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LEISHMANIASIS IN THE IMMUNOCOMPROMISED POPULATION: EVALUATION OF STRATEGIES FOR SCREENING AND MONITORING

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Abstract

Leishmaniasis is a vector-borne disease caused by an intracellular parasite of the genus *Leishmania.*

Among parasitic diseases, human leishmaniasis is second in mortality only to malaria, and due to its severity and its spread in a number of countries, it is considered by World Health Organization (WHO) as a major public health problem.

The clinical features of leishmaniasis include a wide range of manifestation from asymptomatic infections to different levels of disease severity, depending on the species of *Leishmania* involved and on the immune response of the host. *Leishmania* infection can present with six different clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and mucosal leishmaniasis (ML).

The public health impact of leishmaniasis is underestimated in Europe. The risk of emergence of this vector-borne disease in Europe could be a consequence of human intervention on the environment, climate change, immigration and elevated number of immunocompromised individuals. In Italy human leishmaniasis is present in different epidemiological and clinical forms, and in last years the increase of temperature is leading towards the diffusion of sand flies and consequently VL into regions of northern Italy.

A striking example of increase of leishmaniasis cases in northern Italy is represented by the VL outbreak that developed in the province of Bologna in 2012-2013. Following this outbreak, we also detected a high prevalence of asymptomatic *Leishmania* infection in a selected municipality of the Bologna province, which is important considering the risk of reactivation in immunocompromised patients.

In fact, in most cases individuals do not develop clinical symptoms, but *Leishmania* parasites can persist lifelong in the host after an acute infection and easily reactivate under immunosuppressive conditions, causing severe disease with high mortality rate.

The aim of this study was to identify *Leishmania* infection in selected groups of immunocompromised (IC) patients, including newly diagnosed HIV infected individuals, patients receiving kidney transplant (KT) and patients undergoing immunosuppressive therapies for immune-mediated diseases (IMD).
To date, there is no gold standard test to identify cryptic leishmaniasis, and it is difficult to understand if seropositive individuals are infected or it was a prior infection. On the other hand, parasite load can be low and molecular methods can not be used alone to identify asymptomatically infected individuals.
For this reason, our study focused on the validation of a combination of methods to be used for the screening of asymptomatic *Leishmania* infection. The selected methods included high sensitive Real-Time PCR for detection of parasitic kinetoplast (k)DNA in peripheral blood, Western Blot (WB) for detection of specific IgG and Whole Blood Assay (WBA) to evaluate the anti-leishmanial T-cell response by quantifying the production of IFN-γ, IL-2 and IP-10 after stimulation of patients' blood with *Leishmania* specific antigens.

The methods have been validated on a cohort of immunocompetent individuals living in an endemic area of the Bologna province. Among 145 individuals recruited and screened with WB, Real-Time PCR and WBA, 24 subjects tested positive (17%) to one or more methods, thus confirming the high circulation of the parasite in the selected area.

After the set-up of methods on immunocompetent individuals, we screened for *Leishmania* infection 198 immunocompromised patients included in the abovementioned groups. We considered as *Leishmania*-positive each immunocompromised patient that tested positive to at least one test, including WB, Real-Time PCR and/or WBA. Sixty-two out of 198 immunocompromised patients (31%) tested positive to one or more screening tests. The *Leishmania*-positive patients were included in a 1-year follow up by performing Real-Time PCR and WBA every three months.

Given the high prevalence of asymptomatic infection in immunocompromised patients in endemic regions such as Italy, it seems essential to develop a plan for screening and follow-up of *Leishmania* infection. The screening algorithm that we tested in this study appears to be effective to identify accurately quiescent parasitic infection. The study is ongoing to evaluate markers (such as parasitic load and/or production of Th1 soluble factors upon stimulation with leishmanial antigens) that could predict the progress of the disease.

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1. Introduction

Leishmaniasis is a vector-borne disease caused by an intracellular parasite from the genus *Leishmania* (family: *Trypanosomatidae*) transmitted to humans by the bite of a sand fly.

Leishmaniases are endemic in the tropics, subtropics and temperate areas, spreading over 98 countries, with more than 350 million people at risk of developing the disease, and 12 million cases of infection [1].

Among parasitic diseases, human leishmaniasis is second in mortality only to malaria, and due to its potential for lethality and its dispersion in several countries, it is considered by World Health Organization (WHO) as a major public health problem [2].

The spread of leishmaniasis is influenced by several factors, such as climate change, large-scale population migrations and travel and trade to endemic areas [3].

The disease is closely associated with poverty, malnutrition and comorbidities, since many of these factors act on the ability of the immune system to respond appropriately to the presence of the parasite [4].

The clinical features include a wide range of manifestations ranging from asymptomatic infections to different levels of disease severity, depending on the species of *Leishmania* involved and on the immune response of the host. Leishmania infections can present with six different clinical forms: visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and mucosal leishmaniasis (ML) [5].

More than 30 species belonging to the genus *Leishmania* have been identified, all pathogenic to mammals, of which approximately 21 are involved in human infection [5].

1.1 The parasite

Protozoa of the genus *Leishmania* belong to the kingdom Protists, subkingdom Protozoa, phylum Sarcomastigophora, subphylum Mastigophora, class Zoomastigophora, order Kinetoplastida, suborder Trypanosomatinae and family Trypanosomatidae. On the basis of the parasite development patterns within the intestine of the insect vector, two subgenera have been identified: *Leishmania Leishmania*, present in both the Old World (Europe, Africa, and Asia) and the New World (North, Central, and South America), and *Leishmania Viannia*, endemic only in the New World. The subgenera are further subdivided into distinct complexes based on the epidemiological and clinical characteristics of the infection [4].

First attempts at the classification of *Leishmania* were extrinsic or based on eco-biological criteria such as behavior of the parasite in the vector and definitive host, geographic distribution, tropism, and clinical manifestations. In the early 1970s intrinsic characteristics (immunological, biochemical, and genetic parameters) of *Leishmania* were identified and used to develop new types of classification. Methods like species-specific monoclonal antibody evaluation, electrophoretic analysis of isoenzymes, DNA analysis have been adopted as typing systems and accepted as gold standard for identification and characterization of parasite [6].

In 1990, WHO made a subdivision of the two subgenera through the analysis of isoenzymes of the parasite employing multilocus enzyme electrophoresis (MLEE), which is a technique that involves a biochemical characterization of species based on the different gel electrophoretic mobility of a predefined set of isoenzymes. The combined pattern of these proteins constitutes a zymodeme and based on that classification,

leishmanial species are divided into complexes which are characterized by specific pathogenicity (viscerotropic and dermotropic strains) [7].

The subgenus *Leishmania* is subdivided into the following complexes (Fig. 1) [8]:

- *L. donovani*: responsible for visceral and cutaneous forms in the Old and New World, (species: *L. donovani* and *L. infantum*)
- *L. major*: responsible for Old World cutaneous forms, to which belongs the species *L.major*;
- *L. aethiopica-tropica*: responsible for localized and disseminated cutaneous forms in the Old World (species: *L. aethiopica* and *L. tropica*);
- *L. mexicana*: responsible for localized and disseminated cutaneous forms in the New World (*L. mexicana*, *L. amazonensis* and *L. ganhami*)

The subgenus *Viannia* is divided into the following two complexes (Fig. 1):

- *L. braziliensis*: responsible for cutaneous and mucocutaneous forms in the New World (*L. braziliensis* and *L. peruviana*);
- *L. guyanensis*: responsible for cutaneous and mucocutaneous forms in the New World (*L. guyanensis* and *L. peruviana*)

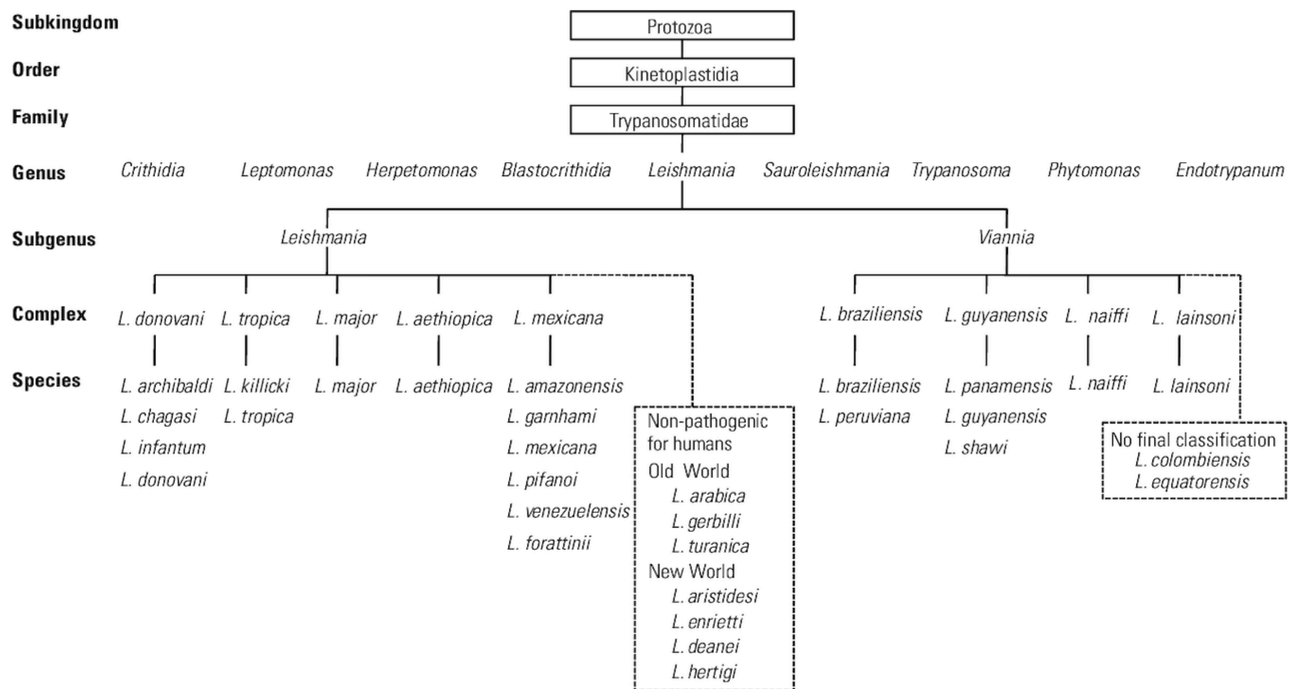


Fig. 1. Taxonomy of Leishmania genus (modified from Banuls et al. 2007)

1.1.1 Morphology

Leishmania parasites are heteroxenous, unicellular hemoflagellate asexual parasites with a diploid genome organized into 34-36 chromosomes [6].

The parasite has two major different cellular morphologies, described as promastigote morphology in the phlebotomine sand fly and amastigote morphology in the mammalian host (Fig. 2) [9].

The basic cellular architecture is fairly conserved between the two *Leishmania* forms, and the shape of cell is defined by cross-linked sub-pellicular corset microtubules. Placed within the cell are the nucleus and the organelles, in particular the single mitochondrion and the Golgi

apparatus. The genetic material of *Leishmania* is composed of genomic DNA delimited in the cell nucleus. In addition, the kinetoplast (kDNA) is located anterior to the nucleus. kDNA is a collection of concatenated DNA located in the mitochondrion, a double membrane-bound organelle with its own genome. Thoroughly, kDNA is organized into hundreds of minicircles of 0.8-1.0 Kb each and a few dozen maxicircles of approximately 23Kb each [10].

Morphologically, the amastigote is characterized by a smaller and more globular shape with a diameter of 2-5 µm and a length of 3-5 µm and with a short immotile flagellum [11].

Contrarily, promastigote is defined by an elongated shape (15-20 µm length) with a long motile flagellum that provides propulsive force capable to facilitate the passage into the phlebotomine digestive tract [12].

Despite amastigotes and promastigotes look definitely different, they preserve the same basic cell structure with the kinetoplast anterior to the nucleus and the flagellum elongated from the basal body.

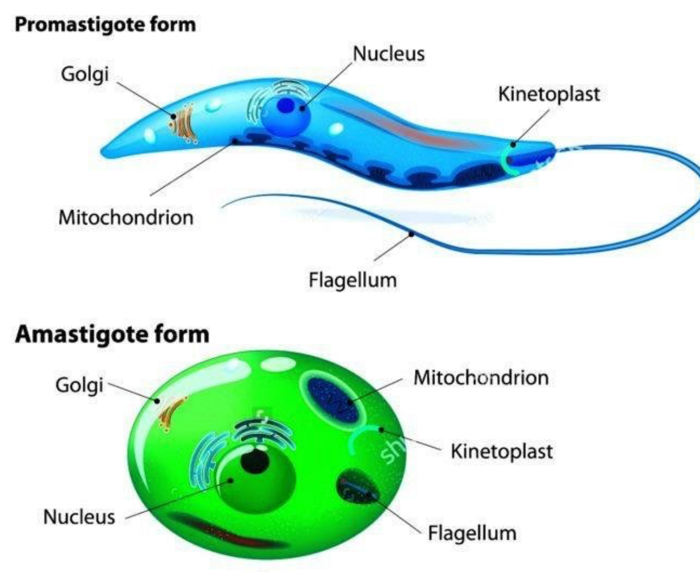


Fig 2. The two forms of the Leishmania parasite. Schematic diagram of a promastigote and amastigotes (modified from Dhiman 2019)

1.1.2 Life cycle

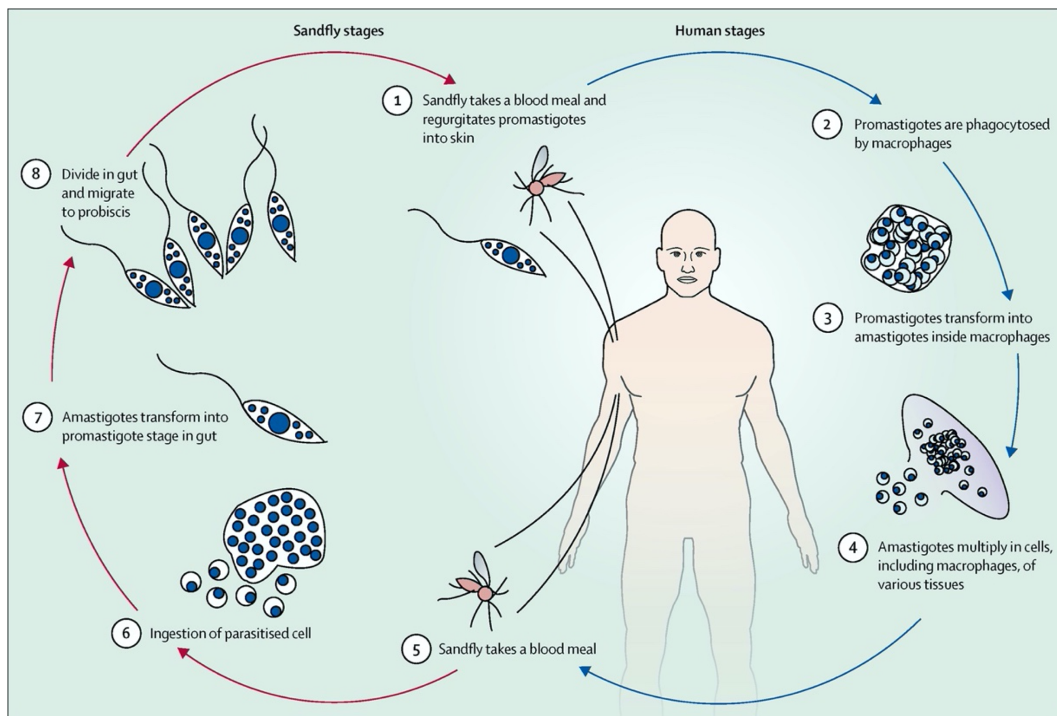


Fig 3: Leishmania life cycle (modified from Burza et al. 2018)

When a *Leishmania*-infected female phlebotomine takes a blood meal, promastigotes are released into the site of the bite (1).

The parasites are phagocytosed by phagocytic cells in the tissues and internalized into a parasitophorous vacuole, which fuses with a lysosome, giving origin to a phagolysosome. The damage caused by the phlebotomine leads to the recruitment of monocytes/macrophages to the bite site, and these are the cells that *Leishmania* infects and where the parasite persists in the host (2).

Within the phagolysosome, promastigotes are transformed into amastigotes, which initiate an intense replicative activity until the vacuole ruptures, resulting in cell lysis. (3) Amastigotes are then released into the extracellular environment and can infect other macrophages, spreading the infection in the host (4).

The biological cycle in the vector begins when the female sand fly becomes infected during a blood meal from an infected mammal by ingestion of amastigotes (5). Within the middle intestinal tract of the insect, rapid transformation of the amastigote into promastigote occurs (6) by body elongation and flagellum formation, with subsequent replication of the protozoa (7). The promastigotes then migrate to the anterior portion of the digestive tract of the sand fly up to the pharynx and proboscis, where they differentiate into metacyclic promastigotes, an infectious form characterized by a tapered appearance and a flagellum with high motor activity (8). The duration of the cycle within the vector is approximately 7-14 days, but it is influenced by environmental temperature and by the sand fly and *Leishmania* species involved [13].

1.2 The vector

Leishmania is transmitted by the bite of hematophagous *Diptera* called phlebotomous.

Although human-biting sandflies exist in various genera (over 800 species), the only demonstrated vectors of human leishmaniasis are species and subspecies of the genus *Phlebotomus* in the Old World (Europe, Asia, Africa) and *Lutzomyia* in the New World (America) [14].

These insects are characterized by a small body of about 2-3 mm in length covered with a dense down and varying in color from pale yellow to black; unlike other *Diptera* they have wings that fit at an angle on the abdomen, allowing them to make only short vertical movements, and are characterized by silent flight [15] (Fig. 4).

The male phlebotomine sand flies are exclusively glycophagous; these insects feed on sugary substances taken from plants and are not involved

in the transmission of the parasite. On the other hand, the female sand flies require a blood meal for the maturation of the eggs, during the meal they introduce saliva and proteophosphoglycans into the host, which contribute to the penetration of the protozoan into the skin [16].
In the Mediterranean region sand flies are mainly active in the evening, at night and in the early morning, they grow in damp and shady places where organic debris is present.
The transmission of the infection occurs throughout the year in tropical and subtropical climates, while in temperate areas the transmission occurs in the hot months, which for the Mediterranean basin correspond to the period between the end of May and the end of October [17].
Because of their wings conformation, sand flies are able to make only small movements and are disturbed by air currents: they move very little away from their breeding sites and lay their eggs in damp soil [18].

Fig. 4: Image of a female sand fly during a blood meal (modified from Munsterann 2019)

Sand flies are widely distributed in Asia, Africa, Australia, Central and South America and Europe. The geographical distribution and density of

the phlebotomine sandflies are related to minimum winter and summer temperatures responsible for latitudinal differences in species distribution. Moreover, the geographical distribution of sandflies is highly linked to temperature, altitude, vegetation and land cover [19, 20]. Phlebotomine sand flies are abundant in the Mediterranean basin, the most present species are *P. perniciosus*, *P. tobbi*, *P. ariasi*, *P. papatasi*, *P. perfiliewi*, *P. sergenti* and *P. neglectus* [21].

In Italy the most reported species is P. perniciosus, a zoophilous and anthropophilous species adapted to domestic and wild habitats (Central Italy, pre-Alpine territories and southern Italy), while *P. perfiliewi* is mainly found in rural areas of northeastern Italy and *P.ariasi* is detected close to the French border [21].

During the last decades an increase in the geographic distribution and density of *P. perniciosus* towards north-continental Italy has been documented and has been associated with climate changes: this species is currently the most efficient vector for the transmission of human and canine leishmaniasis in our country [21-25]. In a recent study conducted in the Emilia-Romagna region (northeastern Italy), a high percentage of *P.perfiliewi* was found in the territory: this species of phlebotomus is therefore the prevalent species in our region [26].

Vertical transmission and injection by needles have been reported, one example is the VL outbreak that occurred in 1990s in Spain among intravenous-drug users [27, 28]. Moreover, transmission of *Leishmania* by organ transplantation, blood transfusion has also been reported [29-33].

1.3 The reservoir hosts

Leishmaniases can be divided in two groups according to the type of infection and cycle of transmission; 1. zoonotic leishmaniasis in which the reservoir are wild animals, commensals or domestic animals, and 2. anthroponotic leishmaniasis, in which the reservoir is human [34]. Despite the fact that each *Leishmania* species falls into the zoonotic or the anthroponotic cycle of transmission, there are cases where the anthroponotic species cause zoonotic transmission [14]. Many species of wild or domestic mammals have been recognized as hosts/reservoirs of *Leishmania*, such as hyraxes, rodents, dogs, cats, foxes, wolves, bats, foxes, primates, armadillos [35-37]. Nevertheless, *Leishmania* reservoirs exhibit temporal and regional variability, for this reason it is fundamental to develop local studies involving ecological, entomological and parasitological analysis to better understand the playing role as reservoir host of different animals in a specific location [36].

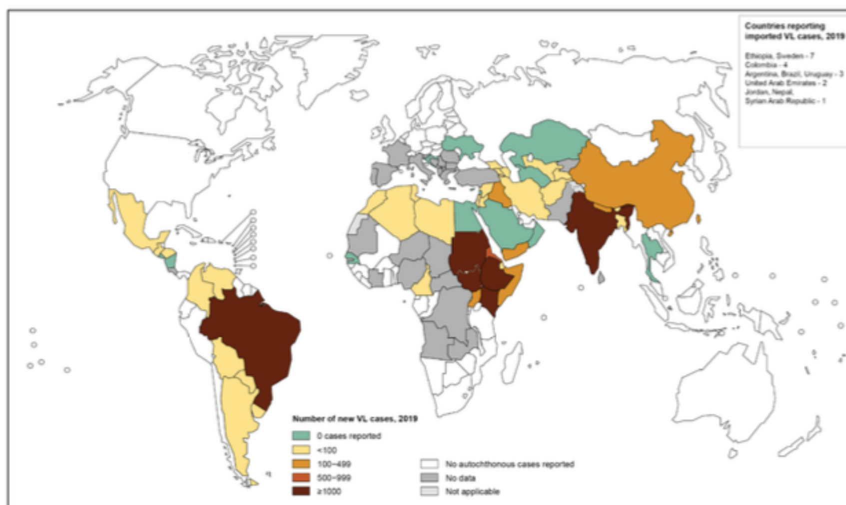
Animal reservoirs are fundamental for maintaining the life cycle of *Leishmania*, additionally they have an important role for transmission of zoonotic and sylvatic infections. The sylvatic transmission of leishmaniasis is the result of established wildlife populations close to areas where vectors circulate, and humans are settled. Dogs and possibly cats are considered responsible of the transmission cycle of the parasite in urban areas and the presence and frequency of these animals may have consequential effect on the leishmaniasis incidence in humans. In areas considered urban or peri-urban, the recurrence of connection between wildlife and human settlements hugely increases the possibility of transmission of *Leishmania* from animals to humans [38]. For example, the case of hares in Madrid and the study conducted in Barcelona on rats

suggest the possibility of transmission of the parasite to sand fly after amplifying in lagomorphs and rodents [39,40,41].
At the moment dogs are considered the most important domestic reservoirs of *L. infantum*, their role as reservoirs has also been assumed because of their connection between wild and peri-domestic environments and because canine leishmaniasis is a chronic infection with high parasite load [14].Not only dogs are considered reservoir hosts of leishmaniasis; other mammals including rodents have been identified as reservoirs showing competence to maintain the life cycle of the parasite [41].

Moreover, studies have been conducted on flying mammals and parasites were isolated in the blood of bats [42,43].
Humans are involved as principal reservoir host when infected by two species causing different clinical disease: VL caused by *L. donovani* and CL caused by *L. tropica*, although these two species of *Leishmania* are considered to be anthroponotic by most studies, there is evidence for their potential zoonotic transmission [44]. The transmission competence of the reservoir species and the availability of the *Leishmania* parasite to the vector are important factors for maintaining the life cycle of *Leishmania* in a specific area.
In conclusion, the epidemiology of leishmaniasis depends on the interaction among the parasite, the vector and the host/s in a specific area. For this reason, the alteration of local ecology by human intervention can change the biotic and abiotic setups in a specific area, increasing the inflow of *Leishmania* vectors and/or inducing the movement of reservoirs from wild areas to urban areas [45,46].
In endemic regions, the proximity of the houses to the natural habitats of the vector and the animal reservoir/s can facilitate the contact between infected sand flies and humans, increasing the risk of infection [47].

1.4 Epidemiology of leishmaniasis

Leishmaniasis is considered a neglected tropical disease and exhibits a worldwide distribution. In 2019, leishmaniasis was declared endemic in 98 countries worldwide by WHO, and the WHO Global Leishmaniasis Programme for 2019 declared 54 countries (68%) endemic for VL and 53 countries (59%) endemic for CL [2]. Globally, there are approximately 350 million individuals at risk for this parasitic infection and approximately 12 million cases of human leishmaniasis, with 50,000-90,000 cases/year of VL and 600,000-1million cases/year of CL worldwide. [14,48,49]. In 2017, 22,145 new cases of VL were reported by WHO, 94% of which were detected in seven countries- Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan- with an estimated mortality of 10-20%, especially in the poorest areas [48]. More than 85% of CL cases occur in Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia, and Syria [49,50]. For MCL, 90% of cases are found in Bolivia, Brazil, and Peru [48, 50] (Fig. 5).



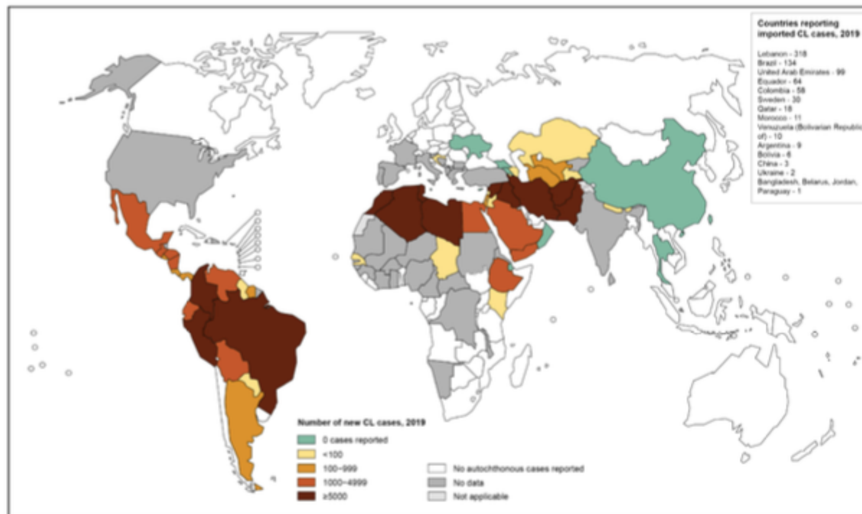


Fig. 5: Global epidemiology of VL and CL, from World Health Organization, Leishmaniasis, Epidemiological situation: epidemiology (https://www.who.int/leishmaniasis/burden/en/).

Overall, leishmaniasis is caused by more than 20 species of *Leishmania*, which are transmitted by almost 30 species of sand flies. Environmental changes and climate are causing an expansion of the geographic range of the vectors and of the regions where cases of leishmaniasis are found [51].

Anthroponotic transmission is distinctive of the *L. tropica* in Asia, North Africa and Middle East e *L. donovani* in East Africa and Indian subcontinent. In the areas with anthroponotic route of transmission, treatment of patients can help the stopping the spread of infection. Conversely, VL caused by *L. infantum* is mainly zoonotic and occurs in the Mediterranean basin, the Middle East, Afghanistan, Iran, Pakistan and Brazil with sporadic cases in Central Asia, China, Mexico, and Latin America [52].

Immunosuppressed individuals and children are at higher risk of clinical manifestation of the disease caused by *L. infantum*, more than immunocompetent adults [53].
The distribution of the infection is influenced by environmental, climatic and socio-sanitary factors such as poverty, malnutrition, migratory phenomena (introduction of non-immune individuals in areas of transmission), impaired immune system (often related to HIV infection), a high density of infected animals, inadequate sanitary conditions or limited resources invested in the control of the parasitosis, consequently impacting mainly on the poorest countries [14].
In addition there are periodic fluctuations in disease incidence driven by climatic factors, vector and reservoir population dynamics, and human movements and behaviors [21, 54].
Over the past 2 decades, a broadening of the geographic distribution of leishmaniasis associated with an increase in disease incidence in endemic areas has been observed, although its impact remains underestimated because reporting is mandatory in only 34% of endemic countries [55]. Even in industrialized countries there is an underestimation of leishmaniasis cases. As an example, the European Region lacks a single European surveillance system, and leishmaniasis is still consider a local infection rather than a global problem. For example, patients infected in Mediterranean area who are diagnosed in continental/northern Europe are not always recorded [48].

1.4.1 Epidemiology of Leishmaniasis in Europe

The public health impact of leishmaniasis has been severely underestimated for many years worldwide. The risk of reintroduction of vector-borne diseases in Europe could be a consequence of travel, trade, global warming and tourism, and these factors could play a fundamental

role in transport of microorganism. [24, 48, 56]. The risk of spreading of infection in Europe is strictly related to 1) the introduction of exotic *Leishmania* species due to increasing worldwide travel of humans and domestic dogs, 2) the spread of VL and CL from Mediterranean areas to temperate non-endemic areas, 3) the re-emergence of disease in endemic areas caused by an increase of immunocompromised individuals [52]. The high prevalence of asymptomatic cases of *Leishmania* infection in Southern Europe indicates that this parasite is a latent public health threat [57-60]. This assumption is demonstrated by the increase of co-infections with human immunodeficiency virus (HIV) and *Leishmania* parasite that has been reported since 1980 [61].

In Europe there are two types of transmission of *Leishmania* infection, zoonotic VL and CL caused by *L. infantum* in the Mediterranean region, and anthroponotic CL caused by *L. tropica* in Greece [52].

Leishmaniasis is endemic in Spain, Portugal, France, Italy, Croatia, Greece, Malta, Cyprus, Turkey, and the Balkans, with the majority of VL cases reported in Albania, Turkey, Italy, and Spain; in nine of these endemic countries, WHO estimated between 2003 and 2008 approximately 410-620 new cases of VL each year [62].

The domestic dog is considered the only reservoir of veterinary importance in Europe, where a large production of prophylactic drugs and treatment for canine leishmaniasis (CanL) is ongoing [63].

The epidemiology of leishmaniasis in Europe has changed in the last 20 years for several reasons related to change of habits and climate. In the first place, the spreading of HIV/*Leishmania* co-infection has been reported and the infection caused by widespread needle has been demonstrated in Southwest Europe by *Cruz et al* [64]. In the second place, leishmaniasis is moving northward in Italy [24].

In addition, the density of vectors of *L. infantum* is increasing in temperate

regions such as northern Spain [65] and central France [66]. The northward spread of the vectors stands attention on the significant risk for the emergence of *Leishmania* infection in non-endemic regions of Europe [63].

1.4.2 Epidemiology of leishmaniasis in Italy

In Italy human leishmaniasis is present in different epidemiological and clinical forms. VL is typical in rural and peri-urban areas along the Tyrrhenian Coast, the low Adriatic Coast, regions in center and southern Italy and in the islands (ie Sicily and Sardinia), in accordance with the biological characteristics of the vectors. Seventy-five percent of the cases have been observed in Lazio, Campania and Sicily [67, 24]. The increase of temperature is leading towards the diffusion of sand flies and consequently VL into regions of northern Italy [24]. Moreover, the global warming and the augmented frequency of immunosuppressed individuals can also multiplicate the cases of leishmaniasis in endemic areas [68].

A striking example of increase of leishmaniasis cases in northern Italy is represented by the VL outbreak that developed in the province of Bologna (Emilia-Romagna region, north-eastern Italy). In the period between November 2012 and May 2013, a relevant increase of autochthonous cases of VL has been reported: in detail in 6 months 14 cases were notified, this data is five-times the annual average estimated in the period between 2008 and 2012 [56].

On the contrary, CL is often underdiagnosed and underreported, the reason might be that it has a benign clinical course and tends to resolve spontaneously without requiring hospitalization. This form of infection is present in rural areas, sub-Apennine, and along the coast mainly in Sicily,

Calabria, Abruzzo, and Sardinia [21].

1.5 Pathogenesis

After transmission of *Leishmania* promastigotes by sand flies, the parasites may remain localized in the site of inoculation, spread to the mucosa of nasopharynx or disseminate to spleen, liver, bone marrow and sometimes to other organs, thus causing 3 clinical forms of leishmaniasis: 1) cutaneous, 2) mucosal or mucocutaneous, 3) visceral.
CL typically occurs at the site of inoculation causing skin lesions, mainly ulcers. The major causative species are: *L. major* and *L. tropica* in southern Europe, Asia and Africa, *L. mexicana* in Mexico and Central and America, *L. brazilienzis* in Central and South America [69].
MCL spreads from the initial skin lesion and lead to partial or total destruction of mucous membranes of the nose, throat and mouth, it typically develops in months to years after the appearance of the primary skin lesion [69].
VL (kala-azar) is the most severe form and commonly fatal if not treated. VL is caused by *L. donovani* or *L.infantum* and it usually occurs in India, Africa, Mediterranean basin, Central Asia, Central and South America and China. *Leishmania* parasites spread from the site of the bite of sand fly to the lymph nodes, the liver, the spleen and bone marrow causing the systemic disease. Leishmania infection is considered an opportunistic infection in immunocompromised patients, but subclinical infections are very common and only a minority of infected patients develop visceral disease [70]. Even in asymptomatic individuals VL can develop because of reactivation of persistent infection, this condition occurs considering several medical conditions that obstacle the immunological control of the

parasite.

The factors that determine disease progression are not completely understood, but experimental evidence demonstrates that virulence of the parasite, and specific conditions of the individual such as nutritional status, age, and genetic and immune factors can contribute to determine the severity of leishmaniasis [53].

The variability of tropism of *Leishmania* is probably the result of genomic differences acquired during evolution.

Unknown genetic traits render some species (such as *L. major*) more adequate to remain localized at the level of the skin resulting in CL, other species (such as *L. donovani*) most often invade organs by localizing in macrophages of the reticulo-endothelial system and causing VL, while *L. infantum* is capable of causing both CL and VL [71]. Moreover, acquired immunosuppressive conditions can regulate parasite replication, for instance HIV infection can increase the risk of developing VL by 100-2,000 times . In addition, HIV/*Leishmania* co-infection could evolve in chronic disease with a high rate of relapses and treatment failure [72,73].

Even though the *Leishmania* parasites infect several cell types in the mammalian host, macrophages and dendritic cells (DCs) play the most important role in the regulation and outcoming of infection. After the inoculation of the parasite, both neutrophils and macrophages are recruited to the site of infection. The first step played by neutrophils is the uptake and internalization of parasite into the phagosome, after that the fusion with lysosomes occurs [74]. The phagolysosomal environment is hostile for many pathogens, but *Leishmania* is one the microorganism able to survive and multiply in such a harsh habitat. Neutrophils are short-lived cells, they play the role of intermediate host cells to enter silently the macrophages bypassing cell activation. In macrophages the parasites lose their flagella and divide by binary cleavage; repeated parasite

replication leads to rupture of the host cell and to infection of "Trojan horses" as they are used by parasites to hide themselves and spread in the reticulo-endothelial system [75,76].

The outcome of the infection depends on the type of immune response that develops in the host. In particular, the control of infection or the evolution of the disease are related to the specific CD4+ T cell response. Within the CD4+ population there are two subpopulations: Th1 and Th2, Th1, if activated, determine a benign evolution of the infection through the release of mediators such as IFN-γ, IL-2, TNF- α that promote the killing of the parasite by macrophage activation. On the contrary, the prevalent production of IL-4, IL-5, IL-6 and IL-10 by the Th2 subpopulation or a mixed Th1/Th2 response are responsible for increased parasite replication and progression of infection to disease [76-79].

Leishmania has evolved strategies to escape host immune control in order to continue the life cycle and establish infection in the host. The parasite can damage the cellular functions of infected macrophages to the point of death, and to inhibit the inducible NO-synthetase enzyme and, consequently, the production of reactive oxygen species (ROS) within the phagolysosome by altering the maturation process. Moreover, *Leishmania* is also able to modulate the cell-mediated immunity by inducing the release of IL-10 and TGF-β, cytokines responsible of inhibition of the cell-mediated immune response, thus allowing a state of persistent infection [9, 80].

However, not even the efficient Th1-induced immunity is sterilizing; the parasites persist in the host in a quiescent state and this condition can potentially lead to reactivation of the infection and establishment of disease if the host enters a condition of immunosuppression [53].

1.6 Clinical manifestations

The clinical features of leishmaniasis vary depending on the parasite's characteristics and on the effectiveness of the immune response of the host. Human leishmaniasis can manifest in three main clinical forms: 1) cutaneous (localized and disseminated), 2) muco-cutaneous (affecting the mucous membranes of the upper respiratory tract) and 3) visceral or kala-azar with systemic involvement, caused by dissemination of parasites *via* the bloodstream to the reticuloendothelial system in lymph nodes, liver, spleen, and bone marrow [81].

1.6.1 Visceral leishmaniasis (VL)

VL, the most severe form of leishmaniasis also known as kala-azar, is a life-threatening disease. VL is caused by *L. donovani* (India and Eastern Africa) and by *L. infantum* (Mediterranean area and South America). If adequate therapy is not initiated in timely manner, leishmaniasis can lead to death [82, 83].
The incubation period is usually long and can extend from 10 days to 24 months, with an average of 2-6 months. VL is characterized by a wide clinical spectrum, that may vary from asymptomatic or sub-clinical leishmanial infection to mild (oligosymptomatic leishmaniasis) to moderate and severe clinical manifestations. The typical manifestation of the disease include fever, hepatomegaly, splenomegaly, pallor (due to severe anemia) and weight loss [14,84] (Fig.6)
Other signs including respiratory problems or gastrointestinal disorders as diarrhea and vomiting are less common [85].
Classical laboratory abnormalities include pancytopenia, high levels of liver enzymes and of bilirubin and hypergammaglobulinemia, while the

presence of neutrophilia might suggest secondary bacterial infection. In the absence of treatment, hemorrhage caused by thrombocytopenia, cachexia and multisystem failure can lead to death.

Relapses are rare in immunocompetent individuals (5,9%), but become frequent in case of immunosuppression such as individuals who receive corticosteroids or chemotherapy, or in co-infection with HIV, or in transplanted individuals [86-88].

A dermal disease known as post-kala-azar dermal leishmaniasis (PKDL) may emerge months or years after successful therapy for VL. This a dermatosis develops in 10-60% of cases as a complication of VL caused by *L. donovani* in areas such as Asia (India, Nepal, and Bangladesh) and East Africa (Sudan and Ethiopia); the clinical spectrum of PKDL is dissimilar between the two regions [81]. PKDL presents as a diffuse skin rash on the face, trunk, and limbs, highly variable in manifestation (nodular, maculopapular or polymorphic rash) and in severity [89,90] (Fig.7). PKDL is the result of an immunologic reaction to the parasites of the genus *Leishmania* that persist within the skin and PKDL patients act as reservoir for transmission of the infection complicating the efforts to eradicate the disease [81]. Mild cases of PKDL in Sudan may resolve spontaneously within 1 year, but in Asia treatment is indicated for all cases [90].

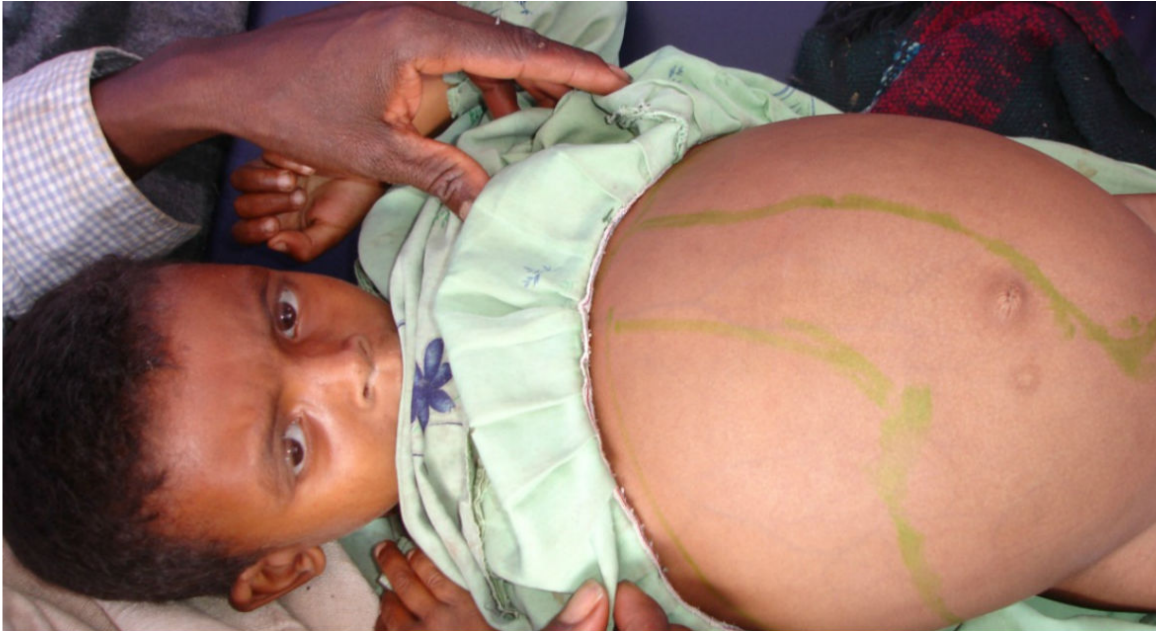


Fig. 6. Hepatosplenomegaly in a VL case. (modified from Roddy Isles 2018)

Fig. 7: Papular rash typical of PKDL (modified from E. Ziklstra 2019)

1.6.2 Cutaneous leishmaniasis (CL)

CL appear with a wide variety of clinical forms, which tend to vary between and within geographic areas, reflecting the different infecting species of parasite, the type of cycle (zoonotic/anthroponotic), the immunologic status of the patient, and perhaps even genetically determined responses of the patient.
CL manifests in the site of the sand fly bite, in increasing order of frequency, the most involved areas are ankles, forearms, hands, legs, cheeks, upper lip, nose and ears (Fig.8). The incubation period is usually some weeks, but it can last for up to years [81].
The classic lesion is characterized by an erythematous painless papule at the site of the bite that slowly grows in dimension to its final size. The size varies from 1 cm to more than 4 cm in diameter in case of plaque lesion and sometimes pruritus may be present. The lesion can develop into a plaque or a nodule, can be ulcerated or nonulcerated [81]. and can last from 5 months to up to 20 years. The structure of the ulcerated lesion is typical; the bottom of the ulcer shows granulation tissue that easily bleeds, and a pink periphery is often hidden by a whitish pseudomembrane. Sometimes, abundant secretion produces an adherent crust [91] (Fig 8).
In the Old World, cutaneous forms of leishmaniasis are caused by *L. infantum*, *L. tropica*, *L. major, L. aethiopica*, and, more rarely, *L. donovani* [92]. *L. infantum* is the major etiologic agent of CL in southern Europe and causes typical lesions formed by single/few nodules with moderate inflammation. Usually, the lesions heal spontaneously over the course of 1 year and appear to confer a sort of immunity. *L. tropica* causes dry ulcers of the skin, often multiple, that do not cause pain and heal spontaneously

in about 1 year leaving scars [91]. CL caused by *L. major* results in highly inflamed and ulcerated lesions that do not cause pain and heal within 2 to 8 months. The lesions could be multiple, especially in immunocompromised individuals. CL caused by *L. aethiopica* results primarily in localized cutaneous nodular lesions, but this species can cause oro-nasal leishmaniasis or diffuse cutaneous leishmaniasis characterized by widely disseminated macules, papules, nodules, or skin plaques or diffuse skin infiltration. In the latter case, the lesions do not heal spontaneously and have episodes of relapse [81].

In the New World, CL is caused by many species of *Leishmania* belonging to the subgenera *Leishmania* and *Viannia*, and it manifests with a wide variety of clinical forms, including localized, disseminated, diffuse, and atypical cutaneous forms, and mucocutaneous forms. Lesions originate at the site of inoculation first as macules then as papules, which ulcerate and expand into a round or oval crateriform lesion, or evolve into a nodular lesion. Lymphatic involvement is common in infections of the *Viannia* subgenus, manifesting as lymphadenopathy or lymphadenitis and secondary mucosal lesions may occur (as described in paragraph) [93, 94].

Moreover, CL can relapse or the scarring lesion could be open again, showing features comparable to those of the first episode, generally, relapses can be mild or more severe than the original lesion [95].

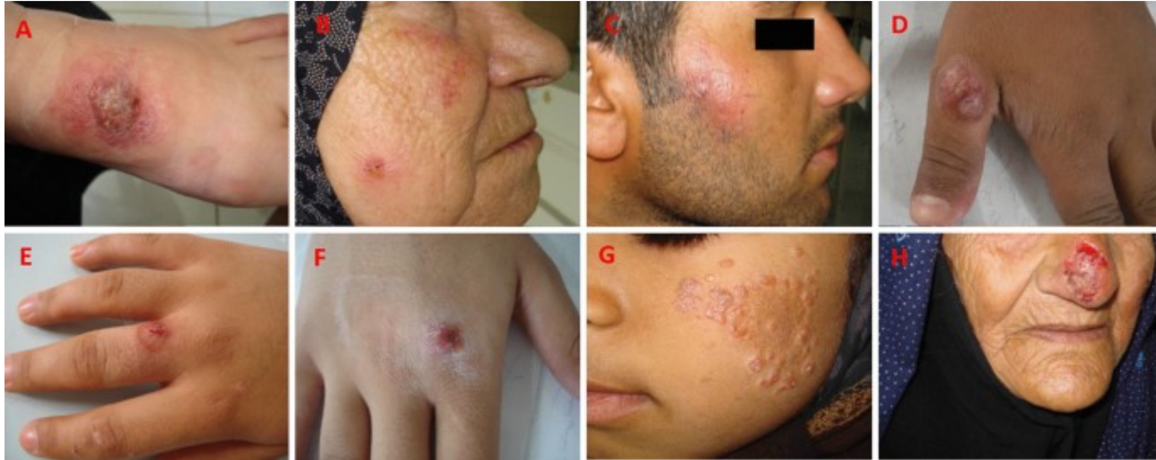


Fig. 8. Examples of lesions of cutaneous leishmaniasis (modified from Karimi T. et al. 2021)

1.6.3 Muco-cutaneous leishmaniasis (MCL)

In endemic areas, such as South America, from 1 to 10% of patients with CL progress into MCL after about 1-5 years from healing of the cutaneous lesion [96].
Mucocutaneous leishmaniasis manifests as nodules and infiltrates of the nasal cartilage, resulting in blockage of the nostrils, destruction of the nasal septum, and nasal collapse (Fig 9). The pharynx, palate, upper lip, and larynx may also be involved, resulting in severe tissue destruction and disfigurement [81, 97].
MCL is typical of the New World with most cases in Bolivia, Brazil, and Peru and it is caused by two species of the subgenus Viannia: *L. braziliensis* and *L. panamensis* [96].
Generally, lesions appear first in the nasal mucous membrane and extend to the mouth the pharynx and the larynx, and to the skin of the nose and lips. Lesions can cause symptoms that vary from malaise and mild pain to

cachexia, the latter develops when the lesions involve the pharynx, larynx and esophagus causing hoarseness and dysphagia [81, 98].

In the first steps of MCL, infiltration of the mucous membranes with superficial ulcers is present, thereafter the ulcers become necrotic [98]. In the case of systemic involvement during the disease, the regional lymphnodes can become painful [99].

MCL almost never heals spontaneously, and secondary bacterial infections are common, with pneumonia being the major cause of death [98].

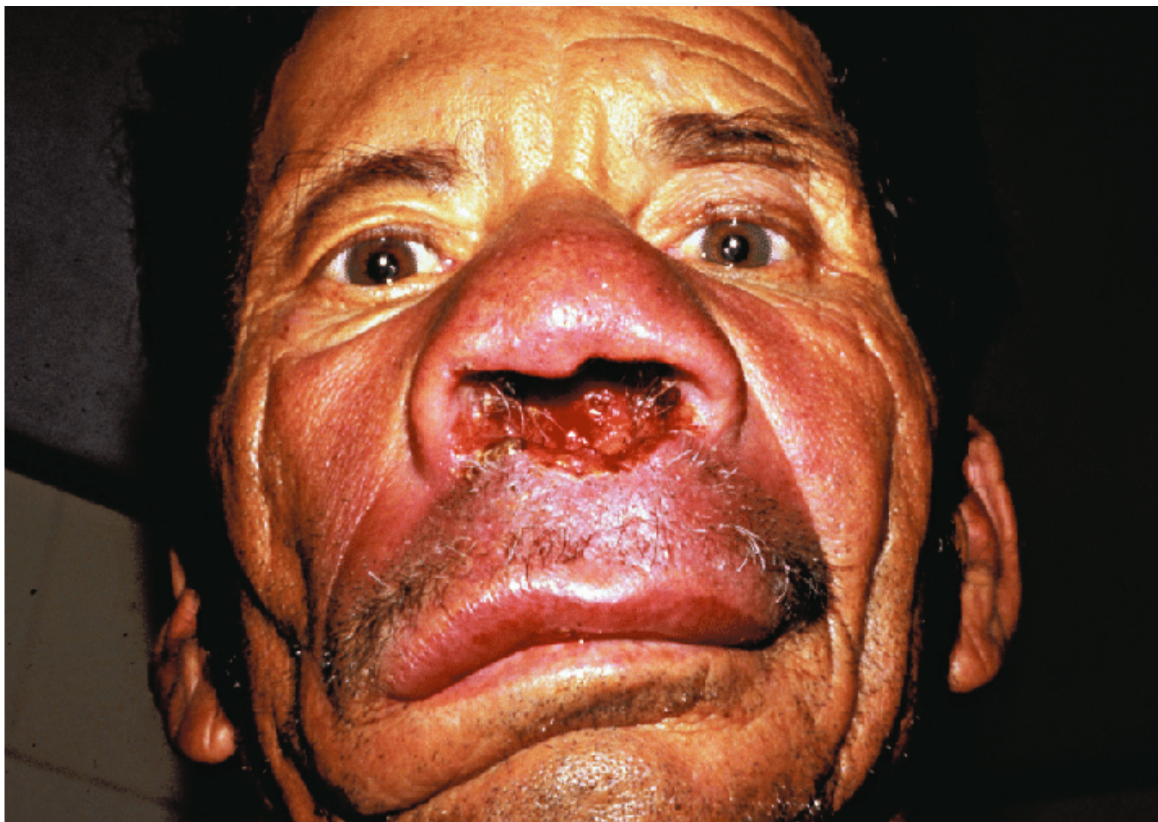


Fig. 9. Ulceration of nasal septum due to MCL (modified from Magill AJ et al. 2011)

1.6.4 Mediterranean mucosal leishmaniasis (ML)

Mucosal leishmaniasis is a rare clinical form included in the group of tegumentary leishmaniasis (CL and MCL), caused by *L.infantum* and present in Mediterranean Europe. The percentage of ML in France in autochthonous cases of Leishmaniasis is 2%, on the other hand in other countries the prevalence is unknown [100]
Clinical manifestations of ML are nodules, granular inflammation, polypoid lesions, the lesions usually involve pharyngeal and laryngeal regions, buccal area and sometimes the nose. ML can be differentiated from MCL because usually is not associated with primary cutaneous lesions, and moreover, MCL commonly involves nasal cavity but this area is less frequently involved in ML. At the moment, the pathogenesis of Mediterranean ML is still unknown and there is no validated guideline for therapy of the rare cases of ML in Mediterranean areas [101].

1.6.5 Asymptomatic *Leishmania* infection

Scientific modeling propose that the asymptomatically infected individuals could be reservoir of parasites driving the infection, despite the fact that their infectiousness to sand flies is not yet completely demonstrated [102]. Studies have reported the ratio of incident asymptomatic infection with *L. donovani* or *L. infantum* to incident clinical manifestations of disease, the data show 1:2in Sudan [102], 5:1 in Ethiopia [102], 4:1 in Kenya [103], 18:1 in Brazil [104], 50:1 in Spain [105], 9:1 in India e Nepal [106] and 4:1 in Bangladesh [107]. These data demonstrate that most individuals infected with *Leishmania* develop an effective immune response and do not show clinical manifestations of disease. Several features of

Leishmania asymptomatic infection are not fully established such as the role of asymptomatic carriers in transmission of infection or the prognosis of asymptomatic leishmaniasis. The mechanism determining the capacity of an infected individual to progress or not progress to clinical form of disease is not fully elucidated, but the *Leishmania*-specific cell-mediated immune response seems to play a fundamental role. Studies from Colombia demonstrated that macrophages from asymptomatic individuals tested positive to a Leishmanin skin test (LST, a test of in vivo parasite-specific delayed-type hypersensitivity) were less permissive to *in vitro* infection than those of individuals presenting recurrent leishmaniasis, suggesting that different individuals may exhibit different clinical susceptibility. LST [108]. Considerable attention has also been paid to cytokines that can modify the immune response through their action on macrophages. The active form of the disease leads to the production of IFN-γ and IL-10, whereas in asymptomatic individuals IL-10 is not produced, but IL-2 and IFN-γ are overproduced [109]. Comparing the number of CD4+ T helper cells and the production of IFN-γ by CD8+ T cells in individuals with asymptomatic infection, individuals with recurrent disease and healthy control donors an increase has been observed in asymptomatic individuals. These data suggest an active role of cytotoxic T cells in inducing resistance to *Leishmania* infection [110].

It is not possible to predict if asymptomatically infected individuals will develop VL and in what moment of their life, because the future of disease is strictly related to a complex interaction between parasite, host and environment. A supplementary challenge is the difficulty in identifying the asymptomatic *Leishmania* infection; in fact serological methods are not accurate, and even molecular methods show fluctuations in test positivity due to the short half-life of the parasite DNA in the blood of the host and also depending on the choice of the target sequence [5]. An IFN-γ Release

Assay (IGRA) on whole blood stimulated with *Leishmania* antigens (SLA) has recently been developed as an alternative to the LST for screening naturally exposed immune individuals [111,112]. The studies conducted on asymptomatic individuals demonstrated that in addition to high levels of IFN-γ, high levels of IL-2 were proved to be the sensitive and specific marker in detecting asymptomatic infection [113]. Considering that the T cells of patients with *Leishmania* infection secrete significant levels of specific cytokines upon stimulation with leishmanial antigens, a Whole Blood Assay (WBA) has been developed. The method is based on the principle of IGRA, reproducing *in vitro* the key immunological process of the natural infection and by measuring the level of specific cytokines after stimulation of blood of the patient with soluble *Leishmania* antigens (SLA). Efforts to improve the detection of asymptomatic infection in endemic regions should be a priority to improve leishmaniasis control, and it is critical to investigate the determinants of asymptomatic infection and those of disease progression. For VL control, the most important question remains whether asymptomatic infected individuals are infectious to the sand flies [112].

Furthermore, the identification of asymptomatic carriers of *Leishmania* is also of fundamental importance when it comes to Transfusion Transmitted Leishmaniasis (TTL): blood donors living in endemic areas may have asymptomatic infection and transmit the parasite through the blood to other individuals. In fact, parasites of the genus *Leishmania* can survive in blood under the storage conditions of blood banks and maintain infectivity, thus being able to infect the cells of a nonimmune individual following blood transfusion [114]. However, pre-storage leukodepletion is obligatory in Italy since 2016 and this technique is significantly efficacious in reducing the risk of *Leishmania* transmission by blood donors [33].

1.7 Leishmaniasis in immunocompromised patients

As demonstrated in the studies about outbreaks of leishmaniasis in Italy [56,115] and Madrid [116], leishmaniasis has the potential to emerge and re-emerge in Europe. The reasons are various, including human intervention on the environment, climate change, immigration and elevated number of immunocompromised individuals [52]. Immunosuppression is one of the main risk factors for the development of clinical forms of the parasitic disease, and it can also influence the course of the disease, causing a worse prognosis and frequent relapses. Evidence indicates that DNA of *L. infantum* is often detectable in peripheral blood of HIV-infected patients, suggesting that in these patients there is an increased infectivity and an overall higher parasite load [117]. With the wide-scale use of highly active antiretroviral therapy (HAART), a gradual decrease in VL incidence has been reported in last years in HIV-infected individuals [117,118]. Despite the fact that immunosuppression has classically been observed in HIV infected individuals, several non-HIV-related immunosuppressive conditions are emerging globally, due to the increasing use of drugs causing an impairment of the immune system [53]. The group of non-HIV-related immunosuppressive conditions ranges from transplantation medicine, to rheumatology, haematology and oncology [119].

Antinori et al. wrote a detailed review of leishmaniasis in organ transplant recipients, demonstrating 79 cases of leishmaniasis in transplant patients up to now globally, a four-fold increment since the 1990s [118]. The time occurrence of leishmaniasis vary based on the transplanted organ, with median times of 6 months after liver transplantation and 19 months after transplant of kidney [119]. Because of the increasing number of organ

transplantation world-wide, and the augmented frequency of travels to and from VL-endemic areas, cases of leishmaniasis in transplant recipients are expected to increase.
Regarding to rheumatology, VL has been associated with the use of several immunosuppressive drugs, as azathioprine, methotrexate, steroids, cyclosporine and cyclophosphamide [120,121].
The introduction of tumor necrosis factor (TNF- α) antagonist drugs has attracted attention recently, initially the studies were focused on the risk of tuberculosis reactivation using these drugs, but there are now several reports on leishmaniasis, occurring after median of 18 months of use [122].

Lastly, many cancer-related cases (in the majority of cases haemotological tumors) of VL have been observed, they were related to the use of chemotherapeutic regimens and monoclonal antibodies.
The clinical signs of leishmaniasis are usually more severe in immuncompromised patients, and uncommon manifestations often occur. Atypical signs consist in detection of parasites in unusual sites such as the intestine, the oral cavity, the lung. In addition, disseminated skin lesion are also more frequent than in immunocompetent individuals. Moreover, in renal transplant recipients VL can cause graft dysfunction [119].
Finally, relapses of leishmaniasis are frequent in immunocompromised patients and mortality is higher than in immunocompetent individuals.

At the moment, an European surveillance and reporting system is not available, and the numbers of leishmaniasis cases in immunocompromised patients are largely underestimated, due to the presence of atypical signs and the challenges in diagnosis (see paragraph “1.6 Leishmaniasis in immunocompromised patients”).

The factors of progression to disease after primary *Leishmania* infection or reactivation of a quiescent leishmanial infection are not completely understood [121]. Generally, *Leishmania* parasite persists in the host after primary infection, and this could lead to parasite replication and disease if immunosuppression is established. Fundamentally, HIV and *Leishmania* infect the same host cells (ie macrophages and dendritic cells), and reciprocally fortify each other's pathogenic effects compromising macrophage activity [122]. Evidence indicates that *Leishmania* co-infection induce immune activation in HIV-positive patients, which persists also after VL therapy or after HAART, leading to more rapid HIV disease progression and low CD4+ c cell count [122].

For non-HIV-related immunosuppressive conditions, several cellular pathways are affected, including T-cell lymphocytes through distinct mechanism such as lymphocyte depletion, impediment of maturation, cell cycling and co-stimulation [123]. In this scheme, VL can relapse from an original latent infection, or a *de novo* infection can develop after the bite of an infected sand fly or, infrequently, a iatrogenic infection can be acquired through infected donor organ or blood [120].

1.8 Diagnosis of leishmaniasis

The diagnosis is based on the evaluation of clinical signs and/or clinic pathological alterations compatible with the disease and on the confirmation of the infection by Leishmania through the use of serological, cellular and molecular techniques.

1.8.1 Diagnosis of VL

The diagnosis of VL is complex because its clinical manifestation resembles some other common diseases such as typhoid fever, malaria and tuberculosis. Specific and accurate laboratory methods become fundamental for differential diagnosis.
Laboratory diagnosis of VL can be performed detecting the parasite (direct diagnosis) or detecting the body's immune response following exposure to the parasite (indirect diagnosis) [124].
In the methods for indirect diagnosis we can includes serological tests, based on detecting specific antibodies:
1) Indirect Fluorescent Antibody test (IFAT), that is considered a very sensitive (96%) and specific (98%) test ;
2) Enzyme Linked Immunosorbent Assay (ELISA): sensitivity and specificity depend on the antigen used, high antibody titres are correlated to active disease [125];
3) Immunoblotting: provide detailed antibody responses to several leishmanial antigens, it is more sensitive than IFAT and ELISA but expensive and time consuming;
4) Direct Agglutination Test (DAT): promastigotes are stained and incubated with sera of patient and agglutination is present after overnight incubation. It shows high specificity and sensitivity, it is simple and inexpensive, but DAT remains positive for a long time after the disease is cured [126];
5) Immunochromatographic strip test (ICT): the antigen rK39 is fixed on a nitrocellulose paper, a drop of serum is smeared over the tip of the strip and the result is ready in few minutes. rK39 ICT show variable sensitivity (60-100%) and specificity (81,2-96,4%) [127].

Direct diagnosis can be carried out using the following methods:
1) Microscopy examination of smears obtained from different biological samples such as bone marrow, spleen, lymph nodes. Smears are stained with Giemsa in order to visualize the parasite under the microscope. At microscopy, the cytoplasm of leishmanial amastigotes is pale blue with a large nucleus stained red. In the same layer of the nucleus, a deep red or rod-like body called kinetoplast is present [128,129]. However, these methods are risky and invasive and may exhibit suboptimal sensitivity [128]. In addition, the microscopic examination for *Leishmania* parasites in liver biopsies, or buffy coat of peripheral blood are used.
2) Culture from different biological samples in order to isolate the parasite. Culture of parasite can improve the sensitivity of detection of the parasite, but *Leishmania* culture is rarely performed in routine clinical practice; this method is time consuming (around 2-4 weeks are needed for parasite grow), expensive, and it needs dedicated expertise.
3) Molecular biology techniques (end-point PCR, Real Time PCR, loop-mediated isothermal amplification, LAMP) for the detection and quantification of *Leishmania* DNA. In recent years PCR-based diagnostic methods with a wide range of targets have been described. A variety of target sequences are used including ribosomal RNA genes, kinetoplast DNA (kDNA), gp63, G&PD, miniexon-derived RNA (medRNA) and β-tubulin gene region. Considering PCR, sensitivity is near to 100% by using spleen biopsy or bone marrow aspirates and it is lower but still excellent by employing peripheral blood samples (70-100%) [130].
However, PCR techniques are complex and expensive to perform, in fact in many VL-endemic countries this method is not applicable in the diagnostic routine. To overcome PCR limitations, in last decades, a novel

molecular technique has been developed, ie LAMP. The method is field-friendly and cost-effective. LAMP exhibits several advantages, including the fact that does not require complex equipment, entails easy DNA extraction including boil and spin, control of amplification is performed by naked-eye and dry reagents such as polymerase enzyme can be used [131].

1.8.2 Diagnosis of CL

The diagnosis of CL is based on clinical manifestations, but requires laboratory confirmation because skin lesions caused by the parasite can be confused with insect bites, impetigo, dermatophyte infections, mycobacterial skin infections, sporotrichosis ì and skin tumors [4]. Several diagnostic methods have been reported with a wide variation in diagnostic accuracy, including direct parasitological tests (microscopy, histopathology and parasite culture) and indirect methods such as serological and molecular diagnostics [128].

The gold standard is direct diagnosis performed on skin specimens taken from the lesion; sampling includes steps as cleaning the lesion and, when ulcerated, removing exudate crusts to obtain a clean base, the surface is then scraped to obtain material the size of a grain of rice to perform cytological examination. Alternatively, a skin biopsy can be performed taking a 3-mm punch or wedge biopsy sample from the site where is present the active lesion. Once tissue is obtained, biopsies are paraffin-embedded, and stained with hematoxylin/eosin or Giemsa. Diagnosis is based on direct visualization of amastigotes with the red rodlike cytoplasmic kinetoplast. Histologic examination has a sensitivity of 70-

80%, whereas cytologic examination has sensitivity ranging from 40-80% [132,133].

Novel methods have been developed also for the diagnosis of CL. Nucleic acid amplification methods, in particular PCR, are reported to have sensitivity higher than 90%, but they are difficult to implement in point of care (PoC) diagnostics and in resource limited settings. Numerous tests targeting several different gene sequences have been established over the last years such as ribosomal DNA internal transcribed spacer 1 sequence or sequence in the kDNA [134,135].

Currently, serological tests for CL are based on indirect fluorescent test, ELISA, Western Blot, DTA, but these methods are not largely employed for the diagnosis of CL because of the poor humoral response and resultant low sensitivity [136].

1.8.3 Diagnosis of leishmaniasis in immunocompromised patients

Symptoms of VL can be present in various conditions that should be included in the differential diagnosis, such as disseminated mycobacterial infections, disseminated histoplasmosis and lymphoma. Furthermore, laboratory findings include pancytopenia, increase of C-reactive protein, ferritin and sedimentation rate. Even though immunocompromised patients have a high risk of progression to clinical manifestation of leishmaniasis, a huge rate of cases remain asymptomatic or manifest subclinical conditions [137]

Several studies indicate that serological methods should be used with caution to diagnose VL in immunocompromised patients because of the limited sensitivity. On the contrary, peripheral blood or buffy-coat smears

and culture seem to be more sensitive in this class of patients than in immunocompetent individuals. Notwithstanding, molecular methods such as PCR to detect DNA of *Leishmania* in peripheral blood or bone marrow aspirates improve sensitivity and specificity in comparison with conventional methods [137]. In addition, if VL relapse is suspected, it should be confirmed by quantitative PCR (qPCR) to evaluate the amount of DNA of *Leishmania* in blood. Moreover, microscopy/culture of bone marrow or buffy-coat can be used to confirm of positivity, conversely serology is not useful in case of relapse.

1.8.4 Screening of asymptomatic *Leishmania* infection

The elements that define whether an asymptomatically infected individual advance to clinical leishmaniasis have not been yet understood, but a *Leishmania*-specific cellular immune response dictate by both parasite and host characteristics seems to play a key role. Serological methods have been considered on their capacity to diagnose clinical VL, but in many studies they have been used to identify asymptomatic infection [138]. Evidence indicates a significant increase of seroprevalence with age in endemic areas, even if antibody titre among asymptomatic individuals is significantly lower than in recent VL cases [138]. *Moreno et al*. suggest that serological tests are unreliable to identify asymptomatic *Leishmania* infection in comparison with molecular methods and they could underestimate the infection rates in the population in study [139]. Molecular methods are highly sensitive and specific and could be used to identifying *Leishmania* infection in individuals with low concentration of parasites and without clinical manifestations. On the other hand, fluctuations in the positivity of these methods have been found due to the

variability of the target sequences and the short half-life of parasite DNA in blood [140]. A prototype of Interferon Gamma Release Assay has recently been improved as a substitute of LST to identify individuals naturally exposed to parasite [141]. *Carvalho et al*. demonstrated that peripheral blood mononuclear cells (PBMC) from individuals with subclinical or asymptomatic infection respond to stimulation with specific *Leishmania* antigens (SLA) producing IL-2, IFN-γ and IL-10 [142]. Another study of *Ibarra-Meneses et al.* shows that IGRA exhibits higher sensitivity and specificity than LST, and it could emerge as suitable method to identify and quantify released cytokines, particularly IFN-γ and IL-2, in individuals potentially exposed to *Leishmania* [143].

1.9 Treatment of leishmaniasis

The WHO considers leishmaniasis to be one of the most important neglected diseases in the world; very little resources are devoted for drug discovery and the available drugs are few, toxic and with only one exception require intravenous/intramuscular administration.

Concerning VL, pentavalent antimonials were until recent the most used drugs for the treatment of leishmaniasis. The mechanism of action of these compounds is still poorly understood, but they appear to act directly on the molecular processes of protozoa and macrophages by inhibiting various metabolic pathways (fatty acid oxidation, glycolysis, and ATP/GTP synthesis) and inducing apoptosis. However, because of their accumulation in the tissues, antimonials can cause severe adverse effect like nausea, vomiting, anorexia, abdominal pain, myalgia, headache, lethargy, cardiac arrhythmia, arthralgia severe pancreatitis, and nephrotoxicity, [144,145]. Antimonials are associated with higher mortality

in malnourished patients, HIV-*Leishmania* co-infected patients and other underlying diseases [137] As antimonials frequently cause adverse effects, hospitalization and close monitoring of patients is required during treatment [146]. The adverse effect and long-term treatment period conduct to treatment noncompliance and abandonment of therapy, causing the emergence of resistant *Leishmania* strains [147]. Liposomal Amphotericin B (LAB) is now considered the first-line drug for VL treatment in industrialized countries. This drug possesses a dual mechanism of action, by stimulating the production of free radicals inducing oxidative stress in the protozoan cells and by altering the parasite plasma membrane permeability by forming pores through an action of inhibition of ergosterol synthesis, an essential constituent of the protozoan plasma membrane [144,145].
LAB has is less toxic than antimonials, but it it is an expensive drug [137].
The anti-*Leishmania* activity of miltefosine seems to be related to its intracellular accumulation following parasite membrane binding and internalization. Miltefosine has the advantage of oral administration, but its use is not indicated for pregnant women, because of teratogenic effect. The efficacy of miltefosine has not been clearly demonstrated in the treatment of European VL [137].
Pentamidine is currently used for treatment, but presents adverse effect such as severe hypoglycemia, diabetes mellitus, myocarditis, hypotension and nephrotoxicity, furthermore, several cases of resistance have been reported [148].
Paromomycin has parental formulations and can cause adverse reaction such as hepato-nephro-oto toxicity. This drug is not commercialized in the WHO European Region [137, 149].
An applicable strategy for treating VL is the combination of drugs, with the administration of two or more drugs with additive effects or synergistic

action to expand pharmacological activity and increase therapeutic efficacy [150]. This mode of action makes it possible to reduce the duration of therapy and the total doses of drugs, leading to an increase in patient compliance to treatment and a containment of toxic effects and costs; it also limits the possibility of selection of resistant protozoa, allowing a prolonged use of the drugs currently used [14].

Regarding CL, no international guidelines are available for treatment. In general, systemic treatment is less commonly used than for VL. Therapeutic alternatives are thermotherapy, cryotherapy, intralesional injections of antimonials, or topical application of paromomycin [151]. Cases of Old World CL are generally treated locally except in cases of multiple, extensive, or potentially disfiguring lesions; in contrast, for New World lesions, systemic treatment is generally recommended. For each patient, the therapeutic decision must be based on the risk-benefit ratio and the species causing infection. In fact, parasites belonging to *Viannia* subgenus can cause MCL, which can lead to mutilating destruction of palate, lips, nasal septum, larynx and pharynx. At the moment there is no way to forecast the development of MCL from a CL primary lesion [137].
Cases of asymptomatic infection are not treated, since the toxicity of available drugs is too high to justify their administration to healthy individuals [40]. Beside this, it is not clear whether the currently available drugs can eliminate the quiescent parasite.

1.9.1 Treatment of leishmaniasis in immunocompromised patients

In general, VL treatment of immunocompromised patients is associated with higher treatment failure, increased relapse rate, augmented drug toxicity and higher risk of treatment-associated mortality as compared with immunocompetent individuals [117].

Based on its excellent safety profile, WHO and international guidelines, propose liposomal amphotericin B as recommended treatment, with a total dose of 40 mg/kg [14].

Studies based on the use of miltefosine are limited. Combination therapy could improve the efficacy of treatment and decrease the emergence of resistance for *Leishmania*. Relapses of VL in HIV-infected patients are progressively more difficult to treat, for this reason the role of combination therapy of LAB with miltefosine and possibly other drugs is recommended to reduce toxicity and increase the efficacy of therapy [137, 152]. However, drug combinations have not yet been standardized for the treatment of VL in immunocompromised patients.

In HIV/VL patients, secondary prophylaxis considerably decreases the relapse rate and should be started after the end of the first course of therapy. The role of secondary prophylaxis in other immunocompromised patients with VL has not been defined yet [137].

2. Aim of the study

Leishmaniasis is a neglected tropical disease; besides, this parasitic disease is of public health concern in the Mediterranean Europe, including Italy. In fact, a multiannual outbreak of the visceral form (VL) has been reported in the Bologna and Modena province, northeastern Italy, and data of our group show that 12.5% of blood donors living in the same area exhibit anti-leishmanial antibodies and/or parasitic DNA in the blood [114]. These findings indicate a high prevalence of asymptomatic *Leishmania* infection in this specific area, which is important considering the risk of reactivation in immunocompromised patients. In fact, most infected individuals do not develop clinical symptoms, but the *Leishmania* parasites can persist lifelong in the host after an acute infection and easily reactivate under immunosuppressive conditions, causing severe disease with high mortality rate.

The aim of this study is to identify *Leishmania* infection in selected groups of immunocompromised (IC) patients, including newly diagnosed HIV infected individuals, patients receiving kidney transplant (KT) and patients undergoing immunosuppressive therapies for immune-mediated diseases (IMD).

In details, this study aimed to:

1) Validate a combination of methods, including detection of parasitic DNA by Real-Time PCR in peripheral blood, detection of specific IgG by Western Blot and evaluation of anti-leishmanial T-cell response by WBA to be used for the screening of asymptomatic *Leishmania* infection. The validation is performed on samples obtained from a cohort of immunocompetent individuals living in an endemic area for

leishmaniasis (Bologna Province, Emilia-Romagna region, northeastern Italy);

2) Recruit the cohorts of IC patients to screen *Leishmania* infection by the serological, molecular and immunological tests validated in part #1 and monitoring of *Leishmania*-positive patients by i) quantification of the parasite load in the blood by quantitative real-time PCR (qPCR) and ii) evaluation of anti-leishmanial T-cell response by WBA.

3. Methods

3.1 Study design and population

This study provides an identification of *Leishmania* infection in different groups of immunocompetent and IC patients.
The study was divided in two parts: 1) validation of methods for the screening of asymptomatic *Leishmania* infection in a cohort of immunocompetent individuals residing in northeastern Italy; 2) recruitment of different cohorts of immunocompromised patients for identification of *Leishmania* infection by the serological, molecular and immunological tests validated in part #1 as well as monitoring of *Leishmania*-positive patients.

Concerning the part #1, we aimed to evaluate three different methods to be used in combination for the screening of asymptomatic *Leishmania* infection in an endemic area of northeastern Italy. For this part of the study, we recruited adult volunteers residing in a selected area of the Pianoro municipality (Bologna Province, Emilia-Romagna Region). As inclusion criteria, only adults were enrolled, individuals that resided in the selected municipality for at least 5 years, and had no history of cutaneous or

visceral leishmaniasis. In addition, the selected individuals had no immunosuppression in their medical history. In this cross-sectional study, blood and sera samples were collected from each participant after signing informed consent.

Part #2 of the study aimed to identify *Leishmania* infection in selected groups of IC patients: 1) autochthonous patients recently diagnosed with HIV infection; 2) autochthonous patients receiving kidney transplant; 3) autochthonous patients undergoing immunosuppressant therapies for immune-mediated diseases. Patients included in the study were enrolled and followed at the Units of Infectious Disease, Nephrology, and Rheumatology at the hospitals of Bologna (Emilia-Romagna, Italy), Firenze (Tuscany, Italy) and Negrar (Veneto, Italy).

Recruited patients were included according to the following criteria:

- Age ≥ 18 years.
- Patients belonging to the hospitals implicated in the study.
- Patients who have been resident in Italy for at least 2 years.
- Patients with newly diagnosed HIV infection (12 months) and/or with CD4 lymphocyte count <350 cells/mm³.
- Patients receiving a kidney transplant, recruited immediately before the transplant.
- Patients with immune-mediated diseases on immunosuppressive therapies. In this longitudinal, non-interventional, multicenter cohort study, blood and sera samples were collected during scheduled visits, as part of the patients' diagnostic care pathway and after signing the informed consent to participate.

In both part#1 and part#2 of the study, blood collected from each patient was divided in 5 ml of blood in EDTA (for molecular assays, PCR), 2 ml of

blood in heparin (for cell-mediated response tests, WBA) and 5 ml of blood without anticoagulant (for serological tests, WB).

The samples were collected and processed within 24 hours at the Microbiology Unit of the University Hospital Sant'Orsola-Malpighi of Bologna.

Each sample was tested employing the following diagnostic methods:

1) Serological screening: Western Blot on serum samples to detect the presence of specific anti-*Leishmania* antibodies;

2) Molecular assay: Real-Time PCR on blood samples to detect the presence of parasitic DNA;

3) Cell-mediated response test: Whole Blood Assay on blood samples to detect specific cytokines produced after stimulation.

For each patient, 1 cryovial containing 1 ml of EDTA blood, 1 cryovial containing 1 ml of serum and 3 tubes containing 500 µl of heparin-embedded blood each, were prepared. The tubes containing heparin-embedded blood have been incubated at 37°C for 20-24 hours and then transferred to cryovials at -80°C. Cryovials containing EDTA blood and serum were stored at -20°C.

3.2 Serological screening: Western Blot (WB)

The detection of specific *Leishmania* antibodies was assessed employing Western Blot IgG diagnostic kit from LDBIO Diagnostics® (Lyon, France). The test allows the visualization of the binding between antibodies in the patient's serum and specific *L. infantum* antigens (of 14 and 16 kD, respectively) adhered to nitrocellulose strips. The test is considered positive in the presence of the bands P14 and P16.

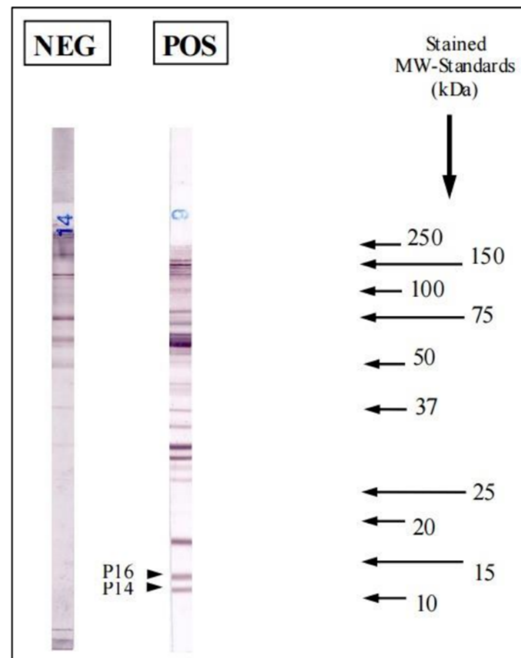


Fig 10. WB strips of negative sample and positive sample with P14 and P16

3.3 Molecular screening: Real Time PCR

Home-made Real-Time PCR able to detect *Leishmania* Kinetoplast(k)DNA on peripheral blood and bone marrow was performed. Nucleic acids were extracted using a semi-automatic DNA extraction system and subsequently a qualitative Real-Time PCR was performed to determine the presence of *Leishmania* DNA.

DNA extraction from peripheral blood was performed using PROMEGA Maxwell® 16 LEV Blood DNA kit on the Maxwell® 16 instrument manufactured by PROMEGA® (Madison, Wisconsin, USA). Extraction was performed in two steps: the first step requires external thermal lysis of the sample (30 minutes), and the second step involves automated purification of the DNA inside the instrument by binding nucleic acid with magnetized silica particles (45 minutes).

At the end of extraction, DNA was amplified employing a Real-Time PCR assay with kDNA of *L. infantum* as molecular target [130]. The target has been chosen to increase the sensitivity of the method, as kDNA has multiple number of copies per cell (from 10^2 to 10^4) [130]. Primers sequences were RV1 (5'-CTTTTCTGGTCCTCCGGGTAGG3') 15 pmol, RV2 (5'- CCACCCGGCCCTATTTTACACCAA-3') 15 pmol (Integrate DNA technologies, Leuven, Belgium) and TaqMan® probe was (5'-TTTTCGCAGAACGCCTACCCGC3') 50 pmol (Thermo Fisher Scientific, Waltham,USA).

REAGENT	VOLUME
H_2O Nuclease Free	6 µl
Premix Ex Taq 2X	12.5 µl
FW Primer (10 µM)	0.5 µl
RV Primer (10 µM)	0.5 µl
Probe (1 µM)	0.5 µl
Sample	5 µl

Tab 1. Schematic diagram of the composition of the reaction mixture for Real Time PCR

To assess the amplification of the extracted DNA, an housekeeping gene expressed in humans cells (β2 microglobulin gene) was run simultaneously.

The instrument used to perform Real-Time PCR was the Rotor-Gene Q® QIAGEN® (Hilden, Germany), the amplification profile shown in Tab. 2 was used.

95°C x 2 min
95°C x 15 sec
60°C x 1 min (45 cycles)

Tab 2. Real-Time PCR amplification profile for the detection of DNA of Leishmania

Samples with Cycle threshold (CT) up to 40 cycles were considered positive.

Moreover, a quantitative Real-Time PCR (qPCR) was performed on the samples tested positive. The qPCR was executed interpolating the CT value obtained with a standard curve. The curve was assessed starting from a culture of *L. infantum* with known concentration of parasites, expressed in terms of parasites/ml (5×10^{6} parasites/ml), making serial dilution up to a final concentration of 5×10^{-2}parasites/ml (Fig.11).

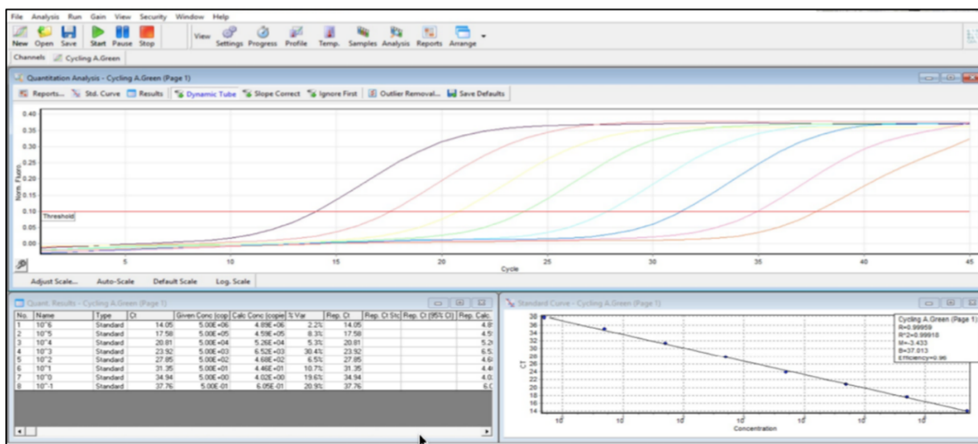


Fig 11. Amplification curves of 8 different points of the standard curve representing the different concentration of Leishmania

This is a semi-quantitative quantification, since the molecular target is a region of the minicircle of kDNA, that is expressed in each protozoan in multiple copies (from 1000 to 10000), making it impossible to have an absolute estimate. Therefore, the final concentration of *Leishmania* in blood samples was expressed as equivalent parasites/ml.

3.4 Evaluation of cell-mediated response test Whole Blood Assay (WBA)

Blood samples were collected from patients in heparin-tubes. Aliquots of blood (500 µl) were incubated in three different tubes:

1. Yellow top tube: negative control;
2. Blue top tube: coated with 10 mg/L of SLA, which stimulates immune cells of whole blood to produce different combination of cytokines;
3. Red cap tube: positive control, containing the mitogen Phytohemagglutinin (PHA), a non-specific activator of the immune response.

After adding the whole blood and mixing meticulously, the tubes were incubated for 24h at 37°C. After centrifugation at 2000g for 10 min, plasma were obtained and stored at -80°C for cytokines analysis. Briefly, the plasma of each individual was incubated with 50 µl of capture beads and after one hour of incubation, 50 µl of detection antibody was included in the mixture and placed 2 hours at room temperature.
Concerning part #2,
IFN-γ, IP-10, and IL-2 were quantified in 50 µl of plasma employing the Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson, Franklin Lakes, NJ, USA) and the method was performed using the BD FACSCanto® cytofluorometer (Becton Dickinson, Franklin Lakes, NJ, USA) (Fig. 12). Conversely, in part #1 of the study only IL-2 was quantified.
Data were acquired employing the Flow Cytometer Analysis Program Array (FCAP 3.0.1, Beckton Dickinson Biosciences, USA). Results for each cytokine were reported as the difference between SLA-stimulated

and control plasma concentrations expressed in pg/ml. The cut-off for each cytokine was measured calculating the area under the ROC (Recevier Operating Characteristic) Curve with a 95% confidence interval and a p-value < 0.05. In the study the ROC Curve and the cut-off for each cytokine were validated by Carrillo and collaborators at the Instituto De Salud Carlos III (Madrid, Spain).

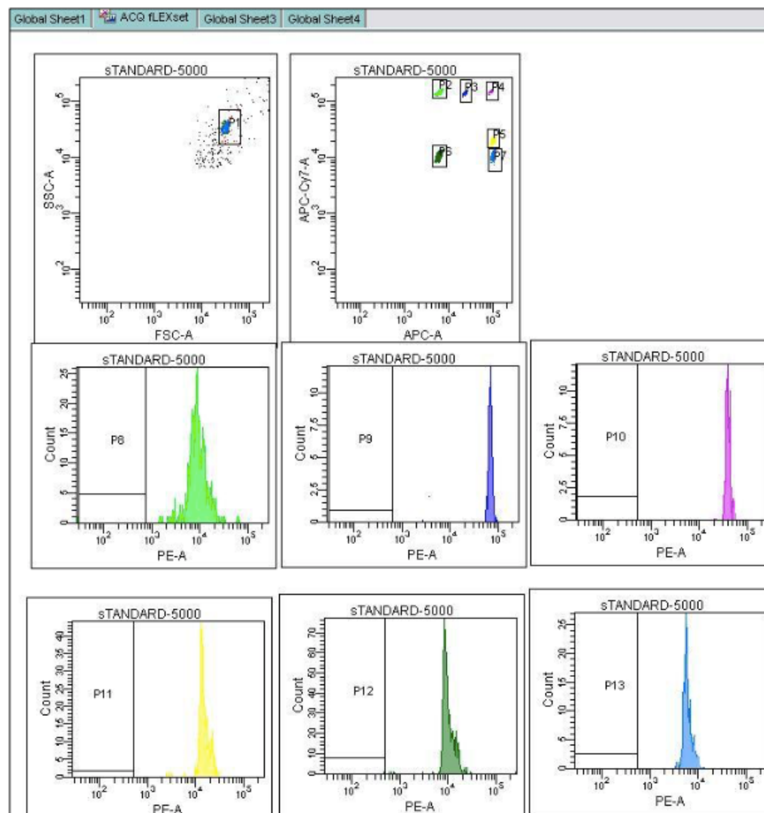


Fig 12. Example of quantification of cytokines employing Flow Cytometer Analysis Program Array

4. Results

The study is divided in two parts: 1) Validation of methods for the screening of asymptomatic *Leishmania* infection in a cohort of immunocompetent individuals residing in northeastern Italy; 2)

Recruitment of different cohorts of immunocompromised patients for identification of *Leishmania* infection by the serological, molecular and immunological tests validated in part #1 and monitoring of *Leishmania*-positive patients.

Concerning the validation of methods for the screening of asymptomatic *Leishmania* infection in a cohort of immunocompetent individuals residing in northeastern Italy (part #1 of the study), we aimed to validate a combination of methods to test 145 volunteers residing in an endemic area for leishmaniasis, ie a selected part of the municipality of Pianoro, in the Bologna province, Emilia-Romagna region, northeastern Italy. The study was conducted in collaboration with the Department of Public Health of the Local Health Autority (Azienda Sanitaria Locale ASL) of Bologna and was approved by the Ethics Committee of the Area Vasta Emilia Centro (CE-AVEC) with protocol number 764/2019.

Regarding to the part #2 of the study, 198 immunocompromised patients were enrolled by the St.Orsola-Malpighi University Hospital of Bologna and the Careggi University Hospital of Florence (Italy) in order to test for asymptomatic *Leishmania* infection and to monitor *Leishmania*-positive patients. Written informed consent was obtained from each patient before sampling. As samples were anonymized, an alphanumeric code was created for each recruited patient, consisting of an acronym indicating the type of immunosuppression ("TRX" for kidney transplant recipients, "HI" for HIV-positive patients, and "REU" for patients with immune-mediated diseases undergoing immunosuppressive therapy) followed by a progressive number.
This study was approved by the Ethics Committee of CE-AVEC on 28/05/2018 (study no. 144/2018/Sper/AOUBO).

4.1 Validation of methods for screening of asymptomatic *Leishmania* infection

The Unit of Hygiene and Public Health, Department of Public Health of Bologna enrolled a cohort of 145 volunteers residing in Pianoro in order to validate and standardize tools for screening of asymptomatic *Leishmania* infection.

The individuals were 67 male (46%) and 78 female (54%), with a mean age of 58 years.

All volunteers were of Caucasian ethnicity; 143 individuals were born in Italy, specifically 102 (70%) were born in the Emilia-Romagna region.

For each individual, 10 ml of peripheral blood were collected and divided into 3 test tubes; one tube with EDTA as anticoagulant, one tube with heparin as anticoagulant, and one tube without anticoagulant.

The samples collected were tested by three techniques: Real-Time PCR to detect and quantify the presence of DNA of *Leishmania* in peripheral blood, WB to identify the presence/absence of antileishmanial IgG in serum and WBA to evaluate the *Leishmania*-specific cell-mediated immune response. Blood samples were transported within 24 hours to the Unit of Microbiology of the St.Orsola-Malpighi University Hospital, Bologna, Italy.

4.1.1 Serological screening (WB)

Among the 145 volunteers enrolled, 15 were positive for the presence of anti-*Leishmania* IgG antibodies in serum by using WB assay. In details, 5 subjects showed positivity to the p14 band, 7 to the p16 band, and 4 to both protein bands present on the strip. Among the 15 individuals who tested positive, 8 were females (53%) while 7 were males (47%). The mean age of the positive individuals was 60 years.

4.1.2 Molecular screening (Real-Time PCR)

Molecular screening was performed to detect the presence of kDNA of *Leishmania* in peripheral blood of the volunteers by Real-Time PCR. The method was validated to determine the presence of kDNA in the peripheral blood of the recruited individuals; the sensitivity of our test was 0.05 parasites/ml. Among the 145 volunteers, 6 individuals resulted positive for the presence of kDNA of *Leishmania*, of which 5 were females (80%) and 1 individual was male (20%), the mean age of the positive individuals was 49 years. Parasite load was quantified in the samples of PCR-positive individuals; results indicated low parasitemia for all the positive volunteers, with a mean parasite load of 1.23×10^{-1} /ml of blood and a mean Ct of 37.83.

4.1.3 Screening for specific cell-mediated immunity (WBA)

The Whole Blood Assay (WBA) was used to assess the cell-mediated response of volunteers after stimulation of whole blood with *Leishmania*-specific antigens (SLA). The method is bases on the quantification of IL-2 production upon SLA stimulation using FACSCanto cytofluorometer. Among the 145 subjects, 12 (8%) were positive with an IL-2 value above

the cut-off of positivity. Among these positive individuals, 5 were females (42%) and 7 (58%) were males with a mean age of 66 years.

4.1.4 Analysis of the screening tools

We arbitrarly considered as positive for cryptic *Leishmania* infection each individual that tested positive to at least one of the three methods; positive individuals were 23 on a total of 145, with a frequence of asymptomatic infection of 17%. There was no concordance when comparing Real Time PCR with WB and WBA, i.e. none of the subjects that tested positive for kDNA in the blood was also positive for the immunological methods, ie serology and WBA. Conversely,10 individuals tested positive for both WBA and WB, while 5 and 2 subjects were positive only for WB or WBA, respectively (Fig. 13). Therefore, the concordance index (Cohen's Kappa) that was calculated by comparing the results of WB and WBA was 0.63 (63%).

The concordance index was interpreted using the Landis scoring grid and substantial concordance was identified between WB and WBA.

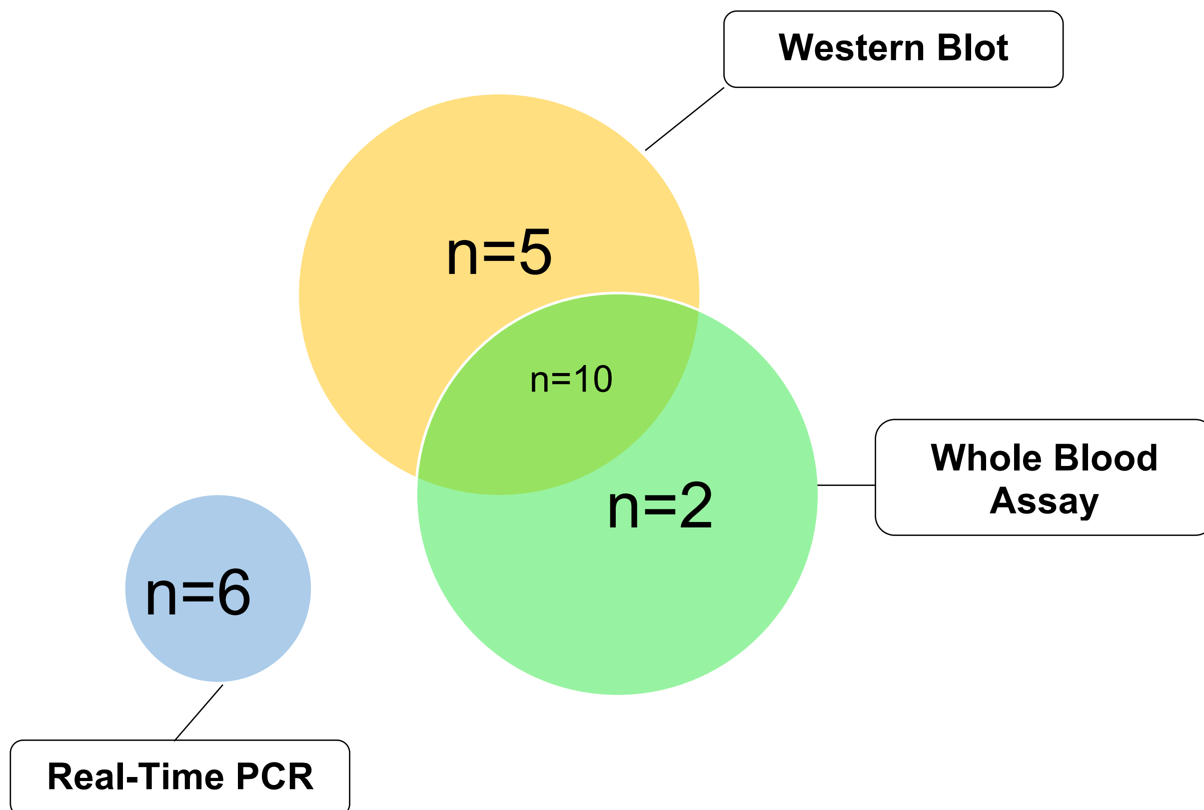


Fig. 13: Type of positive Leishmania markers, n= 23 blood samples from immunocompetent individuals.

4.2 Screening for *Leishmania* infection of immunocompromised patients

The immunocompromised patients recruited in the study by the research unit of Bologna and Firenze were 198, including 118 patients receiving kidney transplant, 76 patients recently diagnosed with HIV infection and/or with CD4+ T cell count <350 cells/mm³, and 4 patients undergoing immunosuppressive therapies for immune mediated diseases. For each individual, 10 ml of peripheral blood were collected and divided into 3 test tubes; one tube with EDTA as anticoagulant, one tube with heparin as anticoagulant, and one tube without anticoagulant.

In the case of patients enrolled in Bologna, the samples were delivered within 24 hours of collection to the researchers participating in the project at the Parasitology Laboratory of the Microbiology Unit of St.Orsola-Malpighi University Hospital of Bologna. On the contrary, in the case of enrollment in Florence, blood samples were stored at -80°C at the Infectious Diseases Unit in Careggi Universitary Hospital, and periodically sent to Bologna for diagnostic tests.
To date, Real-Time PCR and WB have been performed on the whole IC population, while WBA has been evaluated on 155/198 patients (78%).

The immunocompromised patients undergoing diagnostic tests for *Leishmania* infection consisted of 139 males (70%) and 57 females (29%). In 2 cases, the sex of the patient was unknown (1%). The mean age of the tested subjects was 54 years (range 18-81). The majority of patients were born in Italy (168/198, 83.3%), the remaining patients had different nationalities: 6 patients were from Albania (3%), 4 patients from Peru (2%), 2 patients from Moldova (1%), 2 patients from Senegal (1%), 2 patients from Brazil (1%), 2 patients from India (1%), 2 patients from Argentina (1%), 1 patients from Pakistan (1%), 1 patient from Nigeria (0,5%), 1 patient from Russia (0.5%), 1 patient from Ghana (0.5%), 1 patient from the Philippines (0.5%), 1 patient from El Salvador (0.5%), 1 patient from Morocco (0.5%), 1 patient from Poland (0.5%), 1 patient from Serbia (0.5%), 1 patient from Syria (0.5%), 1 patient from Switzerland (0.5%), 1 patient from Chile (0.5%), and 1 patient from Cameroon (0.5%). Regardless the origin, all patients included in the study have been residing in northeastern or central Italy for at least 2 years.

At the time of recruitment, 3 out of 198 patients presented clinical signs of VL, including 2 patients with immune-mediated diseases undergoing

immunosuppressive treatment and one HIV-positive patient; the parasitic disease was confirmed in all 3 patients by the detection of high parasite load in peripheral blood samples employing real-time PCR.

4.2.1 Serological screening in IC patients

The serological screening was performed with WB. Considering the data from immunocompromised patients overall, of 198 patients tested, 38 were positive at WB (19%, Figure 15), of whom 19 showed positivity for the p14 band (50%), 12 for the p16 band (32%), and 7 for both bands (18%).

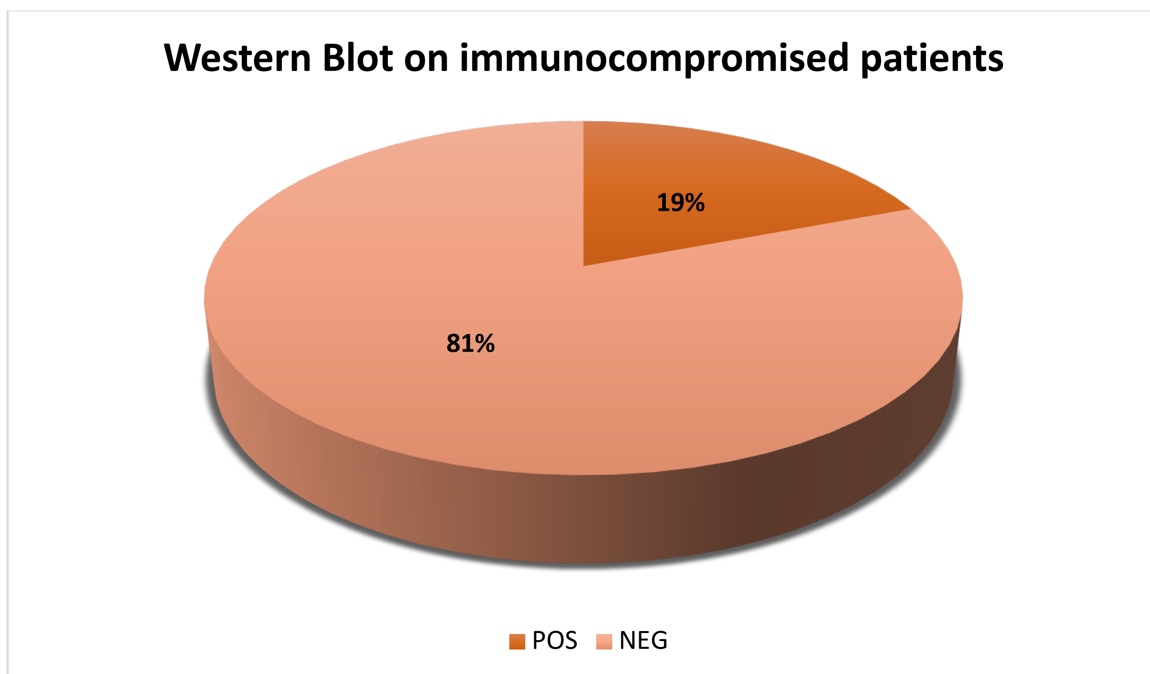


Fig. 14 Graphical representation of the results obtained with WB (n=198 immunocompromised patients). POS; positive, NEG; negative

4.2.1.1 Serological screening in kidney transplant recipients

Anti-*Leishmania* IgG were detected in 24 out of 118 kidney transplant recipients (20%). Specifically, 14 out of 24 (58%) positive samples recognized the 14-kD antigen (p14 band), 7 (29%) sera recognized the 16-kDa antigen (p16 band) and 3 (13%) sera recognized both the specific bands (p14-p16, Figure 15). Following the manufacturers' instructions, sera were considered WB positive if one band or both bands were detectable.

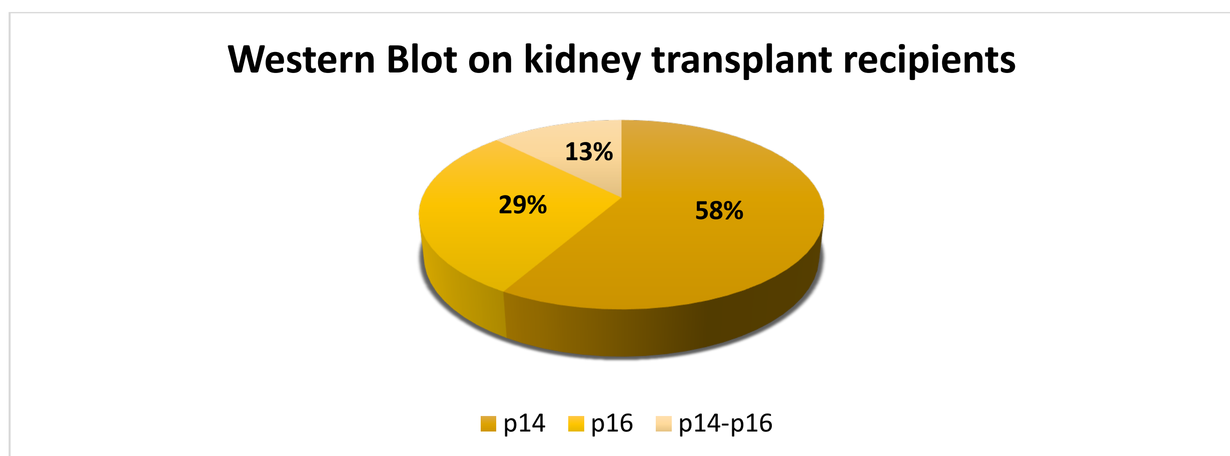


Fig. 15 Graphical representation of the results obtained with WB (n= 118 kidney transplant recipients). POS; positive, NEG; negative

4.2.1.2 Serological screening in HIV-positive patients

Anti-*Leishmania* IgG were detected in 12 out of 76 HIV-positive patients (16%). Specifically, 5 out of 12 (42%) positive samples recognized the 14-kD antigen (p14 band), 5 (42%) sera recognized the 16-kDa antigen (p16 band) and 2 (16%) sera recognized both the specific bands (p14-p16, Figure 16).

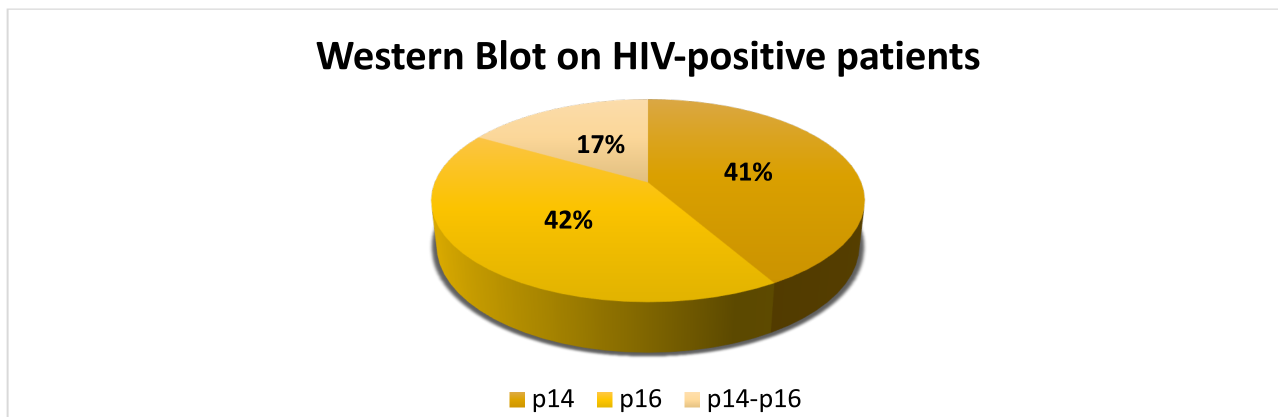


Fig. 16 Graphical representation of the results obtained with WB o(n=76 HIV-positive patients).

4.2.1.3 Serological screening in patients undergoing immunosuppressive therapy for immune-mediated diseases

In the cohort of patients undergoing immunosuppressive therapy for immune-mediated disease, only 4 patients could be recruited so far. Among these patients, 2 tested positive for the presence of anti-*Leishmania* antibody (50%), each single 2 sera (100%) recognized both the specific bands (p14-p16).

4.2.2 Molecular screening in immunocompromised patients (Real-Time PCR)

In order to perform a molecular screening of immunocompromised patients, Real-Time PCR to determine the presence of kDNA in the peripheral blood of the patients was employed. In case of positive result, quantification of the parasite load was carried out. Among the whole population of immunocompromised patients, 8/198 (4%) resulted positive for the presence of *Leishmania* kDNA (Figure 17).

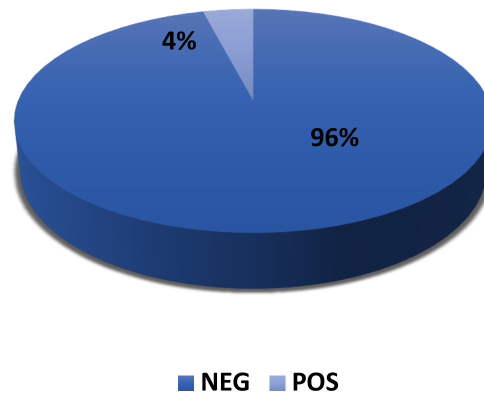


Fig. 17 Graphical representation of the results obtained with Real-Time PCR for the detection of kinetoplast DNA(kDNA) (n=198 immunocompromised patients). POS; positive, NEG; negative

4.2.2.1 Molecular screening in kidney transplant recipients

Leishmania kDNA was detected by Real-Time PCR in 5/118 (4.2%) kidney transplant recipients. The samples that tested PCR-positive have been further analysed to calculate the parasite load; the parasitemia was low in all 5 patients as shown in Table 3.

	Parasite equivalents/ml
TRX35	0.2
TRX67	0.1
TRX68	139
TRX69	0.4
TRX106	0.1

Tab.3: Quantification of kDNA of Leishmania in n=5 peripheral blood samples from kidney transplant recipients that tested positive by Real-Time PCR.

4.2.2.2 Molecular screening in HIV-positive patients

Among 76 HIV-positive patients, only 1 patient tested positive for the presence of kDNA of *Leishmania* (1.3%). This patient exhibited the clinical manifestation of VL disease and had a high parasite load (1230 parasite equivalents/ml).

4.2.2.3 Molecular screening in patients undergoing immunosuppressive therapy for immune-mediated diseases

Two out of 4 patients undergoing immunosuppressive therapy for immune-mediated diseases tested positive by Real-time PCR (50%). Both these patients exhibited the clinical symptoms of VL, and the parasite load in the samples was high in either case (Table 4)

	Parasite equivalents/ml
REU1	21.000
REU2	1220

Tab.4: Quantification of kDNA of Leishmania in n=2 peripheral blood samples from patients undergoing immunosuppressive therapy for immune-mediated diseases that tested positive by Real-Time PCR. .

4.2.3 Screening for *Leishmania* cell-mediated immunity in immunocompromised patients

The WBA was based on the quantification of IFN-γ, IL-2, and IP-10 in the serum of patients after stimulation of peripheral blood with specific *Leishmania* antigens. The quantification of cytokines was assessed using a cytofluorometer.
Samples were considered positive when the amount of any of these analytes exceeded a certain cut-off value, previously calculated with the collaboration of the WHO Collaborating Center for Leishmaniasis, at the Instituto De Salud Carlos III (Madrid, Spain) on different categories of immunocompromised patients. The cut-off values for each analyte are shown in Tables 6a-c. Thirty one out of 155 blood samples obtained from immunocompromised patients (26%) tested positive for one of more cytokines by WBA (Fig. 18, Fig. 19). The concentration of cytokine levels for each WBA-positive patient are reported in Table 6.

TRX	
Cytokine	**Cut-off (pg/ml)**
IFN-γ	21
IL-2	35
IP-10	762

Tab.5a: Cut-off values calculated for IFN-γ, IL-2, and IP-10 in kidney transplant recipients.

HI	
Cytokine	**Cut-off (pg/ml)**
IFN-γ	12
IL-2	32
IP-10	1179

Tab. 5b: Cut-off values calculated for IFN-γ, IL-2, and IP-10 in HIV-positive patients.

REU	
Cytokine	**Cut-off (pg/ml)**
IFN-γ	96
IL-2	67
IP-10	208

Tab. 5c: Cut-off values calculated for each cytokine in patients undergoing immunosuppressive therapy for immune-mediated diseases.

	IP-10 pg/ml	IL-2 pg/ml	IFN-γ pg/ml
TRX3	3731	44	3
TRX4	2473	21	4
TRX5	313	53	0
TRX6	2512	21	1
TRX13	2316	4	0
TRX17	3479	346	69
TRX21	1035	13	0
TRX23	826	22	0
TRX24	923	15	3
TRX32	2900	72	10
TRX34	1157	100	1
TRX35	5043	61	6
TRX43	1725	33	1
TRX44	11114	3401	820
TRX45	3951	25	6
TRX48	1104	15	2
TRX54	9182	39	3
HI15	1353	1	1
HI18	16141	1265	473
HI 12	4700	344	94
HI 14	2285	7	2
HI 24	1758	3	1
HI 28	1265	7	1
HI 35	10625	2	3
HI 48	1497	0	1
HI 52	1207	1	0
HI 53	1721	10	2

HI 63	6050	285	15
HI 80	1551	1	0
REU1	291026	103	67
REU2	8258	1	13

Tab. 6: Concentration of cytokines in sera of immunocompromised patients after SLA stimulation. TRX: patients reciveing kidney transplant, HI: HIV-positive patients, REU: patients undergoing immunosuppressive therapy for immune-mediated diseases. The values highlighted in yellow are above the cut-off level and are considered positive.

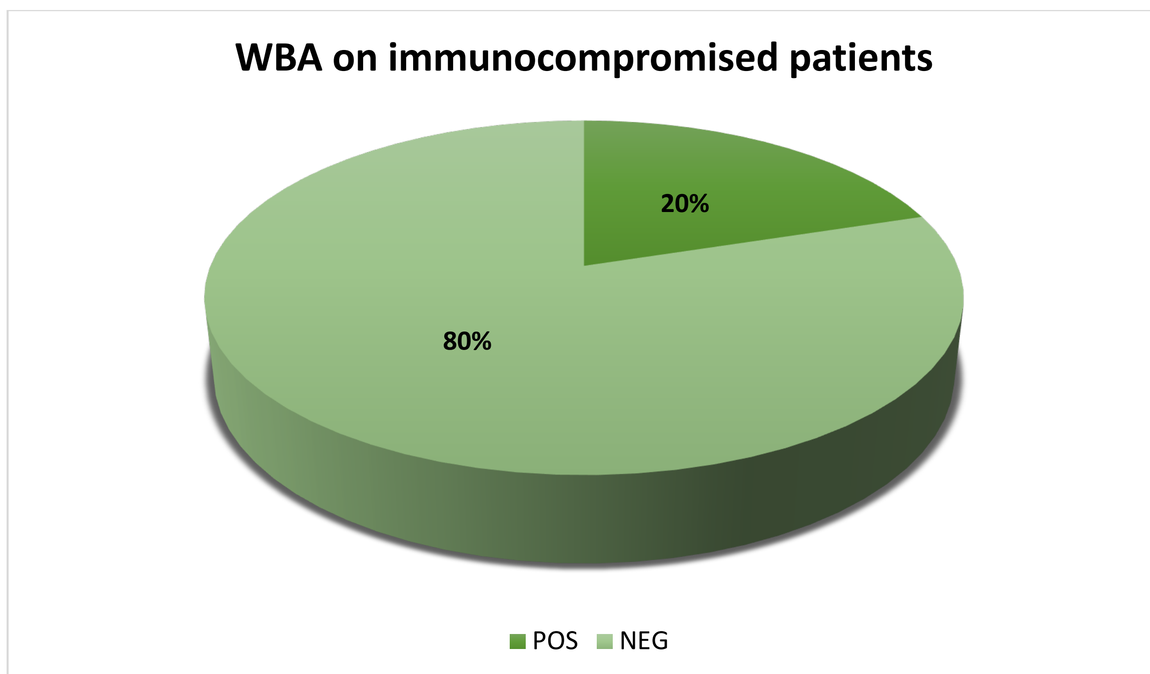


Fig. 18 Graphical representation of the results obtained with WBA (n=155 immunocompromised patients). POS; positive, NEG; negative

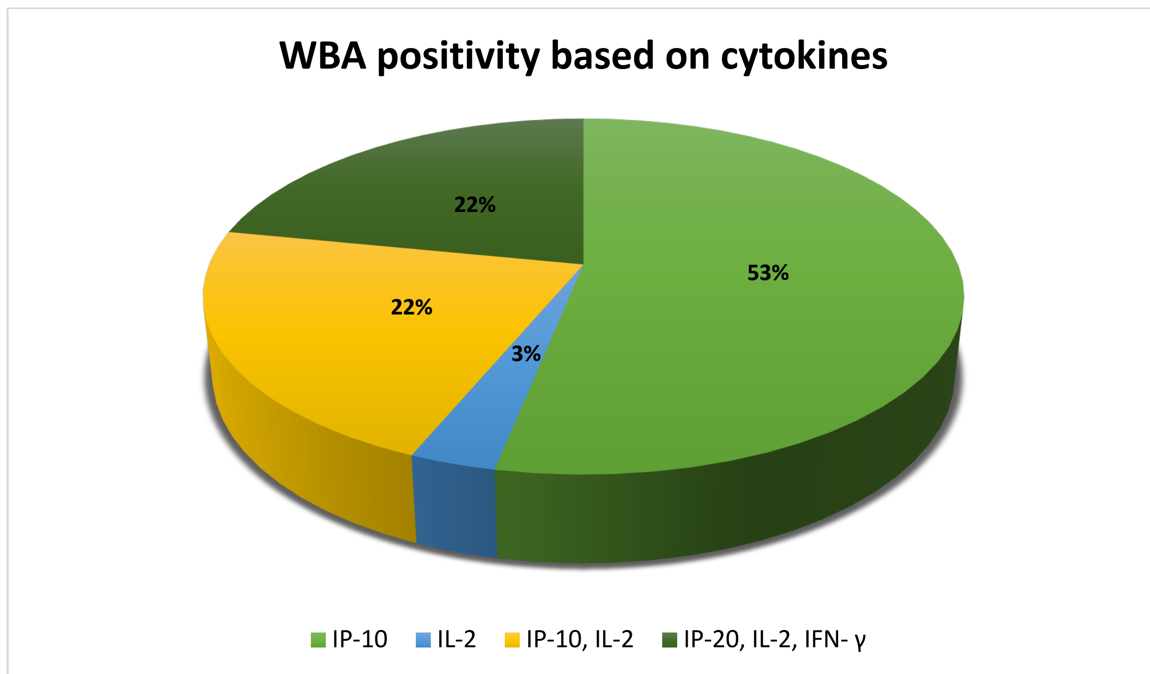


Fig. 19 Graphical representation of cytokines resulted over the value of cut-off on the 31 patients tested positive to WBA

4.2.3.1 Screening for *Leishmania* cell-mediated immunity in kidney transplant recipients

WBA was performed on blood samples from 78 patients receiving kidney transplant, of whom 17 resulted positive (22%, Figure 20).

Among 17 positive samples, 9 were positive for IP-10 (53%), 1 for IL-2 (6%), 0 for IFN-γ, moreover, 6 samples tested positive for IL-2 and IP-10 (35%), and 1 sample for all tested cytokines (6%) (Fig. 20).

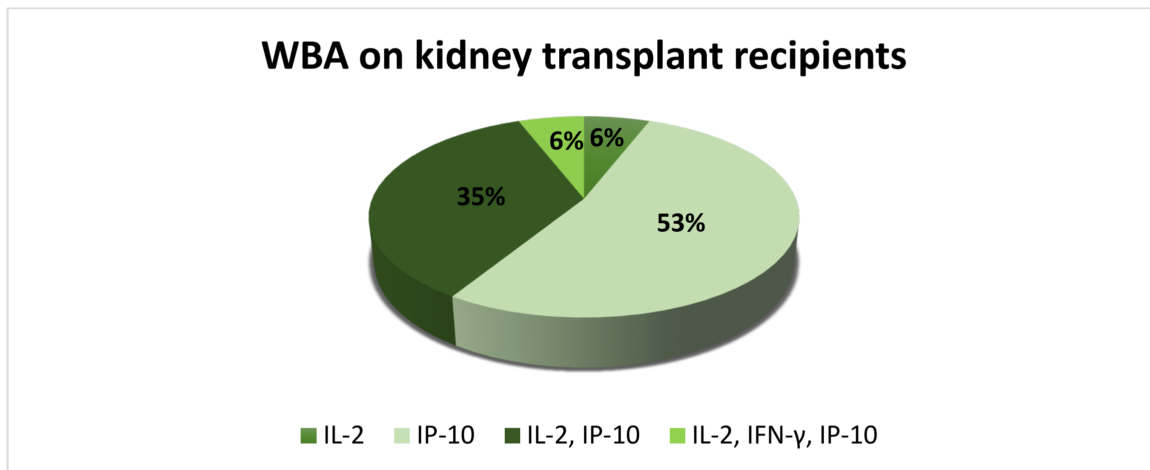


Fig. 20 Graphical representation of the results obtained on 17 kidney transplant recipients that tested positive by WBA.

4.2.3.2 Screening for *Leishmania* cell-mediated immunity in HIV-positive patients

WBA was performed on peripheral blood samples from a cohort of 72 HIV-positive patients and resulted positive in 12 patients (17%). Specifically, 9 patients tested positive for IP-10 (75%), 0 patients tested positive for IL-2 or IFN-γ only, 3 patients resulted positive for all the cytokines (25%, Figure 21).

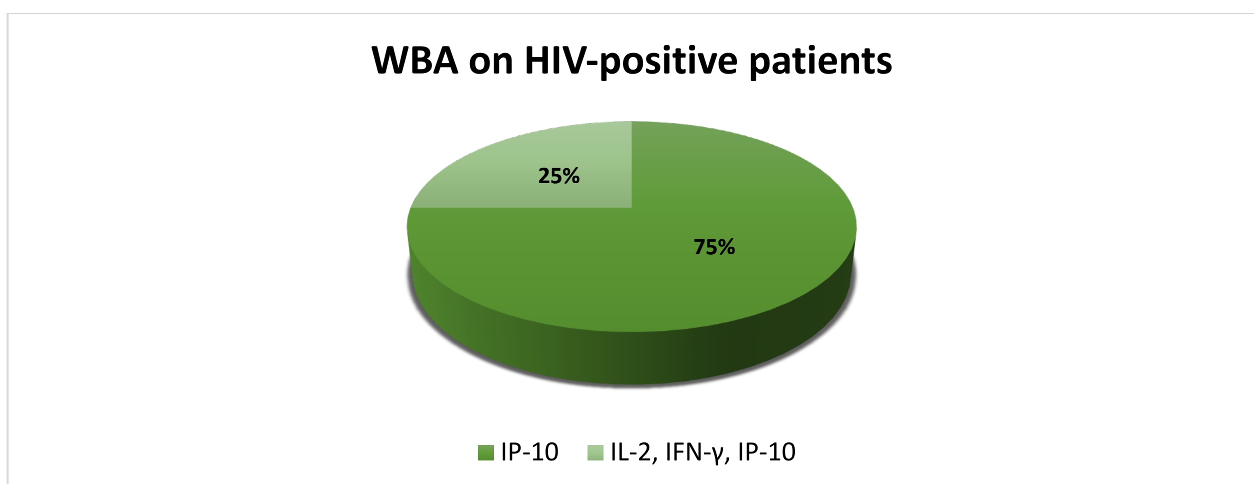


Fig. 21 Graphical representation of the results obtained with WBA on n=12 HIV-positive patients that tested positive by WBA.

4.2.3.3 Screening for *Leishmania* cell-mediated immunity in patients undergoing immunosuppressive therapy for immune-mediated diseases

WBA was performed on the 4 patients undergoing immunosuppressive therapy for immune-mediated diseases. Two patients resulted positive (50%), both the patients manifested clinical symptoms of VL. One patient was positive for IP-10 only, the second patient was positive for IP-10 and IL-2.

4.2.4 Analysis of the screening results

After the validation of the methods that was performed in the first part of the study on immunocompetent individuals, we arbitrarly considered as having *Leishmania* infection each immunocompromised patient that tested positive to one or more diagnostic methods, including WB, Real-Time PCR and WBA. We recruited 198 immunocompromised patients of whom 118 kidney transplant recipients, 76 HIV-positive patients and 4 patients undergoing immunosuppressive therapy for immune-mediated diseases. Sixty-two out of 198 immunocompromised patients (31%) tested positive to one or more screening tests; of these 62 patients, 38 were kidney transplant recipients (61%), 22 were HIV-positive patients (36%) and 2 were patients undergoing immunosuppressive therapy for immune-mediated diseases (3%).

The concordance between the tests was analyzed; only results obtained from 155 patients that were tested with all the three methods could be evaluated to standardize the analysis of results. Of these 155 samples, 57 tested positive with at least one method (37%). The evaluation of concordance between the different tests has been determined using Cohen's Kappa coefficient (Tab.7 a-c).

		WB	
		NEG	POS
PCR	NEG	118	30
	POS	3	4

Tab. 7a: Evaluation of concordance between Real-Time PCR and WB (n=155).
Cohen's kappa= 0.13.

		WB	
		NEG	POS
WBA	NEG	102	23
	POS	21	9

Tab. 7b: Evaluation of concordance between WB and WBA (n=155).
Cohen's kappa=0.11.

		WBA	
		NEG	POS
PCR	NEG	121	28
	POS	3	3

Tab. 7c: Evaluation of concordance between WBA and PCR (n=155)
Cohen's kappa= 0.06.

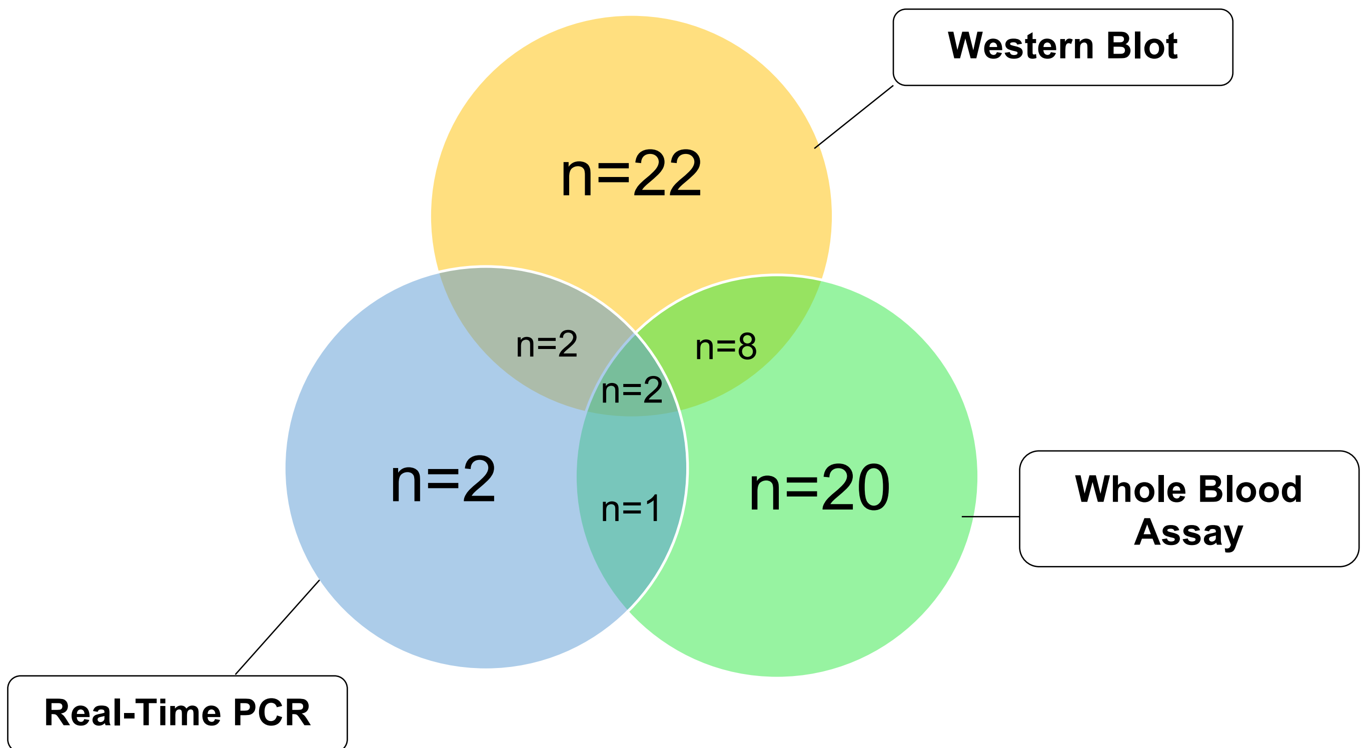


Fig. 22: Type of positive Leishmania markers at the time of enrollment, n= 57 immunocompromised patients

4.3 Monitoring of immunocompromised patients that tested positive for *Leishmania* infection

Of the total of 62 immunocompromised patients that tested positive for *Leishmania* infection, VL was diagnosed in 3 patients at the time of recruitment. Two of these patients are included in the group of patients undergoing immunosuppressive therapy for immune-mediated diseases, and they entered a 3-month monitoring of parasite load and cell-mediated immune response by WBA. These patients are currently negative for the presence of *Leishmania* DNA in the blood at three months follow up, while WBA has not been analyzed yet.

The third patient with VL was a HIV-positive patient that was lost at follow-up due to patient now referring to a different clinical center. Regarding the remaining 59 *Leishmania*-positive immunocompromised patients, 26 patients started the follow up with collection of blood samples and evaluation by real-time PCR and WBA every three months. Currently, none of the 26 patients that came for the first follow up visit (FU1) showed circulating DNA of *Leishmania*.

Moreover, two patients had their second visit (FU2) and the absence of parasitic DNA was confirmed at 6 months after recruitment. The preliminary results of monitoring are reported in Table 8. In addition, 5 out of the 10 monitored patients that tested positive for WBA at recruitment were WBA positive at the first follow up visit. Of these patients, only one performed the second follow up visit and was still positive for WBA.

		Screening test resulted positive	***Real-Time PCR (FU1)***	***WBA (FU1)***	***Real-Time PCR (FU2)***	***WBA (FU2)***
1	**TRX2**	*WB*	*NEG*	*NEG*	*ND*	*ND*
2	**TRX5**	*WBA*	*NEG*	*NEG*	*ND*	*ND*
3	**TRX6**	*WBA*	*NEG*	*POS*	*ND*	*ND*
4	**TRX11**	*WB*	*NEG*	*NEG*	*ND*	*ND*
5	**TRX13**	*WBA*	*NEG*	*NEG*	*ND*	*ND*
6	**TRX14**	*WB*	*NEG*	*NEG*	*ND*	*ND*
7	**TRX17**	*WB+WBA*	*NEG*	*POS*	*NEG*	*POS*
8	**TRX19**	*WB*	*NEG*	*POS*	*ND*	*ND*
9	**TRX21**	*WB+WBA*	*NEG*	*NEG*	*ND*	*ND*
10	**TRX24**	*WBA*	*NEG*	*NEG*	*ND*	*ND*
11	**TRX28**	*WB*	*NEG*	*NEG*	*NEG*	*NEG*

12	**TRX29**	*WB*	*NEG*	*NEG*	*NEG*	*NEG*
13	**TRX35**	*WBA+PCR*	*NEG*	*NEG*	*ND*	*ND*
14	**TRX43**	*WB+WBA*	*NEG*	*NEG*	*ND*	*ND*
15	**TRX46**	*WB*	*NEG*	*NEG*	*ND*	*ND*
16	**TRX48**	*WB+WBA*	*NEG*	*POS*	*ND*	*ND*
17	**TRX51**	*WB*	*NEG*	*NEG*	*ND*	*ND*
18	**TRX52**	*WB*	*NEG*	*NEG*	*ND*	*ND*
19	**TRX54**	*WB+WBA*	*NEG*	*POS*	*ND*	*ND*
20	**TRX68**	*WB+PCR*	*NEG*	*NEG*	*ND*	*ND*
21	**TRX69**	*PCR*	*NEG*	*NEG*	*ND*	*ND*
22	**TRX74**	*WB*	*NEG*	*NEG*	*ND*	*ND*
23	**TRX83**	*WB*	*NEG*	*NEG*	*ND*	*ND*
24	**TRX84**	*WB*	*NEG*	*NEG*	*ND*	*ND*
25	**REU1**	*WB+PCR+WBA*	*NEG*	*ND*	*ND*	*ND*
26	**REU2**	*WB+PCR+WBA*	*NEG*	*ND*	*ND*	*ND*

Tab.8: Monitoring of immunocompromised patients that tested positive for Leishmania infection at the screening test. Follow up was carried out by Real-Time PCR and WBA at three months (FU1) and at six months (FU2) after screening. POS= positive, NEG= negative; ND= not performed.

5. Discussion and conclusion

Leishmaniasis is considered an emerging infectious disease and a public health threat in Europe; because of global warming this infection could spread to non-endemic countries. In Italy, cases of leishmaniasis have been spreading northward in the last decade with an increase of VL cases in north-eastern Italy [114].

It is difficult to calculate the real prevalence of *Leishmania* infection as the majority of infected individuals do not manifest clinical signs of the disease. The identification of asymptomatic carriers is crucial; in fact, the potential reactivation of a quiescent parasitic infection represents a threat to immunocompromised individuals.

Nowadays, the main cause of *Leishmania* reactivation is co-infection with HIV, but severe leishmaniasis has been also observed in non-HIV-related immunosuppressive conditions such as solid organ or bone marrow transplants and rheumatological diseases [53]. Considering the increase of the number of immunocompromised patients in Europe, and the consequent increase of VL cases, the identification of asymptomatic carriers in endemic areas is of importance within the leishmaniasis control and monitoring programs [53,118].

To date, an optimal screening test for asymptomatic *Leishmania* infection has not been identified, and classical diagnostic methods are inefficient in detecting cryptic infection in both immunocompetent and immunocompromised individuals.

The aim of this study was to employ and validate a combination of methods for the screening of asymptomatic *Leishmania* infection in immunocompetent and immunocompromised individuals.

Development and validation of a diagnostic protocol is a process that consists in two parts: 1) determination of feasibility of the methods,

optimization of the methods and determination of the performance; 2) monitoring of protocol performance during ordinary use of the methods.

We started the study selecting from the literature the most sensitive methods for detection of *Leishmania* infection, including detection of parasitic DNA in peripheral blood by Real-Time PCR, detection of specific IgG by Western Blot and evaluation of anti-leishmanial T-cell response by WBA in whole blood upon stimulation with SLA.

We validated a Real-Time PCR with optimal sensitivity in order to detect *Leishmania* DNA in blood of patients. To reach this aim, we selected a proved sensitive molecular target such as kDNA, which is present in about 10^4 copies per parasite [154]. *Lachaud et al*. demonstrated that the sensitivity of kDNA-based PCR is 10^{-3} parasites per tube [155]; in our setting, we obtained a sensitivity of 10^{-2} parasites/ml.

Ultrasensitive PCR can be useful to detect the parasite even in asymptomatic cases [139]. Nevertheless, the demonstration of parasites may difficult in asymptomatic infections; cryptic infections are generally related to very low or undetectable parasitemia and PCR positivity could be fluctuating; for this reason immunological methods are considered an useful addition.

Regarding to serological tests, *Mary et al.* demonstrated a higher sensitivity of WB in comparison to ELISA and IFAT in detection of VL, including HIV-positive patients [156]. In the study of *Mary et al.* the antigens 14-kD and 16-kD appeared to be the best molecules for serodiagnosis of VL because IgG against these antigens were detected in the sera of all patients and no cross-reactivity was present.

Other sensitive and specific methods can be considered to detect cryptic *Leishmania* infection. For example, development of a sensitive method to detect the specific cell-mediated immune response to *Leishmania* could resemble the IGRA test in detection of latent tuberculosis. WBA was found to be an useful tool for screening and epidemiological programs, especially in cases of exposure in immunocompromised individuals where serological test alone are insensitive [157]. At the moment, clinical experience with WBA is limited, but proof of validity of the method for screening of leishmaniasis in endemic countries is present in the literature [158, 159, 110,141, 143].

We selected the abovementioned methods, ie kDNA real-time PCR, WB and WBA and we employed each method on blood samples obtained from a cohort of immunocompetent individuals. We tested 145 individuals residing in Pianoro municipality, an endemic area of Bologna Province (northeastern Italy) and we defined an individual as positive for cryptic leishmaniasis when at least one marker of *Leishmania* infection was present.

Among 145 individuals recruited and screened with WB, Real-Time PCR and WBA, 24 subjects tested positive to one or more methods. The prevalence of *Leishmania* infection in Pianoro was 17%, confirming the high circulation of the parasite in the selected area. In a previous study conducted in 240 blood donors residing in the Valsamoggia municipality of the Bologna province, the prevalence of cryptic *Leishmania* infection was 12.5% [114]. The higher prevalence of *Leishmania* infection in the municipality of Pianoro can be due to the combined use of three different techniques (WB, WBA, and PCR) in the Pianoro study, whereas only PCR and WB were used in the study conducted in Valsamoggia.

Both WB and WBA evaluate the host response to the protozoan parasite, while by using Real Time PCR we detected the DNA of *Leishmania* in the blood. In immunocompetent individuals, there was no concordance comparing the results of Real-Time PCR with the immunological tests as none of the subjects that tested positive for kDNA in the blood was also positive for the specific cell-mediated response and/or for the presence of *Leishmania* antibodies in sera (Figure 22). Conversely, among 24 *Leishmania* positive individuals, 10 volunteers tested positive for both WBA and WB showing a substantial concordance between the two immunological methods as calculated by the Cohen's Kappa index.

The detection of discordant results between direct molecular methods and serological methods in detection of asymptomatic *Leishmania* infection is in line with our previous study [114] Moreover, evidence indicates that in endemic areas *Leishmania* DNA could be present in blood of immunocompetent individuals without detectable specific antibodies. Mary et al. tested 46 asymptomatic subjects living in endemic area of France, *Leishmania* was present in 10 cases with low parasitemia, and no specific antibodies tested by Western Blot [130].

In conclusion, our findings in immunocompetent individuals demonstrate that Real-Time PCR and immunological tests should be employed in conjunction to detect cryptic leishmaniasis. In addition, the employment of two different immunological methods testing different arms of the specific immune response allows to reduce the underestimation of asymptomatic *Leishmania* infection.

After testing 145 immunocompetent individuals and validating a diagnostic test combination, we screened a cohort of 198

immunocompromised individuals for *Leishmania* infection. Among the 198 individuals recruited, 62 were positive for at least one method (31%). In the case of immunocompromised patients, evaluation of concordance between diagnostic methods was performed only in patients who were tested with all 3 methods (155/198).

The concordance between all diagnostic methods was slight, with a Cohen's kappa between 0.13 and 0.06. We observed a substantial difference examining the concordance index between WB and WBA in the population of immunocompetent individuals (0.66) and in the cohort of immunocompromised individuals (0.11); a potential reason is that both humoral and cell-mediated immune response can be impaired in immunosuppressed patients, giving a higher rate of false negative results.

The main limitation of this study is the fact that there is no gold standard to identify cryptic leishmaniasis. Because of the absence of gold standard, it is difficult to know if the seropositive individuals or individuals with specific T cells against *Leishmania* were persistently infected or if it was a prior infection. On the other hand, parasitemia can be low or absent and PCR cannot be used alone to identify those individuals with a quiescent parasitic infection. The addition of other serological tests in association to WB, such as DAT, could have further increased the sensitivity of the anti-leishmanial antibody detection [160]).

Given the high prevalence of asymptomatic infection in immunocompromised patients in endemic regions such as Italy, it seems essential to develop a plan for screening and follow-up of *Leishmania* infection to predict the progress of the disease. It is at present impossible to predict who will develop VL disease among *Leishmania*-positive

asymptomatic immunocompromised individuals. Our study included a first phase of screening of immunocompromised patients and then a one-year follow up of *Leishmania*-positive patients in order to evaluate potential predictive markers of disease progression. Currently, the follow up of positive patients is ongoing.

Our follow up study is in line with the current guidelines as close monitoring of immunocompromised patients that tested positive for cryptic leishmaniasis is recommended by WHO in order to recognize the symptoms of a possible relapse of disease and to promptly start an appropriate treatment [137].

In conclusion, the screening algorithm for cryptic leishmaniasis that we evaluated in this study appears to be effective for the identification of asymptomatic *Leishmania* infection in both immunocompetent and immunocompromised individuals and demonstrates that test combination is required for an accurate identification of quiescent parasitic infection as there is not a single gold standard test that is capable to identify all cases of cryptic leishmaniasis.

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