Bastien P.** Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *Journal of Clinical Microbiology*. (2002); 40, 210–215.
- Laemmli.** U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*. (1970); 227; 680–685.
- Lainson R, Shaw J J.** Evolution, classification and geographical distribution. In Peters W & Killick-Kendrick R (Eds). *The Leishmaniases in Biology and Medicine*. (1987); vol. 1. Orlando: Academic Press, pp: 1-120.
- Lainson R, Ward RD., Shaw JJ.** *Leishmania* in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the *Leishmania mexicana* and *L. braziliensis* complexes. *Proc. Roy. Soc. Lond. B*. (1977); 199:309–320.
- Lajemi M, Demignot S, Borge L, Thenet-Gauci S, Adolphe M.** The use of Fluoresceincadaverine for detecting amine acceptor protein substrates accessible to active transglutaminase in living cells. *Histochem J*. (1997) Aug; 29(8): 593-606.
- Lally F, Smith E, Filer A, et al.** A novel mechanism of neutrophil recruitment in a coculture model of the rheumatoid synovium. *Arthritis Rheum*. (2005); 52: 3460-9.
- Lancaster MV, Fields RD.** Antibiotic and Cytotoxic Drug Susceptibility Assays using Resazurin and Poisoning Agents. (1996); US Patent No. 5,501,959.
- Lanzavecchia A.** Antigen uptake and accumulation in antigen-specific B cells. *Immunol Rev*. (1987); 99: 39–51.
- le Fichoux Y, Quaranta JF, Aufeuve JP, Lelievre A, Marty P, Suffia I, et al.** Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J Clin Microbiol*. (1999) Jun; 37(6): 1953-7.
- Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB.** Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science*. (2002); 297: 1689–1692.
- Leiden, The Netherlands:** Centocor B.V.; 2009. Simponi 50 mg solution for injection in pre-filled pen [summary of product characteristics].
- Leishman WB.** On the possibility of occurrence of trypanosomiasis in India. *British Medical Journal*. (1903); 30, P: 1252.
- Li RC, Schentag JJ, Nix DE.** The fractional maximal effect method: a new way to characterize the effect of antibiotic combinations and other nonlinear pharmacodynamic interactions. *Antimicrob Agents Chemother*. (1993); 37(3): 523-531.
- Liarte DB, Mendonca IL, Luz FC, Abreu EA, Mello GW, Farias TJ.** QBC® for the diagnosis of human and canine American visceral leishmaniasis: preliminary data. *Revista da Sociedade Brasileira de Medicina Tropical*. (2001); 34, P: 577-81.
- Liese J, Schleicher U, Bogdan C.** TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur J Immunol*. (2007) Dec; 37(12): 3424-34.

Liew FY, Li Y, Millott S. Tumour necrosis factor (TNF-alpha) in leishmaniasis. II. TNF-alpha-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. *Immunology.* (1990) Dec; 71(4): 556-9.

Lin Y, Means GE, Feeney RE. The action of proteolytic enzymes on N,N-dimethyl proteins. Basis for a microassay for proteolytic enzymes. *J Biol Chem.* (1969) Feb 10; 244(3): 789-93.

Lindoso JA, Barbosa RN, Posada-Vergara MP et al. Unusual manifestations of tegumentary leishmaniasis in AIDS patients from the New World. *Br. J. Dermatol.* (2009); 160, 311-318.

Liu D, Uzonna JE. Macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol.* (2012) Jun 12; 2: 83.

Lodge R, Descoteaux A. Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*. *Clin Immunol.* (2005) Mar; 114(3): 256-65.

Loewi G, Dorling J, Howard A. Mononuclear cells from inflammatory joint effusions: electron-microscopic appearances and immunoglobulin synthesis. *J Rheumatol.* (1974) Mar; 1(1): 34-44.

Lorand L, Conrad SM. *Mol. Cell. Biochem.* (1984); 58, 9-35.

Lorand L, Parameswaran KN, Velasco PT, Hsu LK, Siefiring GE Jr. New colored and fluorescent amine substrates for activated fibrin stabilizing factor (Factor XIIIa) and for transglutaminase. *Anal Biochem,* Jun. (1983); 131(2): 419-25.

Lorand L. Transglutaminase, Remembering Heinrich Waelsch. *Neurochem. Int.* (2002); 40: 7-12.

Louassini M, Foulquière MR, Benitez R, Adroher FJ. Activity of key enzymes in glucose catabolism during the growth and metacyclogenesis of *Leishmania infantum*. *Parasitol Res.* (1999); 85, 300-306.

Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr Opin Immunol.* (2008); 20: 332–338.

Luque-Ortega JR, Rivas L. Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in *Leishmania donovani* promastigotes. *Antimicrob. Agents Chemother.* (2007); 51(4), 1327-1332.

Lustigman S, Brotman B, Huima T, Castelhana A L, Singh R N, Mehta K, and Prince A M. Transglutaminase-catalyzed reaction is important for molting of *Onchocerca volvulus* third-stage larvae. *Antimicrob Agents Chemother.* (1995) Sep; 39(9): 1913–1919.

Lux H, Heise N, Klenner T, Hart D, Opperdoes F.R. Ether-lipid (alkyl- phospholipid) metabolism and the mechanism of action of ether-lipid analogues in *Leishmania*. *Mol. Biochem. Parasitol.* (2000); 111(1), 1-14.

Machado, C.M., Martins, T.C., et al., Epidemiology of neglected tropical diseases in transplant recipients. Review of the literature and experience of a Brazilian HSCT center. *Rev. Inst. Med. Trop. Sao Paulo* 2009; 51, 309–324.

Maggi P, Gaudio V, Valente M, Latorraca A, Cavaliere RL, Marroni M, et al. Leishmaniasis in patients with chronic renal failure: A diagnostic and therapeutic challenge for the clinician. *Journal of Nephrology*. (2004); 17, P: 296-301.

Makarov KS, Aravind L, and Koonin EU. A superfamily of archael, bacterial and eukaryotic proteins homologous to animal transglutaminases. *Protein Science*. (1999); 8:1714–1719.

Malvern PA, USA: Centocor Ortho Biotech, Inc.; 2009. Remicade® (infliximab) for IV injection [prescribing information].

Maly K, Uberall F, Schubert C, Kindler, E, Stekar J, Brachwitz H, Grunicke H.H. Interference of new alkylphospholipid analogues with mitogenic signal transduction. *Anticancer Drug Des*. (1995); 10(5), 411-425.

Manson-Bahr PEC, and Apted FIC. *Mansons Tropical Diseases*. 8th edition, (1985), ELBS and Baillier Tindall- London- UK, P: 38-41.

Manson-Bahr PEC, Heisch RB, Studies on leishmaniasis in east Africa.III. Clinical features and treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. (1956); 50, P: 465–471.

Manson-Bahr PEC. Diagnosis. In: Peters W. Killick-Kendick, R, editors. *The leishmaniasis in biology and medicine*. (1987); Vol. 2. London: Academic Press; p: 703.

Marcili A, et al. Phylogenetic relationships of *Leishmania* species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes: Taxonomic revision of *Leishmania (L.) infantum chagasi* in South America. *Infect. Genet. Evol.* (2014); 25, 44–51.

Marie I, Guglielmino E. Non-tuberculous anti-TNF associated opportunistic infections. *Rev Med Interne*. (2010) May; 31(5): 353-60.

Mary C., Faraut, F., et al., Reference values for *Leishmania infantum* parasitemia in different clinical presentations: quantitative polymerase chain reaction for therapeutic monitoring and patient follow-up. *Am. J. Trop. Med. Hyg.* 2006; 75, 858–863

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. *Phlebotomine* sandflies and the spreading of leishmaniasis and other diseases of public health concern. *Med Vet Entomol*. (2013); 27: 123-147.

Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, Gramiccia M, Mortarino M, Pietrobelli M, Gradoni L. The northward spread of leishmaniasis in Italy: evidence from retrospective and on-going studies on the canine reservoir and *Phlebotomine* vectors. *Trop. Med. Int. Health*. (2008); 13: 256–264.

Marsden PD. Mucosal leishmaniasis ("espundia" Escomel, 1911). *Trans R Soc Trop Med Hyg.* (1986); 80(6): 859-76.

Martin A, Romito G, Pepe I, Vivo GDe, Merola MR, Limatola A, Gentile V. Transglutaminase-Catalyzed Reactions Responsible for the Pathogenesis of Celiac Disease and Neurodegenerative Diseases: From Basic Biochemistry to Clinic. *Curr Med Chem.* (2006); 13: 1895-1902.

Martin JL, Yates PA, Soysa R, Alfaro JF, Yang F, et al. Metabolic Reprogramming during Purine Stress in the Protozoan Pathogen *Leishmania donovani*. *PLoS Pathog.* (2014); 10(2): e1003938.

Martin-Mola E, Balsa A. Infectious complications of biologic agents. *Rheum Dis Clin North Am.* (2009); 35 (1): 183–99.

Martinez P, de la Vega E, Laguna F, Soriano V, Puente S, Moreno V, et al. Diagnosis of visceral leishmaniasis in HIV-1 infected individuals using peripheral blood smears. *AIDS.* (1993); 7, P: 227-30.

Marty P, Lelievre A, Quaranta JF, Rahal A, Gari-Toussaint M, Le Fichoux Y. Use of the leishmanin skin test and western blot analysis for epidemiological studies in visceral leishmaniasis areas: experience in a highly endemic focus in Alpes-Maritimes (France). *Trans R Soc Trop Med Hyg.* (1994) Nov-Dec; 88(6): 658-9.

Marty, P., Lelievre, A., et al., Detection by Western blot of four antigens characterizing acute clinical leishmaniasis due to *Leishmania infantum*. *Trans. R. Soc. Trop. Med. Hyg* 1995. 89, 690–691.

Mary C, Faraut F, Lascombe L, and Dumon H. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J. Clin. Microbiol.* (2004); 42: 5249–5255.

MARY J. MYCEK AND HEINRICH WAELSCH. The Enzymatic Deamidation of Proteins. *THE JOURNAL OF BIOLOGICAL CHEMISTRY.* (1960) December; Vol. 226, No. 12.

Mathers CD, Ezzati M, Lopez AD. Measuring the burden of neglected tropical diseases: the global burden of disease framework. *PLoS Negl Trop Dis.* (2007); D1:e114.

Maugeri DA, Cazzulo JJ, Burchmore RJ, Barrett MP, Ogbunude PO. Pentose phosphate metabolism in *Leishmania mexicana*. *Mol Biochem Parasitol.* (2003) Aug 31; 130(2): 117-25.

McCarthy-Burke C, Bates PA, and Dwyer DM. *Leishmania donovani*: use of two different, commercially available, chemically defined media for the continuous in vitro cultivation of promastigotes. *Exp. Parasitol.* (1991); 73: 385-387.

McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol.* (2007) Jun; 7(6): 429-42.

Mehrotra S, Fakiola M, Oommen J, Jamieson S, Mishra A, et al. Genetic and functional evaluation of the role of CXCR1 and CXCR2 in susceptibility to visceral leishmaniasis in north-east India. *BMC Med Genet.* (2011); 12: 162.

Mehta K, Rao UR, Vickery AC, Fesus L. Identification of a novel transglutaminase from the filarial parasite *Brugia malayi* and its role in growth and development. *Mol Biochem Parasito.* (1992) Jul; 53(1-2): 1-15.

Meinecke CK, Schottelius J, Oskam L, Fleischer B. Congenital transmission of visceral leishmaniasis (Kala Azar) from an asymptomatic mother to her child. *Pediatrics.* (1999); 104:e65.

Meredith SE, Zijlstra EE, Schoone GJ, Kroon CC, van Eys GJ, Schaeffer KU, el-Hassan AM, Lawyer PG. Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. *Arch Inst Pasteur Tunis.* (1993) Jul-Oct; 70 (3-4): 419-31.

Meulenbroek AJ, Zeijlemaker WP. Human IgG Subclasses: Useful Diagnostic Markers for Immunocompetence. 2. Amsterdam, The Netherlands. *CLB.* (2000).

Miclet E, Stoven V, Michels PA, Opperdoes FR, Lallemand JY, Duffieux F. NMR spectroscopic analysis of the first two steps of the pentose-phosphate pathway elucidates the role of 6-phosphogluconolactonase. *J Biol Chem.* (2001) Sep 14; 276(37): 34840-6.

Miles SA, Conrad SM, Alves RG, Jeronimo SM, Mosser DM. A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania*. *J Exp Med.* (2005); 201(5): 747–754.

Mishra A, Saxina A, Singh S. Chemotherapy of leishmaniasis: past, present and future. *Curr. Med. Chem.* (2007); 14 (10), 1153-1169.

Mittal MK, Rai S, Ashutosh, Ravinder, Gupta S, Sundar S, et al. Characterization of natural antimony resistance in *Leishmania donovani* isolates. *Am J Trop Med Hyg.* (2007); 76:681–688.

Mohan AK, Cote TR, Block JA, Manadan AM, Siegel JN, Braun MM. Tuberculosis following the use of etanercept, a tumor necrosis factor inhibitor. *Clin Infect Dis.* (2004); 39: 295-9.

Montenegro J. CUTANEOUS REACTION IN LEISHMANIASIS. *Arch Derm Syphilol.* (1926); 13(2): 187-194.

Moral L, Rubio EM, Moya M. A leishmanian skin test survey in the human population of l'Alacantí region (Spain): implications for the epidemiology of *Leishmania infantum* infection in southern Europe. *Trans R Soc Trop Med Hyg.* (2002) Mar-Apr; 96(2): 129-32.

Moravej A, Rasouli M, Kalani M, Asaei S, Kiany S, Najafipour S, et al. IL-1beta (-511T/C) gene polymorphism not IL-1beta (+3953T/C) and LT-alpha (+252A/G) gene variants confers susceptibility to visceral leishmaniasis. *Mol Biol Rep* (2012); 39(6): 6907–14.

MORSIANI M, and SOFFRITTI E. Modification of Hematopoiesis during Acute Experimental Intoxification with 6-Aininonicotinamide. *Boll. Soc. Ematologia,* (1956); 4:13-

15.

Motazedian M, Fakhar M, Motazedian MH, Hatam G, Mikaeli F. A urine-based polymerase chain reaction method for the diagnosis of visceral leishmaniasis in immunocompetent patients. *Diag Microbiol Infect Dis.* (2008); 60(2): 151–4.

Mottram C J., and Coombs H G. *Leishmania mexicana*: Subcellular distribution of enzymes in amastigotes and promastigotes. *Experimental Parasitology.* (1985) June; Volume 59, Issue 3, Pages 265-274.

Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* (1989); 7: 445–480.

Murphy KP, Travers P, Walport M, Janeway C. Janeway's immunobiology. 7. New York: Garland Science; 2008.

Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. *Lancet,* (2005); 366:1561-1577.

Murray HW, Hariprashad J. Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. *J Exp Med.* (1995); 181(1):387–391.

Murugaiyan G, Mittal A, Lopez-Diego R, Maier LM, Anderson DE, Weiner HL. IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J Immunol* (2009) 183: 2435–43.

Mwenechanya R, Kovářová J, Dickens NJ, Mudaliar M, Herzyk P, Vincent IM, Weidt SK, Burgess KE, Burchmore RJS, Pountain AW, Smith TK, Creek DJ, Kim DH, Lepesheva GI, Barrett MP. Sterol 14 α -demethylase mutation leads to amphotericin B resistance in *Leishmania mexicana*. *PLoS Negl Trop Dis,* Jun. (2017); 16:11(6):e0005649.

Nahrendorf M, Hu K, Frantz S, Jaffer FA, Tung CH, Hiller KH, Voll S, Nordbeck P, Sosnovik D, Gattenlohner S, Novikov M, Dickneite G, Reed GL, Jakob P, Rosenzweig A, Bauer WR, Weissleder R, Ertl G. Factor XIII deficiency causes cardiac rupture, impairs wound healing, and aggravates cardiac remodeling in mice with myocardial infarction. *Circulation.* (2006); 113: 1196–1202.

Nandy, A. Addy, M. maji, A. K. Guha, S. K. Banerjee, D. and Chaudhuri, D. Recurrence of kala azar after PKDL: Role of co-factors. *Tropical Medicine and International Health.* (1998); 3, P: 76-78.

Nicoll WS, Sacci JB, Rodolfo C, Di Giacomo G, Piacentini M, Holland ZJ, Doerig C, Hollingdale MR, Lanar DE. *Plasmodium falciparum* liver stage antigen-1 is cross-linked by tissue transglutaminase. *Malar J.* (2011) Jan 21; 10: 14.

Nicolle Ch, Blaizot C. Virulence des cultures de *Leishmania infantum*. Sensibilite du chacal au kala azar tunisien. *Bull Soc Path Exot* (1911); 5: 721-723.

Nieto A, Domínguez-Bernal G, Orden JA, De La Fuente R, Madrid-Elena N, Carrión J. Mechanisms of resistance and susceptibility to experimental visceral leishmaniasis: BALB/c mouse versus Syrian hamster model. *Vet Res.* (2011) Feb 23; 39.

Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, Saigo K, Morinobu A, Koshiba M, Kuntz KM, Kamae I, Kumagai S. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Ann Intern Med.* (2007) Jun 5; 146(11): 797-808.

Nonaka Masahiko, Tanaka Haruo, Okiyama Atsusi, Motoki Masao, Ando Hiroyasu, Umeda Koichi & Matsuura Akira. Polymerization of Several Proteins by Calcium-Independent Transglutaminase Derived from Microorganisms, *Agricultural and Biological Chemistry.* (1989); 53:10, 2619-2623.

North Chicago, IL, USA: Abbott Laboratories; 2009. Humira (adalimumab) injection, solution [prescribing information].

Okwor I, Uzonna JE. The immunology of *leishmania*/HIV coinfection. *Immunol Res.* (2013); 56: 163-171.

Olliaro P, Sudar S. Anthropometrically derived dosing and drug costing calculations for treating visceral leishmaniasis in Bihar, India. *Trop. Med. Int. Health.* (2009); 14: 88-92.

Olszewski et al. TNF Trafficking to Human Mast Cell Granules: Mature Chain-Dependent Endocytosis. *The Journal of Immunology.* (2007); 178: 5701–5709.

Opperdoes FR, Borst P. Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome. *FEBS Lett.* (1977); 80:360–364.

Osman OF. Visceral Leishmaniasis: the PCR and Direct Agglutination Test for Diagnosis and Management. PhD Thesis, University of Amsterdam, The Netherlands, (1998).

Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, et al. Incidence of Symptomatic and Asymptomatic *Leishmania donovani* Infections in High-Endemic Foci in India and Nepal: A Prospective Study. *PLoS Negl Trop Dis.* (2011); 5: e1284.

Ouaissi M, Ouaissi A J. Histone Deacetylase Enzymes as Potential Drug Targets in Cancer and Parasitic Diseases. *J Biomed Biotech.* (2006); 2006(2): 13474.

Oz M, Lorke DE, Hasan M, Petroianu GA. Cellular and Molecular Actions of Methylene Blue in the Nervous System. *Med Res Rev.* (2011); 31(1): 93-117.

Ozon C, Marty P, Pratlong F, Breton C, Blein M, Lelièvre A, Haas P. Disseminated feline leishmaniasis due to *Leishmania infantum* in Southern France. *Vet Parasitol.* (1998) Feb 28; 75 (2-3): 273-7.

Pampiglione S, La Placa M, Schlick G. Studies on mediterranean Leishmaniasis. I. An outbreak of visceral leishmaniasis in Northern Italy. *Trans R Soc Trop Med Hyg.* (1974); 68(5): 349-59.

Pasternack R, Dorsch S, Otterbach JT, Robenek IR, Wolf S, Fuchsbaue HL. Bacterial pro-transglutaminase from *Streptovorticillium mobaraense*--purification,

characterisation and sequence of the zymogen. *Eur J Biochem.* Nov. (1998); 1:257(3):570-6.

Pavli A, Maltezos HC. Leishmaniasis, an emerging infection in travelers. *Int. J. Infect. Dis.* (2010); 14(12), e1032-e1039.

Pedersen BK, Febbraio MA. "Muscle as an endocrine organ: focus on muscle-derived interleukin-6". *Physiological Reviews.* (2008) Oct; 88 (4): 1379–406.

Perlia CP, Kofman S, Sky-Peck H, Taylor SG. Clinical use of 6-aminonicotinamide in patients with disseminated neoplastic disease. *Cancer* (1961); 14(3): 644-648.

Peruhype-Magalhaes, V., Martins-Filho, O.A., et al., Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor- α (+) monocytes are hallmarks of active human visceral Leishmaniasis due to *Leishmania chagasi* infection. *Clin. Exp. Immunol.* 2006;146, 124–132

Peters N. C., Egen J. G., Secundino N., Debrabant A., Kimblin N., Kamhawi S., Lawyer P., Fay M. P., Germain R. N., Sacks D. *In vivo* imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science.* (2008); 321, 970–974.

Pigott DM, Bhatt S, Golding N, et al. Global distribution maps of the leishmaniases. *Elife,* (2014); 3.

Pisano JJ, Finlayson JS, Peyton MP. Cross-link in fibrin polymerized by factor 13: epsilon-(gamma-glutamyl)lysine. *Science.* (1968) May 24; 160(3830): 892-3.

Pitta MG, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, Argiro L, el Kheir M, Bucheton B, Mary C, El-Safi SH, Dessein A. IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. *J Clin Invest.* (2009) Aug; 119(8): 2379-87.

Plenge RM. Rheumatoid arthritis genetics: 2009 update. *Curr Rheumatol Rep.* (2009) Oct; 11(5): 351-6.

Poepl W, Herkner H, Tobudic S, Faas A, Auer H, Mooseder G, et al. Seroprevalence and asymptomatic carriage of *Leishmania spp.* in Austria, a non-endemic European country. *Clin Microbiol Infect.* (2013) Jun; 19(6): 572-7.

Poteet E, Winters A, Yan LJ, Shufelt K, Green KN, Simpkins JW, et al. Neuroprotective actions of Methylene Blue and its derivatives. *PLoS ONE.* (2012); 7(10): e48279.

Pozio E, Gradoni L, and Gramiccia M. La leishmaniose canine en Italie de 1910 a 1983. *Ann. Parasitol. Hum. Comp.* (1985); 60: 543–553.

Pratesi F, Dioni I, Tommasi C, et al. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis.* (2013) Jul; 73(7): 1414-22.

Puig L, Pradinaud R. *Leishmania* and HIV co-infection: dermatological manifestations. *Ann. Trop. Med. Parasitol.* (2003); 97 (Suppl. 1), 107-114.

PULLMAN B, PULLMAN A. On the 6-aminonicotinamide antagonism of DPN-dependent enzymatic systems. *Cancer Res.* (1959) Apr; 19(3 Pt 1): 337-8.

Quirke AM, Lugli EB, Wegner N, Hamilton BC, Charles P, Chowdhury M, Ytterberg AJ, Zubarev RA, Potempa J, Culshaw S, Guo Y, Fisher BA, Thiele G, Mikuls TR, Venables PJ. Heightened immune response to autocitrullinated *Porphyromonas gingivalis* peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. *Ann Rheum Dis.* (2014) Jan; 73(1): 263-9.

Rahman P, Elder JT. Genetic epidemiology of psoriasis and psoriatic arthritis. *Ann Rheum Dis.* (2005); 64 (suppl II): ii37–9; discussion ii40-1.

Rakotomanga M, Blanc S, Gaudin K, Chaminade P, Loiseau P.M. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. *Antimicrob. Agents Chemother.* (2007), 51(4), 1425-1430.

Ramautar R, de Jong GJ. Recent Developments in Liquid-Phase Separation Techniques for Metabolomics. *Bioanalysis.* (2014); 6: 1011–1026.

Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum.* (2003) Oct; 48(10): 2741-9.

Rao RU, Mehta K. Transglutaminases, thioredoxins and protein disulphide isomerase: diverse enzymes with a common goal of cross-linking proteins in lower organisms. *Indian J Exp Biol.* (2004) Mar; 42(3): 235-43.

Raza K, Falciani F, Curnow SJ, et al. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis Res Ther.* (2005); 7(4): R784–95.

Ready PD. Biology of phlebotomine sandflies as vectors of disease agents. *Annu Rev Entomol.* (2013); 58: 227–250.

Ready PD. Leishmaniasis emergence in Europe. *Euro Surveill.* (2010); 15(10): 19505.

Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *Lancet Infect Dis.* (2007) Sep; 7 (9): 581-96.

Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. *J Clin Microbiol.* (2007) Jan; 45(1): 21-5.

Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. *Trans R Soc Trop Med Hyg.* (2004) Feb; 98(2): 102-10.

Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol.* (2002) May 1; 168(9): 4620-7.

Robert HG, Asish KS, Siddhartha D, and Alan TR. Biochemistry of the *Leishmania* Species. *MICROBIOLOGICAL REVIEWS.* (1988) Dec; p. 412-432.

Roessner U, Bowne J. What is metabolomics all about?. *Biotechniques*. (2009) Apr; 46 (5): 363-5.

Rogers ME, Chance ML, Bates PA. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. *Parasitology*. (2002); 124: 495–508.

ROMANÍ-COSTA V, SÁNCHEZ C, MOYÁ F, ESTANY C. Leishmaniasis visceral con relación a la administración de infliximab. *En-ferm Infec Microbiol Clin*. (2004); 22: 310.

Ross R. Note on the bodies recently described by Leishman and Donovan. *British Medical Journal*. (1903) 2, P: 1261-2.

Ruda GF, Wong PE, Alibu VP, Norval S, Read KD, Barrett MP, Gilbert IH. Aryl phosphoramidates of 5-phospho erythronhydroxamic acid, a new class of potent trypanocidal compounds. *J Med Chem*. (2010) Aug 26; 53(16): 6071-8.

Sacks DL, Perkins PV. Identification of an infective stage of *Leishmania* promastigotes. *Science*. (1984); 223, P: 1417-9.

Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis. *J Vector Borne Dis*. (2016) Jan-Mar; 53(1): 8-16.

Salotra P, Sreenivas G, Poque GP. Development of a species- specific PCR assay for detection of *leishmania donovani* in clinical samples from patients with Kala-azar and Post-Kala-azar dermal leishmaniasis. *Journal of Clinical Microbiology*. (2001); 39, P: 849-54.

Santarém N, Cordeiro-da-Silva A. [Diagnosis of visceral leishmaniasis ©FORMATEX, \(2007\).](#)

Satchwell TJ, Shoemark DK, Sessions RB, Tøye AM. Protein 4.2: a complex linker. *Blood Cells Mol Dis*. (2009); 42: 201–210.

Schaefer KU, Kurtzhals JAL, Gachihi GS, Muller AS, Kager PA. A prospective sero-epidemiological study of visceral leishmaniasis in Baringo district, Rift Valley Province, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. (1995); 89(5): 471–475.

Schallig HD, Oskam L. Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. *Trop Med Int Health*. (2002) Aug; 7(8): 641-51.

Schellekens GA, de Jong BA, van den Hoogen FH, et al. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest*. (1998); 101: 273 – 281.

Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum*. (2000) Jan; 43(1): 155-63.

- Schlein Y, Warburg A, Schnur LF, Le Blancq SM, Gunders AE.** Leishmaniasis in Israel: reservoir hosts, sandfly vectors and *leishmanial* strains in the Negev, Central Arava and along the Dead Sea. *Trans R Soc Trop Med Hyg.* (1984); 78(4): 480-4.
- Schönian G, Kuhls K, & Mauricio I.** Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. *Parasitol.* (2011); 138, 405–425.
- Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, Jaffe CL.** PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis.* (2003); 47(1): 349-58.
- Schuerwegh AJ, Ioan-Facsinay A, Dorjée AL, et al.** Evidence for a functional role of IgE anticitrullinated protein antibodies in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* (2010); 107: 2586 – 2591.
- Scott DL, Kingsley GH.** Tumor necrosis factor inhibitors for rheumatoid arthritis. *N Engl J Med.* (2006); 355: 704-12.
- Seaman J, Mercer AJ, Sondorp HE, and Herwaldt BL.** Epidemic visceral leishmaniasis in Southern Sudan: treatment of severely debilitated patients under wartime conditions with limited resources. *Annual International Medicine.* (1996); 124, P: 664-672.
- Sehgal G, Zhang K, Todd RF 3rd, Boxer LA, Petty HR.** Lectin-like inhibition of immune complex receptor-mediated stimulation of neutrophils. Effects on cytosolic calcium release and superoxide production. *J Immunol.* (1993); May 15; 150(10): 4571-80.
- Sengupta PC.** History of kala-azar in India. *Indian medical gazette.* (1947); 82, P: 281-6.
- Sereno D, Monte Alegre A, Silvestre R, Vergnes B, Ouaiissi A.** *In vitro* antileishmanial activity of nicotinamide. *Antimicrob Agents Chemother.* (2005); 49: 808-812.
- Sereno D, Vergnes B, Mathieu-Daude F, Cordeiro da Silva A, Ouaiissi A.** Looking for putative functions of the *Leishmania* cytosolic SIR2 deacetylase. *Parasitol Res.* (2006); 100:1-9.
- Shahrara S, Huang Q, Mandelin AM, Pope RM.** TH-17 cells in rheumatoid arthritis. *Arthritis Res Ther.* (2008); 10: R93.
- Sheffeld VC, and Seegmiller RE.** *J. Embryol. Exp. Morph.* (1980); 59, 217.
- Shi J, van de Stadt LA, Levarht EW, et al.** Anticarbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. *Arthritis Rheum.* (2013); 65: 911 – 915.
- Shoda H, Fujio K, Shibuya M, et al.** Detection of autoantibodies to citrullinated BiP in rheumatoid arthritis patients and pro-inflammatory role of citrullinated BiP in collagen-induced arthritis. *Arthritis Res Ther.* (2011); 13: R191.
- Signorini M, Cassini R, Drigo M, Frangipane di Regalbano A, Pietrobelli M, Montarsi F, Stensgaard AS.** Ecological niche model of *Phlebotomus perniciosus*, the main vector of canine leishmaniasis in northeastern Italy. *Geospat Health.* (2014); 9: 193-201.

Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res.* (2002); Suppl 3: S265–S272.

Singh OP, Sundar S. Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects. *Frontiers in immunology.* (2014); 5: 296.

Singh RN, Mehta K. Purification and characterization of a novel transglutaminase from filarial nematode *Brugia malayi*. *Eur J Biochem.* (1994) Oct 15; 225(2): 625-34.

Singh S, Dey A, and Sivakumar R. Applications of molecular methods for *Leishmania* control. *Exp. Rev. Mol. Diagn.* (2005); 5, 251-265.

Singh S, Mohapatra DP, Sivakumar R. Successful replacement of foetal calf serum with human urine for *in vitro* culture of *Leishmania donovani*. *Journal of Community Diseases.* (2000), 32, P: 289-94.

Singh SP, Reddy DC, Rai M, & Sundar S. Serious underreporting of viscera leishmaniasis through passive case reporting in Bihar, India. *Tropical Medicine and International Health.* (2006); 11, P: 899–905.

Sinha PK, Jha TK, Thakur CP, Nath D, Mukherjee S, Aditya AK, Sundar S. Phase 4 pharmacovigilance trial of paromomycin injection for the treatment of visceral leishmaniasis in India. *J Trop Med.* (2011); 645203.

Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching and identification. *Anal Chem.* (2006); 78: 779–787.

Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. *Lancet.* (2007); 370: 1861-74.

Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. *Nat Rev Drug Discov.* (2003); 2: 473-88.

Smyrna GA, USA: UCB, Inc.; 2009. Cimzia® (certolizumab pegol) [prescribing information].

Snir O, Widhe M, Hermansson M, et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. *Arthritis Rheum.* (2010); 62: 44 – 52.

Spriggs DR, Deutsch S, Kufe DW. Genomic structure, induction, and production of TNF-alpha. *Immunol Ser.* (1992); 56: 3–34.

Srivastava L, Chakravarty AK, Kumar A, Kumar P. Comparison of Elisa and Indirect immunofluorescence in sero-epidemiology of Kala-azar. *Indian Journal of Medical Microbiology.* (1984) June; 79, P: 744-8.

Srivastava P, Gidwani K, Picado A, Van der Auwera G, Tiwary P, et al. Molecular and serological markers of *Leishmania donovani* infection in healthy individuals from endemic areas of Bihar, India. *Trop Med Int Health.* (2013) May; 18 (5): 548-54.

Srivastava Saumya, Prem Shankar, Jyotsna Mishra and Sarman Singh Possibilities and challenges for developing a successful vaccine for leishmaniasis *Parasites & Vectors*. (2016); 9: 277.

Srividya G, Kulshrestha A, Singh R, Salotra P. Diagnosis of visceral leishmaniasis: Developments over the last decade. *Parasitol Res.* (2012); 110: 1065–78.

Stauch A, Sarkar RR, Picado A, et al. Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. *PLoS Negl Trop Dis.* (2011) Nov; 5(11): e1405.

Sternbergs S, and Philips FS. 6-Aminonicotinamide and Acute Degenerative Changes in the Central Nervous System. *Science.* (1958); 127:644-46.

Stockholm, Sweden: Biovitrum AB; 2009. Kineret 100 mg solution for injection [summary of product characteristics].

Street JC, Mahmood U, Ballon D, Alfieri AA, Koutcher JA. ¹³C and ³¹P NMR investigation of effect of 6-aminonicotinamide on metabolism of RIF-1 tumor Cells *in Vitro*. *J Biol Chem.* (1996); 271(8): 4113-4119.

Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin AC et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics.* (2007); 3(3): 211–221.

Sundar S, and Rai M. Laboratory Diagnosis of Visceral Leishmaniasis. *Clinical and diagnostic laboratory immunology.* (2002); P: 951-958.

Sundar S, Chatterjee M. Visceral leishmaniasis - current therapeutic modalities. *Indian. J. Med. Res.* (2006); 123(3), 345-352.

Sundar S, Mehta H, Chhabra A, Singh V, Chauhan V, Desjeux P, Rai M. Amphotericin B colloidal dispersion for the treatment of Indian visceral leishmaniasis. *Clin. Infect. Dis.* (2006); 42(5), 608-613.

Sundar S, Reed SG, Singh VP, Kumar PCK, and Murray HW. Rapid accurate field diagnosis of visceral leishmaniasis. *Lancet*, (1998); 351: 563-565.

Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, Boelaert M, Dujardin JC, Chakravarty J. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis.* (2012); 55: 543-550.

Suzuki A, Yamada R, Chang X, Tokuhira S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet.* (2003); 34: 395– 402.

Symmers, W.S.C. Leishmaniasis acquired by contagion, a case of marital infection in Britain. *Lancet.* (1960); v. 16, p.127-132.

Symmons DP, Bankhead CR, Harrison BJ, et al. Blood transfusion, smoking, and obesity as risk factors for the development of rheumatoid arthritis: results from a primary care-based incident case-control study in Norfolk, England. *Arthritis Rheum.* (1997); 40: 1955-61.

Takahashi N, Takahashi Y, & Putnam FW. Primary structure of blood coagulation factor XIIIa (fibrinolygase, transglutaminase) from human placenta, *Proc. Natl Acad. Sci.* (1986); USA 83: 8 0 1 9 2 8023.

Tautenhahn R, Boettcher C, Neumann S. Highly sensitive feature detection for high resolution LC/MS. *BMC Bioinformatics.* (2008); 9: 504.

TEKTONIDOU MG, SKOPOULI FN: Visceral leishmaniasis in a patient with psoriatic arthritis treated with infliximab: reactivation of a latent infection? *Clin Rheumatol.* (2007) Oct 26; [Epub ahead of print].

Thakur CP. Socioeconomics of visceral leishmaniasis in Bihar (India). *Transactions of the Royal Society of Tropical Medicine and Hygiene.* (2000); 94, P: 156–157.

Theiss AL, Simmons JG, Jobin C, Lund PK. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. *J Biol Chem.* (2005) Oct 28; 280(43): 36099-109.

Thomas H, Beck K, Adamczyk M, Aeschlimann P, Langley M, Oita RC, Thiebach L, Hils M, Aeschlimann D. Transglutaminase 6: a protein associated with central nervous system development and motor function. *Amino Acids.* (2013); 44: 161–177.

Thousand Oaks, CA, USA: Amgen Inc. and Wyeth Pharmaceuticals; 2009. Enbrel® (etanercept) for subcutaneous injection [prescribing information].

Tovar J, Cunningham ML, Smith AC, Croft SL, Fairlamb AH. Down-regulation of *Leishmania donovani* trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival. *Proc. Natl. Acad. Sci.* (1998); USA 95, 5311–5316.

Towbin H, et al. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U S A.* (1979); 76: 4350-4354.

Tretter L, Horvath G, Holgyesi A, Essek F, Adam-Vizi V. Enhanced hydrogen peroxide generation accompanies the beneficial bioenergetic effects of methylene blue in isolated brain mitochondria. *Free Radic Biol Med.* (2014); 77: 317-330.

Tsai YH, Lai WF, Wu YW, Johnson LR. Two distinct classes of rat intestinal mucosal enzymes incorporating putrescine into protein. *FEBS Lett, Sep.* (1998); 18: 435(2-3): 251-6.

Tsuchiya T, Matuoka A, Sekita S, Hisano T, Takahashi A, Ishidate MJr. Human embryonic cell growth assay for teratogens with or without metabolic activation system using microplate. *Teratog Carcinog Mutagen.* (1988); 8: 265-272.

Ueda K, Fukushima M, Okayama H, Hayaishi O. Nicotinamide adenine dinucleotide glycohydrolase from rat liver nuclei. *J Biol Chem.* (1975); 250: 7541-7546.

Upchurch HF, Conway E, Patterson MK Jr, Birckbichler PJ, Maxwell MD. Cellular transglutaminase has affinity for extracellular matrix. *In Vitro Cell Dev Biol.* (1987) Nov; 23(11): 795-800.

Uxbridge, UK: Bristol-Meyers Squibb Pharma EEIG; 2009. Orenzia® 250 mg powder for concentrate for solution for infusion [summary of product characteristics].

van der Poll T, Keogh CV, Guirao X, Buurman WA, Kopf M, Lowry SF. "Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia". *The Journal of Infectious Diseases*. (1997) Aug; 176 (2): 439–44.

van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. *Mol Biochem Parasitol*. (1992) Mar; 51(1): 133-42.

van Gaalen FA, van Aken J, Huizinga TW, Schreuder GM, Breedveld FC, Zanelli E, van Venrooij WJ, Verweij CL, Toes RE, de Vries RR. Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis. *Arthritis Rheum*. (2004) Jul; 50(7): 2113-21.

van Griensven J, Carrillo E., Lopez-Velez R., Lynen L., Moreno J. Leishmaniasis in immunosuppressed individuals. *Clin Microbiol Infect*. (2014); 20: 286–299.

Van Hellemond JJ, Tielens AGM. Inhibition of the respiratory chain results in a reversible metabolic arrest in *Leishmania* promastigotes. *Mol Biochem Parasitol*. (1997); 85:135-138.

van Kuijk AW, Reinders-Blankert P, Smeets TJ, Dijkmans BA, Tak PP. Detailed analysis of the cell infiltrate and the expression of mediators of synovial inflammation and joint destruction in the synovium of patients with psoriatic arthritis: implications for treatment. *Ann Rheum Dis*. (2006); 65: 1551–1557.

van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, Laskay T. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J. Immunol*. (2004); 173, 6521–6525.

Varani S, Cagarelli R, Melchionda F, Attard L, Salvadori C, Finarelli AC, Gentilomi GA, Tigani AR, Rangoni R, Todeschini R, Scalone A, Di Muccio T, Gramiccia M, Gradoni L, Viale P, Landini MP. Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. *Rapid Communications*. www.eurosurveillance.org.

Varnes ME. Inhibition of pentose cycle of A549 cells by 6-aminonicotinamide: consequences for aerobic and hypoxic radiation response and for radiosensitizer action. *NCI Monogr*. (1988); (6): 199-203.

Veitch NJ, Maugeri DA, Cazzulo JJ, Lindqvist Y, Barrett MP. Transketolase from *Leishmania mexicana* has a dual subcellular localization. *Biochem J*. (2004) Sep 1; 382(Pt 2): 759-67.

Vergnes B, Sereno D, Madjidian-sereno N, Lemesre JL, Ouaisi A. Cytoplasmic SIR2 homologue overexpression promotes survival of *Leishmania* parasites by preventing programmed cell death. *Gene*. (2002); 296:139-150.

Vincent IM, Barrett MP. Metabolomic-based strategies for anti-parasite drug discovery. *J Biomol Screen*. (2015) Jan; 20(1): 44-55.

Volpe E, Servant N, Zollinger R, et al. A critical function for transforming growth factor-beta, interleukin-23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol.* (2008); 9: 650-7.

von Stebut E, Belkaid Y, Jakob T, Sacks DL, Udey MC. Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-*Leishmania* immunity. *J Exp Med.* (1998) Oct 19; 188 (8): 1547-52.

Vossenaar ER, van Venrooij WJ. Citrullinated proteins: sparks that may ignite the fire in rheumatoid arthritis. *Arthritis Res Ther.* (2004); 6(3): 107-11.

Vossenaar ER, Zendman AJW, van Venrooij WJ, Pruijn G. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays.* (2003); 25: 1106–1118.

Wallis RS, Broder M, Wong J, Beenhouwer D. Granulomatous infections due to tumor necrosis factor blockade: correction. *Clin Infect Dis.* (2004); 39: 1254-5.

Washizu K, Ando K, Koikeda S, Hirose S, Matsuura A, Akagi H, Motoki M, Takeuchi K Molecular cloning of the gene for microbial transglutaminase from *Streptovorticillium* and its expression in *Streptomyces lividans*. *Biosci Biotechnol Biochem.* (1994); 58: 82–87.

Weber A, Wasiliew P, Kracht M. "Interleukin-1 (IL-1) pathway". *Sci Signal.* (2010); 3 (105): cm1.

Wegner N, Wait R, Sroka A, et al. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α -enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* (2010); 62: 2662-72.

Welwyn Garden City, UK: Roche Registration Ltd; 2008. MabThera 100 mg (10mg/ml) concentrate for solution for infusion [summary of product characteristics].

Welwyn Garden City, UK: Roche Registration Ltd; 2009. RoActemra 20 mg/ml concentrate for solution for infusion [summary of product characteristics].

Westrop GD, Williams RA, Wang L, Zhang T, Watson DG, Silva AM, Coombs GH. Metabolomic Analyses of *Leishmania* Reveal Multiple Species Differences and Large Differences in Amino Acid Metabolism. *PLoS One.* (2015); Sep 14; 10(9): e0136891.

Wherry EJ. T cell exhaustion. *Nat Immunol.* (2011); 12: 492–499.

WHO. Control of leishmaniasis. Report of a WHO Expert Committee. Geneva. (1990). Available at http://apps.who.int/iris/bitstream/10665/39337/1/WHO_TRS_793.pdf

WHO. Burden and distribution. Website. <http://www.who.int/leishmaniasis/burden/en/>. Accessed November 10, (2015).

WHO. CONTROL OF THE LEISHMANIASES. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, Geneva, 22–26 March (2010). Available at http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf

WHO. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, Geneva, March 22–26, 2010. *World Health Organ Tech Rep Ser.* (2010); 949: 1–186. Available at http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf.

WHO. Leishmania/HIV co-infection - Epidemiological analysis of 692 retrospective cases. *Weekly Epidemiological Record*, (1997), Vol 72, 8 (pp 49-56). Available at <http://www.who.int/docstore/wer/pdf/1997/wer7208.pdf?ua=1>.

WHO. Leishmaniasis fact sheet, September, (2016). <http://www.who.int/mediacentre/factsheets/fs375/en/>.

WHO. *Manual on visceral leishmaniasis control.* (1996), Geneva, Switzerland. Available at http://apps.who.int/iris/bitstream/10665/63637/1/WHO_LEISH_96.40.pdf.

WHO. Media Centre. Leishmaniasis. Fact sheet, updated September (2016). www.who.int/mediacentre/factsheet/fs375/en/.

WHO. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, Geneva, 22–26 March 2010. Available at http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf.

WHO. Status Endemicity of cutaneous and visceral leishmaniasis worldwide, 2013. Available at http://www.who.int/leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL_CL_2013.pdf?ua=1.

WHO. Sustaining the drive to overcome the global impact of neglected tropical diseases: Second WHO report on neglected tropical diseases, (2013). [http://www.who.int/neglected_diseases/9789241564540/en/].

Wieder T, Reutter W, Orfanos CE, Geilen CC. Mechanisms of action of phospholipid analogs as anticancer compounds. *Prog. Lipid Res.* (1999); 38(3), 249-259.

Winchester R, Minevich G, Kane D, Bresnihan B, Greenberg D, FitzGerald O. Heterogeneity of the psoriasis phenotype revealed by HLA class I haplotype associations in psoriatic arthritis and psoriasis. *Clin Immunol.* (2008); 127(Suppl 1): S88–S89.

WOLF A, and COWEN D. Pathological Changes in the Central Nervous System Produced by 6-Aminonicotinamide. *Bull. N.Y. Acad. Med.* (1959); 36:814-17.

Wolfe F, Michaud K, Anderson J, Urbansky K. Tuberculosis infection in patients with rheumatoid arthritis and the effect of infliximab therapy. *Arthritis Rheum.* (2004); 50: 372-9.

Wrenger C, Shetter I, Liebau E. Oxidative stress in human infectious diseases – present and current knowledge about its druggability. *Intech.* (2013); 228, <http://dx.doi.org/10.5772/53758>.

Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. *J. Biol. Chem.* (2004); 279(38), 39925-39932.

- Xia J, Sinelnikov IV, Han B, Wishart DS.** MetaboAnalyst 3.0 - making metabolomics more meaningful. *Nucleic Acids Res.* (2015); 43: W251-W257.
- Xynos ID, Tektonidou MG, Pikazis D, Sipsas NV.** Leishmaniasis, autoimmune rheumatic disease, and anti-tumor necrosis factor therapy, *Europe. Emerg Infect Dis.* (2009) Jun; 15(6): 956-9.
- Yang L, Anderson DE, Baecher-Allan C, et al.** IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature.* (2008); 454: 350-2.
- Zackheim HS.** Topical 6-aminonicotinamide plus oral niacinamide therapy for psoriasis. *Arch Dermatol.* (1978); 114(11) 1632-1638.
- Zackheim HS.** Treatment of psoriasis with 6-aminonicotinamide. *Arch Dermatol.* (1975) Jul; 111(7): 880-2.
- Zanger P, Gabrysch S.** Leishmaniasis in the era of tumor necrosis factor alpha antagonist therapy--a research agenda for Europe. *Euro Surveill.* (2013) Jul 25; 18(30): 20542.
- Zanger P, Kötter I, Kremsner PG, Gabrysch S.** Tumor necrosis factor alpha antagonist drugs and leishmaniasis in Europe. *Clin Microbiol Infect.* (2012) Jul; 18(7): 670-6.
- Zatman LJ, Kaplan NO, Colowick SP, Ciotti MM.** The isolation and properties of the isonicotinic acid hydrazide analogue of diphosphopyridine nucleotide. *J Biol Chem.* (1954); 209: 467-484.
- Zatman LJ, Kaplan NV, Colowick SP.** Inhibition of spleen diphosphopyridine nucleotidase by nicotinamide, an exchange reaction. *J Biol Chem.* (1953); 200: 197-212.
- Zhao GC, Zhu JJ, Chen HY.** Spectroscopic studies of the interactive model of methylene blue with DNA by means of β -cyclodextrin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.* (1999); 55: 1109-1117.
- Zhu J, Yamane H, and Paul WE.** "Differentiation of effector CD4+ T cell populations. *Annual Review of Immunology.* (2010); vol. 28, pp. 445–489.
- Zijlstra EE et al.** Kala-azar in displaced people from southern Sudan: epidemiological, clinical and therapeutic findings. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* (1991); 85, P: 365–369.
- Zijlstra EE et al.** rK39 enzyme-linked immunosorbent assay for diagnosis of *Leishmania donovani* infection. *Clinical Diagnosis Laboratory Immunology.* (1998); 5, P: 717–720.
- Zijlstra EE, el-Hassan AM, Ismael A, & Ghalib HW.** Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *American Journal of Tropical Medicine and Hygiene.* (1994); 51, P: 826–836.
- Zijlstra EE, El-Hassan AM, Ismael A, Ghalib HW.** Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *American Journal of Tropical Medicine and Hygiene.* (1994); 51(6): 826–836.

Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, & el-Hassan AM. Post-kala-azar dermal leishmaniasis. *Lancet Infectious Diseases*. (2003); 3, P: 87–98.

Zijlstra EE, Nur Y, Desjeux P, el-Hassan EAM, and Groen J. Diagnosing visceral leishmaniasis with the recombinant k39 strip test: experience from the *Sudan*. *Tropical Medicine and International Health*. (2001); 6, P: 108-113.

Zink AR, Spigelman M, Schraut B, Greenblatt CL, Nerlich AG, Donoghue HD. Leishmaniasis in ancient Egypt and Upper Nubia. *Emerg. Infect. Dis.* (2006); 12(10), 1616-1617.

Zwingenberger K, Harms G, Pedrosa C, Omena S, Sandkamp B, & Neifer S. Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon- γ production. *Clinical Immunology and Immunopathology*. (1990); 57: 242-249.