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Original article

# Diagnostic model of visceral leishmaniasis based on bone marrow findings. Study of patients with clinical suspicion in which the parasite is not observed

Alfredo Bermejo Rodriguez[a,*], Jose Manuel Ruiz Giardin[b], Jesus Garcia Martinez[b], Juan Victor San Martin Lopez[b], Adela Castaneda de la Mata[b], Daniel Lopez Lacomba[b], Jeronimo Jaqueti Aroca[b], Stefan Walter[b]

[a] Universidad Rey Juan Carlos, Madrid, Spain
[b] Hospital Universitario de Fuenlabrada, Madrid, Spain

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ABSTRACT

*Background:* Visceral Leishmaniasis (VL) is a serious protozoal disease endemic in diverse areas, including the southern area of Madrid (Spain), where an outbreak was detected in 2009. The objective of this work is to analyze bone marrow alterations in VL patients and elaborate a diagnostic model with the aim to improve the early detection of this disease. The usual diagnostic methods, as the observation of the parasite on a bone marrow aspirate, have frequent false negatives, and the high sensitivity methods, as PCR and ELISA, are delayed or are not always available.

*Methods:* This observational study evaluated bone marrow parameters of adult patients with clinical suspicion of VL, in which a bone marrow aspiration was performed but *Leishmania* was not directly observed, during the period 2009-2014. The patients finally diagnosed of VL by other methods (VL group, n = 41), and the patients in which the VL was not diagnosed (non-VL group, n = 20) were compared. A multivariant model was elaborated and externally validated.

*Results:* The final multivariant model includes percentage of myeloid series, percentage of plasma cells and quantification of megakaryocytes in the bone marrow, with an area under the ROC curve of 0.87 (0.78-0.96). The model performed well in the external validation.

*Conclusion:* In cases of VL suspicion and when the parasite is not observed in the bone marrow aspiration, the proposed model could be useful in discriminating between patients with and without VL, allowing to take a therapeutic decision while awaiting the definitive diagnosis.

## 1. Introduction

Protozoa of complex *Leishmania donovani - Leishmania infantum* are transmitted from animal reservoirs to humans by bite of sandflies of the *Phlebotomus* species. Most infections are asymptomatic, but some patients can develop clinical Visceral Leishmaniasis (VL)[1] or Kala-azar [1]. Typical clinical manifestations are prolonged fever, loss of weight, anorexia and splenomegaly. Known changes usually found in serum chemistry and blood counts are hypergammaglobulinemia and pancytopenia [1,2].

VL is endemic in areas of northern Africa, the Middle East, the Indian subcontinent, Central America, South America and southern Europe, including Spain [1]. The parasite that causes Kala-azar in countries bordering the Mediterranean primarily affects young children and infants. The species responsible of VL in Spain is *Leishmania infantum,* with an annual incidence of 0.4/100.000 inhabitants and year, affecting mainly children, HIV-infected patients [3] or the immunosuppressed. Since 2009, one outbreak has been detected in the southern area of Madrid, localized in and around the city of Fuenlabrada. In this area, incidence has reached 15 cases/100.000 inhabitants and year, affecting immunocompetent individuals. This is the largest series of cases described in Europe [4–6].

VL patients should be treated as soon as possible, usually with Amphotericin-B (drug with some toxic effects in its conventional form

* Corresponding author.
*E-mail address:* alfredo.bermejo@salud.madrid.org (A. Bermejo Rodriguez).
[1] VL: Visceral Leishmaniasis

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and expensive in its liposomal form). Early diagnosis of VL is crucial, so that we can start this treatment, reducing morbidity and mortality [7,8].

The parasite presents itself in two forms in its vital cycle: amastigote (without flagellum) and promastigote (flagellated). We find it as amastigote inside the macrophages of the Reticuloendothelial System (RES), usually in the liver, spleen, bone marrow and lymph nodes [9–11].

VL diagnosis is based on direct microscopic observation of the parasite on bone marrow aspirate, showing small, round or oval bodies 2–4 μm in diameter with indistinct cytoplasm, a nucleus, and a small rod -shaped kinetoplast. Although this method is rapid, there are however frequent false negatives, in a range from 10 to 95%, in different countries [11–21]; splenic aspirate is also rapid and more sensitive, but is a riskier procedure, especially in thrombocytopenic patients [1,20–22]. The more sensitive and specific technique is PCR of parasite DNA, but it is not currently available in many hospitals and it can delay the onset of treatment by some days [1,23]. Other serological procedures (such as ELISA) increase sensitivity of bone marrow aspiration, but also delays diagnosis [7,23].

Several bone marrow alterations have been described in VL patients: erythroid hyperplasia, dysplastic changes (especially dyserythropoiesis), hemophagocytosis and plasmacytosis [12,15,19,24–30]. The outbreak situation detected in our area justifies the research and the study of the alterations presented by VL patients in their bone marrow, in order to improve the early diagnosis of the disease.

The objectives of this work are:

1. Detect and describe distinctive patterns presented by VL patients in their bone marrow.
2. Elaborate and validate a diagnostic model to predict the presence of *Leishmania* in cases of VL in which the parasite is not directly observed in the bone marrow aspirate.

The combination of the bone marrow characteristics observed in VL patients, could increase the diagnostic sensitivity for VL with respect to the sole observation of the parasite in the bone marrow aspiration, keeping time advantage with respect to high sensitivity and specificity techniques like PCR.

## 2. Material and methods

### 2.1. Ethical concerns

This study was approved by the ethical committee of Hospital Universitario de Fuenlabrada. Informed consent from the patient was required to perform the bone marrow aspiration.

### 2.2. Design and case definitions

Data of patients over 15 years old treated at the Hospital de Fuenlabrada, in which a bone marrow aspiration was performed by clinical suspicion of VL during the period 2009–2014, were analyzed. Clinical suspicion was established in our context by the presence of fever of intermediate duration (defined as non-localized fever occurring in the community, lasting from one to four weeks, and having no diagnostic orientation after basic clinical, analytical and radiological evaluations are completed) [31] and/or splenomegaly (spleen size > 12 cm by ultrasonography), and at least one cytopenia (defined as Hemoglobin < 120 g/L or Leucocytes < $4 \times 10^3$/μL or Platelets < $150 \times 10^3$/μL).

The work was structured in two parts, first the diagnostic model elaboration and second the validation of this model. Data for the **model elaboration** were collected from the following groups of patients (see Fig. 1):

1. **Diagnosed VL group:** all patients in which *Leishmania* amastigotes were not observed in the bone marrow, but were diagnosed of visceral Leishmaniasis by one of the following methods approved by the World Health Organization (WHO) [1]:
   a. Isolation of the parasite from peripheral blood mononuclear cells in NNN medium culture.
   b. DNA detection by PCR in blood or bone marrow.
   c. Positivity by serology by rapid test rK39 or ELISA test (IgG + IgM).
2. **Non-VL group**: all patients with clinical suspicion of visceral Leishmaniasis but without confirmation of the disease by any of the diagnostic methods stated before.

Data from a different group of patients, in which bone marrow aspiration was also performed by clinical suspicion of VL, were also collected in order to validate the model. This validation group was formed by the following patients (see Fig. 1):

1. Patients diagnosed by direct microscopic observation of *Leishmania* in the bone marrow (during the same period 2009–2014).
2. All the patients with clinical suspicion of *Leishmania* in which bone marrow aspiration was performed in the period 2015–2017.

The exclusion criteria in both groups were:

1. Absence of evaluable bone marrow aspiration.
2. Absence of any diagnostic certainty technique (PCR, ELISA or culture) when bone marrow observation was negative.
3. Cases of relapsed VL.

### 2.3. Data collection and statistical analysis

16 bone marrow parameters were collected in all the groups, including quantitative and qualitative parameters. These parameters were evaluated by a single hematologist in each case (among three possible observers, who followed homogeneous criteria based in the ICSH guidelines and other reference publications [32–34]). The analyzed quantitative bone marrow parameters were quantitative cellularity (range 0–5), quantitative megakaryocytes (range 0–5), percentage of myeloid series and erythroid series, myelo-erythroid ratio and percentage of lymphocytes, plasma cells, eosinophils and macrophages. The differential count was performed on 400 bone marrow nucleated cells, stained with Wright dye, under x400 magnification. The range of cellularity was assessed in at least 5 areas of bone marrow particles (x200 magnification), depending on the proportion of hemopoietic cells and fat: 0 (0% cells), 0.5 (10%), 1 (20%), 1.5 (30%), 2(40%), 2.5 (50%), 3 (60%), 3.5 (70%), 4 (80%), 4.5 (90%) and 5 (100%). Megakaryocyte quantification was assigned by the average number of megakaryocytes per field (x200 magnification) in 5 fields, according to the following rule: 0 (0 megakaryocytes/field), 1 (1–2), 2 (3–4), 3 (5–6), 4(7–8) and 5 (≥9). The analyzed qualitative parameters (presence of morphological abnormalities in bone marrow cells) were hemophagocytosis, erythrocyte hemophagocytosis, leukocyte and platelet hemophagocytosis, dyserythropoiesis, dysmegakaryopoiesis, dysgranulopoiesis and megaloblastosis.

The data indicated above, together with other laboratory data, clinical data and demographic data, were registered in a database specific to this study. All basic parameters of the hemogram were registered, together with the diagnostic results of PCR of *Leishmania* DNA, the microbiological cultures and the *Leishmania* serology by rapid immunochromatographic test rk39 or ELISA. The clinical data included were the presence of fever or splenomegaly, the relevant clinical conditions (HIV infection, immunosuppressive therapy, neoplasia or autoimmune disease) and the final diagnosis if VL was ruled out.

Mean, standard deviation, median and quartiles were described in the continuous variables for VL and non-VL groups. Both groups were

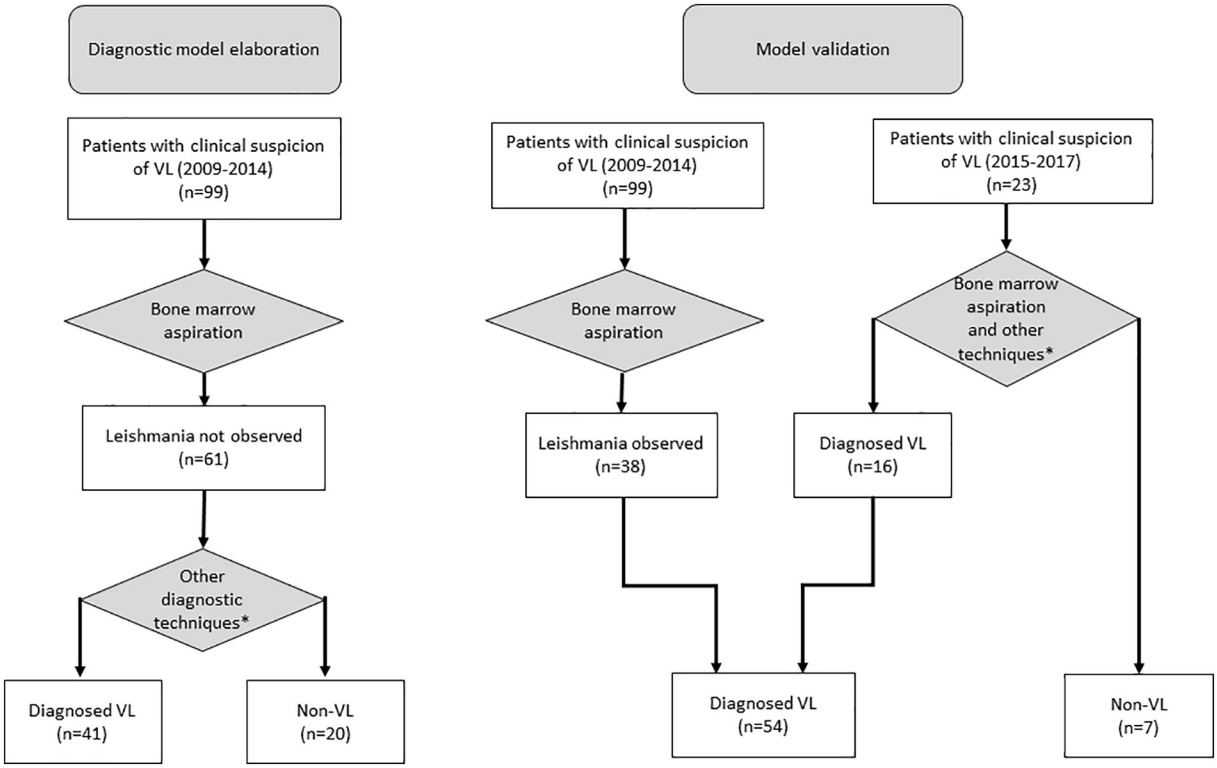


**Fig. 1.** Groups of patients enrolled for the study. *Other diagnostic techniques: PCR,ELISA and/or cultures.

compared for all variables by the non-parametric test U Mann-Whitney, although they were parametric. Absolute and relative frequencies were described for both groups in the categorical variables, and $\chi 2$ tests were performed to evaluate association with diagnosis. Univariant logistic models and areas under the ROC curves analyses were performed with the objective to evaluate discriminatory power of each parameter.

The variables significantly associated ($p$-value $< .05$) were analyzed and we included some of them in the final multivariable model, after following a backward modeling technique. The discriminating power of this model was evaluated by the area under ROC curve and its calibration by the Hosmer-Lemeshow test.

A nomogram from our model was developed, with the aim of simplifying the calculation of the likelihood of a patient having VL. And finally, a predictive score for VL was also developed. The discriminative power of this score was evaluated by the area under ROC curve and its calibration by the Hosmer-Lemeshow test.

An internal validation of the model by bootstraping techniques was performed, and also an external validation in the validation sample, evaluating the area under ROC curve and performing replicates by bootstraping techniques.

The statistical analysis programs used were Medcalc 11.4.2.0® and R 3.5.0®.

## 3. Results

From the original 140 patients registered in our databases, 18 patients were ruled out following the exclusion criteria. For the investigation sample, data of 61 patients with evaluable bone marrow were finally collected: 41 patients of the VL group and 20 of the non-VL group. For the validation sample, data of 61 patients were also collected: 54 of the VL group and 7 of the non-VLN group. These data are shown in Fig. 1.

### 3.1. Demographic and clinical data

In the model elaboration group, mean age of the VL patients was 44.7 years (range 15–95) and 85% were male. 60% of patients were Spaniards and the rest from abroad, remarkably 34% of sub-Saharan Africans. With respect to immunosuppression factors, 12% of patients had HIV infection and 12% other immunosuppression factors, mainly immunosuppressive therapies.

Patients not diagnosed with VL presented a similar age distribution (mean age 51 years, range 15–86), 70% were male and 80% Spaniards. The most frequent diagnosis of non-VL patients were viral infections (including CMV, chronic HBV and HCV), bacterial infections (*Mycobacterium tuberculosis* complex, *Listeria* and *Salmonella*), autoimmune diseases (Systemic Lupus), neoplasia (Hodgkin Lymphoma, Myelodysplastic Syndrome) and others (Still disease and Familial Mediterranean fever).

In the model validation group, the mean age of the VL patients was 44.2 years (range 15–88) and 70% were male. In this case, sub-Saharan Africans were 48% of individuals, and only 39% were Spaniards. 18% of patients had HIV infection and 13% other immunosuppression factors.

### 3.2. Diagnostic data

Using *Leishmania* PCR as reference technique, diagnostic sensitivity for Leishmaniasis of bone marrow aspiration direct observation reached 48% of the 79 patients diagnosed during the period 2009–2014; these results were informed the same day. Rapid rK39-based test used in our media had a low sensitivity (51%) but the specificity was 97%. ELISA test had a 97% sensitivity and 86% specificity with a delay time of 6 days. Mean delay time of *Leishmania* PCR was 7 days in our hospital.

### 3.3. Analytical data

The analysis of the 16 parameters evaluated in the model

**Table 1**
Univariant analysis of bone marrow quantitative parameters (model elaboration group).

| Bone marrow parameter | VL ($n$ = 41) * Mean ± Standard deviation | Non-VL ($n$ = 20) * Mean ± Standard deviation | VL (n = 41) * Median (P25-P75) | Non-VL (n = 20) * Median (P25-P75) | $p$-value (Mann-Whitney test) | Area under ROC curve |
|---|---|---|---|---|---|---|
| Cellularity quantitative (0–5/5) | 3.6 ± 0.4 | 3.5 ± 0.5 | 4.0 (3.5–4.0) | 3.5 (3.0–4.0) | 0.22 | 0.59 |
| Myeloid series % | 43.2 ± 10.3 | 56.0 ± 10.1 | 44.0 (35.5–49.0) | 56.0 (46.7–63.1) | < 0.01 | 0.79 |
| Erythroid series % | 38.8 ± 11.8 | 29.3 ± 11.9 | 38.0 (31.5–44.5) | 26.7 (21.5–36.5) | 0.01 | 0.70 |
| Myelo erythroid ratio | 1.3 ± 0.7 | 2.5 ± 2.0 | 1.2 (0.7–1.5) | 2.1 (1.4–2.9) | < 0.01 | 0.74 |
| Plasma cells % | 4.7 ± 2.8 | 2.3 ± 1.8 | 4.5 (3.0–6.5) | 1.5 (1.0–3.7) | < 0.01 | 0.77 |
| Macrophages % | 1.6 ± 1.6 | 0.7 ± 0.8 | 1.0 (0.5–2.5) | 0.5 (0.5–0.7) | 0.02 | 0.66 |
| Lymphocytes % | 8.3 ± 4.8 | 7.1 ± 3.6 | 6.5 (5.0–10.5) | 6.7 (4.2–9.2) | 0.57 | 0.54 |
| Eosinophils % | 4.9 ± 7.9 | 6.5 ± 6.0 | 3.0(2.0–5.0) | 4.8 (3.0–7.0) | 0.06 | 0.34 |
| Megakaryocytes quantitative (0–5/5) | 3.9 ± 0.6 (n = 40) | 3.4 ± 0.7 (n = 17) | 4.0 (4.0–4.0) ($n$ = 40) | 3.0 (3.0–4.0) ($n$ = 17) | < 0.01 | 0.69 |

Univariant analysis of bone marrow qualitative parameters (model elaboration group)

| Bone marrow parameter | VL (n = 41) Frequency (%) | Non-VL (n = 20) Frequency (%) | $p$-value (Chi-square test) | Area under ROC curve |
|---|---|---|---|---|
| Hemophagocytosis | 48.7 | 45.0 | 0.78 | 0.51 |
| Hemophagocytosis of erythrocytes | 39.0 | 40.0 | 0.94 | 0.50 |
| Hemophagocytosis of leukocytes and platelets | 29.2 | 5.0 | 0.03 | 0.62 |
| Dysgranulopoiesis | 4.8 | 0.0 | 0.31 | 0.50 |
| Dyserythropoiesis | 41.6 | 25.0 | 0.29 | 0.59 |
| Dysmegakaryopoiesis | 14.6 | 0.0 | 0.07 | 0.50 |
| Megaloblastosis | 9.7 | 5.0 | 0.52 | 0.52 |

*n indicated between () if different from above.

elaboration group is shown in Table 1.

Comparing the quantitative bone marrow parameters between the confirmed VL group and the non-VL group, the patients of the VL group presented significantly higher values of erythroid series %, plasma cells %, macrophages % and quantitative megakaryocytes, and lower values of myeloid series % and myelo-erythroid ratio.

Dyserythropoiesis and dysmegakaryopoiesis were qualitative changes more frequent in the VL group, although differences were not significant. Phagocytosis of erythroid cells was similar in both groups, whereas phagocytosis of platelets and leukocytes was more common in the VL group.

Of the parameters significantly associated ($p$-value < .05) with *Leishmania* diagnosis in the univariant analysis, myeloid series %, erythroid series % and myelo-erythroid ratio were strongly correlated, so that the one with the highest discriminant power, myeloid series %, was selected. The 4 significant parameters selected for the multivariable logistic regression model were myeloid series %, plasma cells %, macrophages % and megakaryocytes quantitative. Following the backward modeling technique, the final model included only 3 variables: myeloid series %, plasma cells % and megakaryocytes quantitative. The probability formula of this model was:

$$P_{(Leishmania)} = \frac{1}{1 + e^{-(1.14 - 0.12*MS + 0.40*PLA + 1.17*MEG)}}$$

MS: myeloid series %. PLA: plasma cells %. MEG: megakaryocytes quantitative.

The area under the ROC curve of this model was 0.87 (0.78–0.96). The evaluation of calibration of the model by Hosmer-Lemeshow test showed a $p$-value of 0.145 (> 0.05) that indicated absence of significant differences between observed and predicted probabilities. We developed a nomogram from our model, that predicts the likelihood of a patient having VL (see Fig. 2). For example, a patient with myeloid series 60%, plasma cells 1.5% and megakaryocytes quantitative 2 has a 0.05 probability of having leishmaniasis. Opposite, a patient with myeloid series 40%, plasma cells 7% and megakaryocytes quantitative 4 has a 0.98 probability of having leishmaniasis (see Fig. 2).

Finally, we also developed a predictive score for VL. The points assigned to each parameter were:

1. Myeloid series < 50%: 1 point
2. Plasma cells 2–5%: 1 point. Plasma cells ≥5%: 2 points.
3. Megakaryocytes quantitative > 3: 1 point.

The score ranges from 0 to 4 points. The probabilities of VL were 0% for score 0, 50% for 1, 60% for 2, 88% for 3 and 100% for 4. For the same examples of the nomogram, the score of a patient with myeloid series 60%, plasma cells 1.5% and megakaryocytes quantitative 2 is 0, and the score of a patient with myeloid series 40%, plasma cells 7% and megakaryocytes quantitative 4 is 4.

The area under ROC curve of this score was 0.85 (0.75-0.95) and the Hosmer-Lemeshow test indicated absence of significant differences between observed and predicted probabilities ($p$-value 0.57).

The model was internally validated by bootstraping techniques. The area under ROC curve adjusted for overoptimism was 0.85 (0.75–0.94), very similar to the apparent area under curve. The calibration of this technique was validated by evaluation of the calibration slope (0.99) and by calculation of the Hosmer Lemeshow test in every bootstraping sample (none with significant differences).

### 3.4. External validation

Bone marrow parameters analyzed in the validation group are shown in Table 2.

When applying the VL predictive model in these patients, the area under ROC curve was 0.92 (0.81–1.00). Replicates by bootstraping techniques (2000) rendered a smooth curve with an area under ROC curve of 0.88 (0.57–0.93).

## 4. Discussion

Comparing VL patients with patients suspected but not confirmed VL, the significant differential parameters between the two groups are the following: increased erythroid series, increased plasma cells, increased macrophages, increased megakaryocytes and decreased myeloid series and myelo-erythroid ratio, as well as presence of hemophagocytosis of platelets and leukocytes. Based on this data, the diagnostic model developed can orientate the diagnosis of VL in cases without

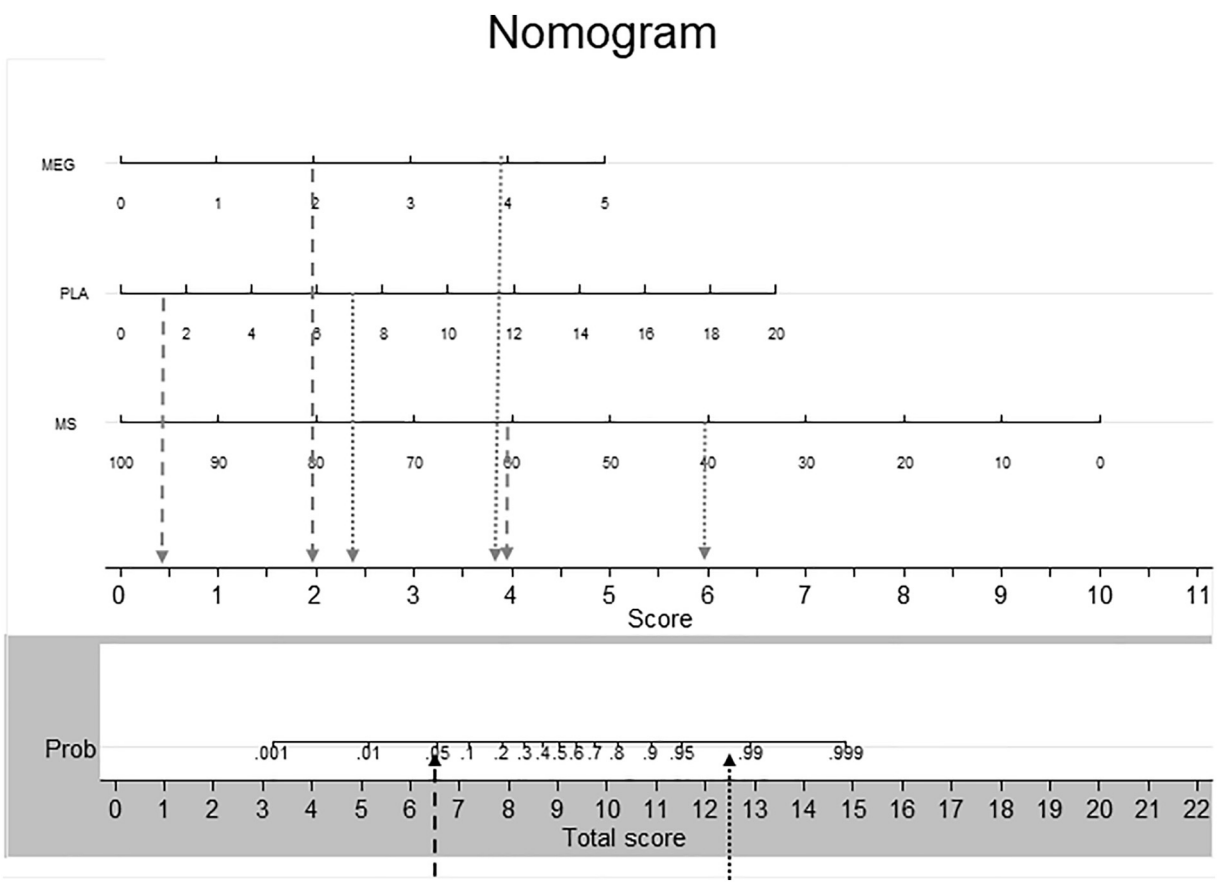


**Fig. 2.** Nomogram of VL predictive model. From the value of each parameter (MEG, PLA or MS), draw the vertical towards "Score" line and write down the value of each parameter in that line. Add the 3 values and take the result to the "Total score" line. Draw the vertical of that sum over the "Prob" line and the result is the probability of VL(0–1). The first and the second examples of the text are drawn with segmented and dotted arrows, respectively.

direct evidence of the presence of *Leishmania*.

Many of the descriptive data found in VL patients are consistent to those published previously. Different authors describe bone marrow changes close to our findings, including increased cellularity, increased erythroid series (with parallel decrease of myeloid series and inversion of myelo-erythroid ratio), increased plasma cells, increased macrophages and presence of hemophagocytosis [12,19,25–27,29,30,35]. Megaloblastic and dysplastic changes described by diverse authors [36,37] were less frequent in our cases. Other changes, as the increase of megakaryocytes are not described in these previous studies. Our work not only confirms many of the bone marrow alterations previously described in VL patients, but also highlights the most important differential parameters.

As our model is based on the proportions of the bone marrow cells, the use of the immunophenotyping by flow cytometry to obtain these data could be of great value. Immunophenotyping is a more objective and reproducible technique in the characterization of the different cell populations in the bone marrow. Unfortunately, this technique was performed only in some of our samples. In these cases, the cellular proportions by immunophenotyping corresponded to those obtained in the cytological counts, but in different ranges, showing higher myeloid series proportion and lower plasma cells proportion, due to dilution by peripheral blood or by processing techniques. Immunophenotyping is also a more expensive technique and it must be referred to other laboratories in many hospitals. Anyway, the elaboration of a diagnostic model based in cell counts obtained by flow cytometry could be very interesting. Even recognizing the limitations of bone marrow cytology (limited number of cells observed, subjectivity of the observer and variability of criteria between centers) our results indicate that it is as a fast and useful technique in most laboratories to orientate the diagnosis

**Table 2**
Descriptive analysis of bone marrow quantitative parameters in validation group.

| Bone marrow parameter | VL ($n$ = 54) * Mean ± Standard deviation | Non-VL ($n$ = 7) * Mean ± Standard deviation | VL (n = 54) * Median (P25-P75) | Non-VL (n = 7) * Median (P25-P75) |
|---|---|---|---|---|
| Cellularity quantitative (0–5/5) | 3.8 ± 0.3 | 3.6 ± 0.8 | 4.0 (3.5–4.0) | 4.0 (3.5–4.0) |
| Myeloid series % | 39.9 ± 12.0 | 60.6 ± 10.9 | 40.5 (31.0–48.5) | 64.5 (52.0–69.1) |
| Erythroid series % | 36.5 ± 10.9 | 23.6 ± 9.8 | 35.7 (30.0–43.0) | 21.5 (16.2–32.6) |
| Myelo erythroid ratio | 1.3 ± 1.0 | 3.3 ± 2.2 | 1.0 (0.8–1.4) | 3.0 (1.5–4.1) |
| Plasma cells % | 7.9 ± 4.3 | 4.3 ± 2.1 | 7.5 (5.0–9.5) | 4.5 (2.3–5.8) |
| Macrophages % | 1.9 ± 2.8 | 0.2 ± 0.2 | 1.0 (0.5–2.5) | 0.0 (0.0–0.5) |
| Lymphocytes % | 10.8 ± 6.4 | 8.7 ± 3.5 | 8.5 (7.0–12.0) | 8.5 (6.1–14.1) |
| Eosinophils % | 3.4 ± 3.2 | 2.2 ± 1.0 | 2.5 (1.5–4.5) | 2.0 (1.6–3.2) |
| Megakaryocytes quantitative (0–5/5) | 3.8 ± 0.7 | 3.4 ± 0.9 | 4.0 (3.0–4.0) | 3.0 (3.0–4.0) |

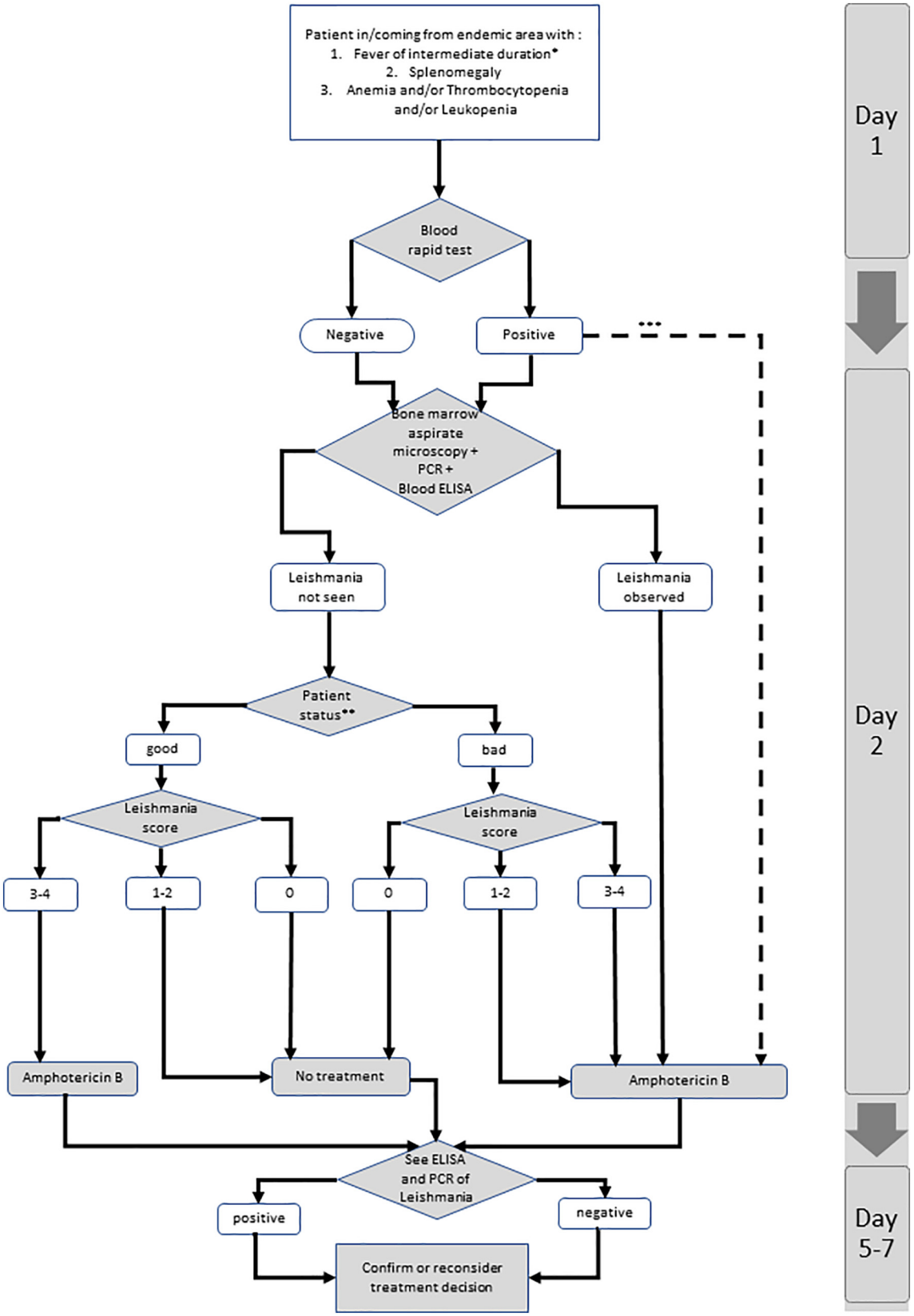


**Fig. 3.** Proposed management flow chart for patients with clinical suspicion of Leishmaniasis. *Once ruled out common causes like viral infections, Q fever, rickettsiosis, brucellosis, malaria and endocarditis. **Bad status is defined by at least one of the following: hemodynamic instability, symptomatic anemia or thrombocytopenia, severe neutropenia (< 500/$\mu$L), presence of SIRS (Systemic Inflammatory Response Syndrome), bad tolerated fever or coexisting morbidities. ***If blood rapid test is positive and patient present bad status, start treatment with Amphotericin B.

of VL when the parasite is not observed.

VL diagnosis is made by the demonstration of the parasite presence in human tissues or by its immunological consequences. But this is not always achieved in an adequate time to correctly treat this potentially lethal disease. The usual diagnostic methods present diverse limitations. As pointed out previously, direct observation of the parasite can result in false negatives (bone marrow observation) [11–21] or risks (splenic aspiration) [1,20–22]. PCR of the parasite in bone marrow tissue or others and some serologic test as ELISA are delayed in time. Rapid serologic tests, like rK39, render good results with high sensitivity and specificity [38], but have not performed well in our media, with lower sensitivity, perhaps in relation with the species of *Leishmania infantum* affecting our patients, although this hypothesis has not been confirmed by other authors [39]. We also hypothesize that this low sensitivity could also be related to the short evolution time of the disease in our patients, in whom the rK39 test was performed rapidly due to the high suspicion of leishmaniasis in our environment. The proposed diagnostic model can help to improve the diagnostic picture of VL, especially in terms of time, while reducing the sensitivity problems of the simple microscopic search for *Leishmania* in the bone marrow aspiration.

In patients with suspicion of VL, our diagnostic model can stratify the probability of having VL when the parasite is not observed in the bone marrow aspirate, helping to take a therapeutic decision in an appropriate time while waiting for the *Leishmania* PCR or the ELISA results. This score, integrated in a diagnostic workflow, could be useful in endemic areas where rapid serologic techniques with high sensitivity or PCR are not available, and would help in the decision of treating a patient when the presence of *Leishmania* cannot be demonstrated rapidly.

We propose a diagnostic workflow (Fig. 3) in patients with high suspicion of VL (fever of intermediate duration, splenomegaly and cytopenia/s), starting with a rapid blood test for VL, a blood sample for ELISA test and a bone marrow aspirate plus sample for PCR of *Leishmania*. If *Leishmania* is observed in the bone marrow, the patient will receive treatment with Amphotericin B; we will also start that treatment if a rapid blood test is positive and the patient presents bad clinical status. Otherwise, we calculate the *Leishmania* score and evaluate the patient status. Bad status is defined by at least one of the following: hemodynamic instability, symptomatic anemia or thrombocytopenia, severe neutropenia (< 500/μL), presence of SIRS (systemic inflammatory response syndrome), bad tolerated fever or coexisting morbidities. The patients with low probability of having *Leishmania* (score 0 points) will not be treated. The patients with intermediate probability (1–2 points) will be treated only if they present bad status, whereas the patients with better clinical status will wait for the ELISA test and the PCR of *Leishmania* result. All patients with high probability score (3–4 points) will be treated with Amphotericin B. When having the PCR of *Leishmania* result, the gold standard test, the treatment decision could be changed if the diagnosis is different. If we had a negative ELISA test (and the rapid test is also negative), we can also rule out the leishmaniasis.

Finally, we must point out that this model has been developed with patients during an outbreak, epidemiological context not usual in our country. Although it has been internally and externally validated, we think there is a need for external validation in other epidemiological circumstances, both in our country and in other countries.

## 5. Conclusions

In conclusion, in cases of clinical suspicion of Leishmaniasis, the following bone marrow parameters can be considered useful in discriminating between patients with and without VL: decrease of **myeloid series %** or increase of **erythroid series %**, increase of **plasmatic cells** %, increase of **macrophages** %, increase of **megakaryocytes** and presence of **hemophagocytosis of leukocytes and platelets**.

The elaborated model includes three discriminative marrow parameters (**myeloid series %**, **plasmatic cells** % and **megakaryocytes quantification**) to establish the probability of having VL in case the *Leishmania* parasite is not observed in the bone marrow.

Therefore, in cases in which VL is suspected and the parasite is not observed in the marrow aspiration, the evaluation of the score of this model could be considered either to exclude the diagnosis or to orientate it positively. In this last case, we could take a therapeutic decision in an appropriate time while waiting for the *Leishmania* PCR or the ELISA results.

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## Declaration of Competing Interest

All authors declare no conflicts of interest relevant to this article.

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