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Current clinical, laboratory, and treatment outcome characteristics of visceral leishmaniasis: results from a seven-year retrospective study in Greece

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SUMMARY

Objectives: Visceral leishmaniasis (VL) is re-emerging in endemic areas. The epidemiological, clinical, laboratory, and treatment outcome characteristics in a large cohort of VL patients is described herein.
Methods: The cases of 67 VL patients (57% male, mean age 56 years) treated in two Greek hospitals over the last 7 years were identified and evaluated retrospectively.
Results: Forty-six percent of patients reported contact with animals. Seventeen patients (25%) were immunocompromised, and 22% were co-infected with another pathogen. Sixty-four percent of patients had fever, 57% had weakness, 37% had sweats, 21% had weight loss, and 13% had a dry cough, while 6% developed haemophagocytic syndrome. The median duration of symptoms was 28 days. Fifty-eight percent of patients had splenomegaly, 49% had hepatomegaly, and 36% had lymphadenopathy. The diagnosis was established by positive PCR in peripheral blood (73%) and/or bone marrow specimens (34%). Sixty-one patients (91%) received liposomal amphotericin (L-AMB). Six patients (10%) did not respond or relapsed but were eventually cured after a second cycle of L-AMB. During a 6-month follow-up, the overall mortality was 9%, although none of these deaths was attributed to VL.
Conclusions: VL is still a common disease in endemic areas, affecting immunocompetent and immunocompromised patients. Its diagnosis is challenging, and molecular techniques are valuable and helpful tools to achieve this. Treatment with L-AMB is safe and very effective.

1. Introduction

Visceral leishmaniasis (VL) is an endemic and potentially life-threatening disease in the tropics, subtropics, and Mediterranean basin, including Greece. It is characterized by a broad range of clinical and laboratory findings, such as fever, cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia, and hypoalbuminemia.[1]

Leishmaniasis is a mandatory notifiable disease in Greece.[2] *Leishmania infantum* is the species responsible, while the most common vectors are *Phlebotomus neglectus*, *Phlebotomus tobbi*, and *Phlebotomus perfiliewi*.[3,4] Nevertheless, the true prevalence of VL is probably underestimated, as infectious diseases are generally under-reported in many countries, including Greece.[5,6]

Data on VL in Greece are scarce. Therefore, the aim of this study was to report the epidemiological and clinical characteristics, risk factors, diagnostic tools, treatment, and outcome of VL among patients treated in two tertiary care Greek hospitals during the last 7 years.[2,3,7–9]

2. Patients and methods

All adult patients (age >14 years) with well-established VL diagnosed at the Department of Medicine of the University General Hospital of Larissa, and the Infectious Diseases Unit of the Pathophysiology Department of the General Hospital of Athens "Laikon" from January 1, 2007 to December 31, 2013, were evaluated. The patients' electronic records/written charts were

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reviewed for demographic characteristics, underlying diseases, laboratory parameters, treatment outcome, and the mortality rate at 24 weeks after diagnosis. The clinical manifestations and potentially concurrent infections were also recorded.

An index patient was defined according to international criteria as a confirmed VL case in the presence of clinical and laboratory manifestations compatible with VL (i.e., two or more of persistent fever >38 °C, hepatosplenomegaly, substantial weight loss, anaemia, leukopenia, polyclonal hypergammaglobulinemia, or lymph node enlargement), along with a positive result for two of the following three tests: serology (immunofluorescence antibody test), demonstration of parasite by smear in tissue samples (i.e., bone marrow smear, lymph node), or molecular techniques.[1,10]

Regarding molecular techniques, DNA extraction was performed from 2 ml of blood or 1 ml of bone marrow, which was suspended in 1 ml phosphate buffered saline (PBS) and layered over Biocoll Separating Solution (Biochrom, Germany). After centrifugation at 1800 rpm for 30 min, the interface was collected and suspended in 8 ml of PBS. The suspension was centrifuged for 10 min at 2000 rpm and the supernatant was discarded. The pellet was re-suspended in 0.2 ml of PBS. DNA extraction was then performed using the QIAamp DNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The DNA was eluted in 0.1 ml of buffer AE. A simple PCR reaction amplifying a fragment of the SSU rRNA gene of Leishmania was used for detection, as described previously.[11] For *Leishmania* species identification, another previously described PCR method was also available.[12]

The patient was considered as immunosuppressed when there was a history of malignant disease (haematological malignancy or solid tumour) or a history of inherited or acquired immunodeficiency (e.g., splenectomy, HIV infection) or was under treatment with immunosuppressive agents (e.g., corticosteroids, azathioprine, methotrexate, and chemotherapeutic or biological agents). With regard to corticosteroids, immunosuppression was considered if the patient had received a dose of ≥5 mg of prednisone or equivalent, every day, for at least the last 30 days. Anaemia was defined when haemoglobin was <12 g/dl in females and <13 g/dl in males. Leukopenia and neutropenia were defined when leukocyte and neutrophil counts were less than 4×10^9/l and 1×10^9/l, respectively. Thrombocytopenia was defined when the platelet count was less than 140×10^9/l. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), C-reactive protein, albumin, and gamma-globulins, and the erythrocyte sedimentation rate (ESR), were determined using standard techniques.

The treatment schedule for liposomal amphotericin (L-AMB) was administered according to the US Food and Drug Administration (FDA)-approved regimen for immunocompetent (3.0 mg/kg on days 1 to 5, 14, and 21) and immunosuppressed patients (3–5 mg/kg daily or intermittently for 10 doses (days 1–5, 10, 17, 24, 31, and 38) up to a total dose of 40–60 mg/kg).

Clinical response was assessed at the completion of treatment and was defined as cure (defervescence, restoration of laboratory parameters, or significant reduction in spleen size) or failure (persistent or worsening of clinical and laboratory findings). Relapse was defined as the reappearance of signs and symptoms of the disease within 6 months, in association with the identification of the parasite in a bone marrow smear, after initial successful treatment.

The study was approved by the institutional review boards of the two tertiary medical centres. All participants gave their informed consent.

3. Results

Over the 7-year study period, 67 patients were identified with well-established VL (56 in Larissa and 11 in the Athens medical centre); their mean ± SD age was 56.1 ± 19.5 years and 38 were males. The age distribution of the patients is shown in Figure 1. The main clinical, physical, and laboratory findings of the patients are shown in Table 1. Forty-four patients (66%) were living in rural areas and 31 (46%) had been in frequent contact with animals (strays or domestic dogs). At diagnosis, 17 patients (25%) were considered immunocompromised; seven of them were under immunosuppression with corticosteroids, azathioprine, methotrexate, or anti-tumour necrosis factor regimens due to autoimmune rheumatic diseases, seven had an active underlying malignancy, and one patient each had a history of splenectomy, hypogammaglobulinemia, and HIV infection. Moreover, nine patients (13%) suffered from diabetes mellitus.

The median duration of symptoms at diagnosis was 28 days (range 1–240 days). Overall, 56 patients (84%) reported low-grade fever, whereas 43 (64%) had a fever >38 °C. Thirty-eight patients (57%) reported weakness, 14 (21%) loss of weight, and nine (13%) a dry cough. Of interest, one patient presented only with fever and a single intranasal bleeding lesion and two patients presented with prolonged fever and neurological symptoms (acute polyradiculitis and sensory-motor axonal neuropathy, respectively). Two patients were recorded as asymptomatic, as the first patient had only splenomegaly and the second had enlarged inguinal lymph nodes.

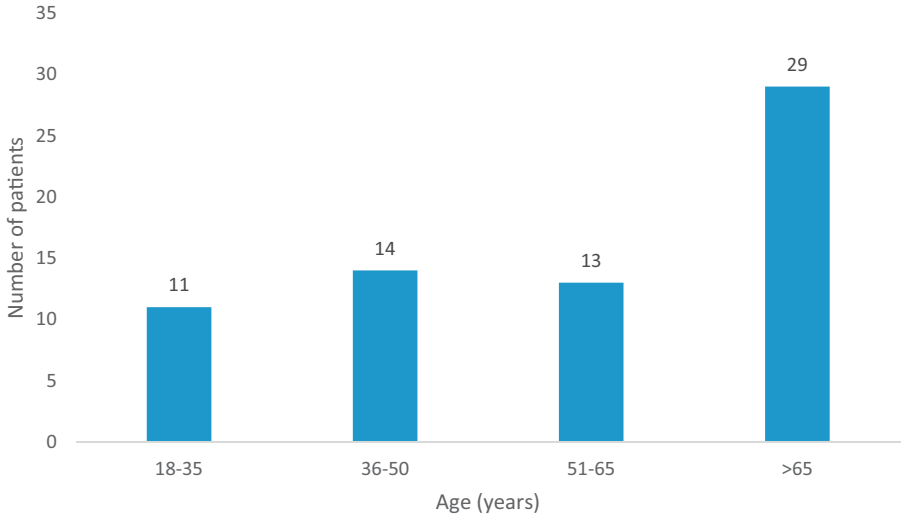

Figure 1. Distribution of patients with visceral leishmaniasis according to age.

Table 1
Clinical and laboratory characteristics of the 67 patients with visceral leishmaniasis

Characteristic	No. (%) of patients
Clinical symptoms	
Fever	43 (64)
Weakness	38 (57)
Sweats	25 (37)
Rigors	15 (22)
Loss of weight	14 (21)
Cough	9 (13)
Abdominal pain	8 (12)
Rash[a]	7 (10)
Headache	7 (10)
Bleeding	3 (4)
Diarrhoea	2 (3)
Neurological symptoms[b]	2 (3)
Physical findings	
Splenomegaly	39 (58)
Hepatomegaly	33 (49)
Lymphadenopathy	24 (36)
Ascites	3 (4)
Peripheral oedema	2 (3)
Hyperpigmentation	1 (1)
Laboratory findings	
Elevated inflammation markers[c]	54 (81)
Complete blood count	
Anaemia	49 (73)
Thrombocytopenia	29 (43)
Leukopenia/neutropenia	27 (40)/12 (18)
Pancytopenia	23 (34)
Polyclonal hyperglobulinemia	40 (60)
Elevated transaminases	28 (42)
Low serum albumin	25 (37)
Microscopic haematuria	18 (27)
Positive non-organ specific autoantibodies	15 (22)
Low complement level	10 (15)
Positive rheumatoid factor	5 (7)
DIC	3 (4)
Cryoglobulinemia	1 (1)

DIC, disseminated intravascular coagulation.
[a] Haemorrhagic ($n=5$), erythema nodosum ($n=1$), urticaria ($n=1$).
[b] Acute polyradiculitis ($n=1$), sensory-motor axonal neuropathy ($n=1$).
[c] Increased C-reactive protein and/or erythrocyte sedimentation rate.

In addition, at diagnosis, 15 patients (22%) suffered simultaneously from other infections (co-infection), including five patients with a well-established second zoonosis (brucellosis in three and Q-fever in two), two patients each with lower respiratory infection, urinary tract infection, primary bacteraemia, and intra-abdominal infection, as well as one patient each with osteomyelitis and bacterial meningitis. Of note, four patients (6%) developed haemophagocytic syndrome (HS) with a high fever, pancytopenia, splenomegaly, haemophagocytosis in bone marrow, hypertriglyceridemia, and high serum ferritin levels (range 636–23 782 mg/dl). Physical examination revealed the presence of splenomegaly (39/67, 58.2%), hepatomegaly (33/67, 49.3%), and lymphadenopathy (24/67, 35.8%).

In the majority of cases, the diagnosis was confirmed by positive PCR specific for *Leishmania* species in peripheral blood (49 out of 54 patients tested, 91%) and/or bone marrow specimens (23 out of 32 patients tested, 72%). In all patients with VL for whom specification of the parasite was requested, *L. infantum* was exclusively identified. Furthermore, the diagnosis was established by direct microscopy on bone marrow smears in 26 out of 48 patients tested (54%) and positive serology by immunofluorescence in 15 out of 37 patients tested (41%).

Sixty-one patients (91%) received L-AMB (total dose 21 mg/kg for the immunocompetent and 40 mg/kg for the immunocompromised patients). Treatment side effects such as rigors, retrosternal pain, dyspnoea, rash, and deterioration in renal function developed in 12 patients (20%). Interestingly, one patient developed drug-related myocarditis with a very low ejection fraction and acute pulmonary oedema that were reversed after L-AMB withdrawal. Another patient experienced a tumour lysis-like syndrome during treatment, with a transient deterioration in renal function and a parallel elevation of uric acid levels without any other metabolic or electrolyte disturbances. Consequently, due to serious side effects under treatment with L-AMB, three patients were treated with pentamidine (4 mg/kg every other day for up to 15 doses) due to the lack of any other alternative drug regimen for VL in the study hospitals (i.e. pentavalent antimonial drugs, paromomycin, or miltefosine). Unfortunately, one of these patients developed renal impairment and irreversible insulin-dependent diabetes mellitus.

Six (10%) patients in total did not respond to treatment or relapsed within 6 months (two of them from the immunocompromised group). All six of these patients received a second cycle of L-AMB and were eventually cured. Following therapy, five immunocompromised patients were given secondary prophylaxis with intermittent administration of L-AMB (3 mg/kg every 21 days) to prevent relapses due to the underlying disease (duration range 1–2.5 years). One of them – a patient with underlying myelodysplastic syndrome – relapsed 10 months after the initial diagnosis, with a parallel transformation to acute myelogenous leukaemia, and died a few days later. Of note, five patients did not receive any treatment: three refused to be treated and two died of their underlying malignant disease before the initiation of treatment. One patient was lost to follow-up. Overall, six patients (9%) died during a 6-month follow-up period (crude mortality) due to bacterial infections (four patients) and underlying malignancy (two patients). Interestingly, there was no significant difference regarding the demographic, clinical, laboratory, and treatment outcome characteristics between immunocompetent and immunocompromised patients in this study (Table 2).

4. Discussion

It was found that the disease burden of VL still carries a notable morbidity in Greece, a Mediterranean country endemic for VL. A remarkable proportion of patients were immunocompromised. Co-infection with other pathogens was also common. In the majority of cases (85%), the diagnosis of VL was established using molecular techniques. Treatment with L-AMB was safe and effective.

Molecular-based diagnostic methods are indeed highly sensitive and specific assays for the detection of Leishmania DNA[13] and are considered the cornerstone of the clinical algorithm for the primary diagnosis of VL,[10,14] especially in the case of HIV co-infection.[10,13,14] A lack of detailed standardization and global homogenization and validation, together with the reduced specificity in endemic areas, are the main disadvantages of these useful diagnostic tools,[15] whereas PCR positivity does not distinguish asymptomatic infection from true active disease. The definitive diagnosis of VL requires the demonstration of parasites by smear in affected organ tissues, although serological tests such as the indirect fluorescent antibody test and detection of rK-39 antigen are also useful diagnostic tools. Unfortunately, due to the retrospective design of this study, the detection of rK-39 antigen was not available, although this method is quite cheap and has high sensitivity and specificity (>98%).[16]

Recent reports have demonstrated a post-war re-emergence and a current increasing incidence of VL in Greece following the continued increase in canine seroprevalence during the past decade.[2,17,18] One major factor contributing to increasing rates of VL is immunosuppression; modern treatment modalities for malignant, autoimmune, and hereditary diseases, including chemotherapy, biological agents,[19] corticosteroids, radiation therapy, and haematopoietic stem cell and solid organ transplantation, as well as HIV infection, have greatly increased the numbers of immunocompromised patients, who are vulnerable to VL. In this

Table 2
Clinical and laboratory characteristics of the 67 patients with visceral leishmaniasis according to their immune status (immunocompetent vs. immunocompromised)

Characteristic	Immunocompetent ($n=50$)	Immunocompromised ($n=17$)	p-Value
Clinical symptoms			
Fever	31 (62)	12 (71)	NS
Weakness	28 (56)	10 (59)	NS
Sweats	21 (42)	4 (23)	NS
Rigors	12 (24)	3 (18)	NS
Loss of weight	10 (20)	4 (24)	NS
Cough	8 (16)	1 (6)	NS
Abdominal pain	6 (12)	2 (12)	NS
Rash	6 (12)	1 (6)	NS
Headache	6 (12)	1 (6)	NS
Physical findings			
Splenomegaly	29 (58)	10 (59)	NS
Hepatomegaly	24 (48)	9 (53)	NS
Lymphadenopathy	17 (34)	7 (41)	NS
Laboratory findings			
Elevated inflammation markers[a]	42 (84)	12 (71)	NS
Complete blood count			
Anaemia	36 (72)	13 (76)	NS
Thrombocytopenia	20 (40)	9 (53)	NS
Leukopenia	23 (46)	4 (24)	NS
Pancytopenia	16 (32)	7 (41)	NS
Polyclonal hyperglobulinemia	29 (58)	11 (65)	NS
Elevated transaminases	20 (40)	8 (47)	NS
Low serum albumin	19 (38)	6 (35)	NS
Microscopic haematuria	12 (24)	6 (35)	NS
Positive non-organ specific autoantibodies	13 (26)	2 (12)	NS
Treatment response	46 (94)	13 (86)	NS

NS, non-significant.
[a] Increased C-reactive protein and/or erythrocyte sedimentation rate.

context, 521 accumulated cases of VL were recorded by the Hellenic Centre for Disease Control and Prevention from 2004 to 2013.[20] Nevertheless, it should be pointed out that this number may not be accurate, as infectious diseases are generally under-reported in Greece.[5]

In this large cohort study it was found that the clinical features of VL vary widely from asymptomatic to full-blown disease characterized by fever, organomegaly, hypergammaglobulinemia, and peripheral blood cytopenias.[1,10,11] An atypical clinical presentation is very challenging for clinicians as it can lead to a considerable delay in the final diagnosis. The absence of fever in VL is rarely described in immunocompetent patients, but it usually occurs in terms of HIV infection or drug-related immunosuppression.[21] Indeed, 11 patients (16%) in the present series did not report fever, of whom the majority (73%) were immunocompromised.

VL is also a not uncommon and important cause of reactive HS and should be considered seriously in patients coming from endemic areas.[22] Four patients (6%) in the present study developed HS due to VL. A high clinical suspicion, as well as the use of modern, high-yield diagnostic tools such as PCR, may lead to the early diagnosis of VL-associated HS, minimizing unnecessary hospitalization and potentially harmful investigations and treatments for HS.[22,23]

Bacterial co-infections were frequent (22%) in the study cohort, including five patients (7.5%) with a second zoonosis (brucellosis or Q-fever), which is not widely reported in the literature. The extensive overlap in geographical distribution of many zoonoses, close environments and climates, and animal migration may contribute to this phenomenon.[24] Advanced VL, especially in the context of secondary neutropenia, is often complicated by bacterial infections that are a major cause of mortality during hospitalizations.[25] Indeed, high rates of co-infection (41–60%) have already been reported in previous studies, with urinary infections being the most common, followed by blood, respiratory, gastrointestinal, and skin infections.[26,27]

Of interest, VL is associated with the development of several non-organ specific autoantibodies (22% in the present study population).[10,28,29] These markers, together with other laboratory immune findings, such as polyclonal hypergammaglobulinemia and low serum complement levels, can be present without any other profound clinical manifestation of autoimmune disease.[28–31] The true clinical significance of autoimmune serological findings is condensed in the diagnostic dilemmas or treatment failures that they can engender. Several patients with VL were initially treated with corticosteroids or cyclophosphamide as they were misdiagnosed with autoimmune hepatitis or systemic lupus erythematosus.[29,31] Therefore, apart from the diagnostic dilemmas, a high level of clinician vigilance is required, especially in areas endemic for VL, before deciding to administer immunosuppression.[29,30]

Finally, regarding treatment, agents with efficacy against VL include amphotericin B, pentavalent antimonial drugs, paromomycin (a parenteral aminoglycoside), and miltefosine (the first oral drug for the treatment of VL).[1,2,10] Nevertheless, L-AMB is the drug with the highest therapeutic efficacy and the most favourable safety profile.[10,32] Conventional amphotericin B also has a high efficacy and is actually the drug of choice in resource-limited countries, e.g., India, although it is associated with a higher risk of renal toxicity and other side effects. In this study L-AMB was administered as the first-line drug, similarly to many other Mediterranean countries,[33] with an initial treatment response of approximately 90%. In addition, all of the patients who did not respond or relapsed were eventually cured after the administration of a second cycle of L-AMB. The incidence of adverse events was relatively low and in most cases these were non-serious.[10,34] Interestingly, one patient developed drug-related myocarditis[35] and another developed a subclinical tumour lysis-like syndrome, and these have rarely been reported as side effects in patients with VL.[36] In contrast, one of the three patients who were consequently treated with pentamidine developed renal function deterioration and irreversible insulin-dependent diabetes mellitus due to pancreatic toxicity, which are both very well-described adverse events.[37,38] Pentamidine isethionate was the first drug tried as second-line treatment. It was used widely by district hospitals in

areas endemic for kala-azar in India until the last decade. However, due to its diminishing efficacy (80%) over a period of time and serious toxicity, its use has been limited.[39]

Treatment failure and relapse rates are particularly high in cases of impaired cellular immunity, especially in HIV co-infection.[40] The usual strategy in these cases is to re-treat with L-AMB, as the parasite does not necessarily develop resistance to this drug even after repeated treatments or prophylactic use.[10,41] Secondary prophylaxis reduces the rate of recurrences, although optimal regimens and the duration of prophylaxis have not yet been defined.[10,42,43] In this study, the patient who relapsed despite secondary prophylaxis was HIV-negative but profoundly immunosuppressed due to an underlying active haematological malignancy. Moreover, it is well-known that VL is nearly always lethal without treatment.[1,10] Even with treatment, fatality rates can be 10% or higher due to far-advanced disease, an underlying illness, or drug-related toxicity. In this study cohort, the 6-month mortality rate was 9%, and although mortality could not be directly attributed to VL, this probably reflects the frailty of the host immune status.

In conclusion, the diagnosis of VL remains challenging as the disease is characterized by diverse and many atypical manifestations. Molecular methods contribute significantly to a prompt and timely diagnosis of VL, which is essential to reduce the marked morbidity and mortality that this disease still carries. Indeed, early recognition of special clinical and laboratory characteristics of VL appears critical for the effective management of affected patients, as the overall treatment efficacy of L-AMB is very good.

Conflict of interest: All authors have nothing to declare.

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