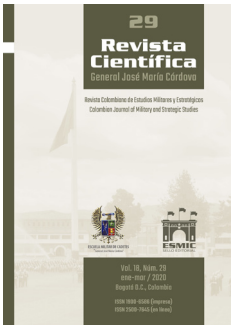

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Parasitic load of *Leishmania* spp. in Colombian military personnel with cutaneous leishmaniasis: a case study

Carga parasitaria de *Leishmania* spp. en personal militar colombiano con leishmaniasis cutánea: estudio de caso

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Abstract. Military operations occurring in areas where *Leishmania* spp. is endemic continually expose the military personnel of the Colombian National Army to contracting leishmaniasis. Medical treatments for this disease represent high costs in military logistics. The objective of this study was to quantify the parasitic load of *Leishmania* spp. in patients diagnosed with cutaneous leishmaniasis, using real-time PCR *kDNA* minicircle amplification. Also, to identify the specific species using PCR amplification and sequencing of the *HSP70* and *MPI* molecular markers to monitor and determine the effectiveness of meglumine antimoniate and pentamidine isethionate treatment, and its relation to other clinical-epidemiological data. In addition to demonstrating the efficacy of treatments and their characteristics, the risk of future outbreaks of mucocutaneous leishmaniasis was identified in certain cases, and differences between the medical resolution according to the clinical-epidemiological variables were explained multifactorially.

Keywords: armed forces; *Leishmania* spp.; National Army of Colombia; parasitology; qPCR

Resumen. El personal del Ejército Nacional de Colombia se expone continuamente a contraer leishmaniasis debido a las operaciones que tienen lugar donde este parásito es endémico, lo cual ocasiona altos costos por tratamientos médicos. El objetivo de este estudio fue cuantificar la carga parasitaria mediante la amplificación del minicírculo del *kDNA* por PCR en tiempo real e identificar molecularmente la especie de *Leishmania* spp. en pacientes con diagnóstico para leishmaniasis cutánea, a partir de amplificación por PCR y secuenciación de los marcadores moleculares *HSP70* y *MPI*, para monitorear y determinar la efectividad al tratamiento con antimoniato de meglumina e isetionato de pentamidina, así como su relación con otros datos clínico-epidemiológicos. Además, se demuestra la eficacia de los tratamientos con sus características, se identificó el riesgo de futuros brotes de leishmaniasis mucocutánea en ciertos casos y se refieren las diferencias encontradas entre las variables clínico-epidemiológicas explicadas multifactorialmente.

Palabras clave: *kDNA*; Ejército Nacional de Colombia; *Leishmania* spp.; Fuerzas Armadas; parasitología; qPCR

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Introduction

Leishmaniasis is a tropical vector-borne disease (VBD) caused by infection with any of 20 different species of protozoan parasites belonging to the genus *Leishmania*. It can affect the skin (cutaneous leishmaniasis [CL]), mucous membranes (mucocutaneous leishmaniasis [MCL]), or viscera (visceral leishmaniasis [VL]) (Herwaldt, 1999; Ul Bari, 2006). In the Americas, it is transmitted to humans through the bite of infected female blood-sucking insects of the genus *Lutzomyia*.

Colombia is the second country after Brazil, with the highest number of CL cases in the continent. In 2017, a total of 7764 cases were reported in Colombia, and it was estimated that 56% of the population was at risk of contracting the cutaneous form of the disease (World Health Organization [WHO], 2019; Organización Panamericana de la Salud [PAHO], 2019). Cutaneous leishmaniasis is currently the parasitic disease that most affects the foot soldiers of the Colombian National Army. According to a report by the *Sistema Nacional de Vigilancia en Salud Pública* (Sivigila; National Public Health Surveillance System) and the *Salud Operacional de la Dirección de Sanidad Ejército* (Operational Health of the Army Health Directorate), 40,185 cases of CL were reported, between 2008 and 2018, in active military personnel, followed by 559 cases of mucocutaneous leishmaniasis (Instituto Nacional de Salud [INS], 2019).

An adequate diagnosis of leishmaniasis will determine an appropriate treatment that, with follow-up and monitoring, will bring about an excellent prognosis of the disease. Therefore, diagnostic tests and follow-up treatment must be sensitive, specific, fast, and affordable. Real-time polymerase chain reaction (qPCR) is a molecular method of clinical diagnosis that meets the above criteria and can quantify the amplification products of a target DNA through monitoring at each quantification cycle. This technique can be used to monitor certain anti-leishmaniasis pharmacological treatments and relate the number of parasite DNA equivalents, also called the parasitic load. To do so, the internal control of a molecular marker, such as *GAPDH*, β-microglobulin, *18S rRNA*, or *ERV 3* is used to normalize the parasitic load or the number of *Leishmania* parasites DNA equivalents relative to the number of human cells DNA equivalents (Romero et al., 2010; De Paiva-Cavalcanti et al., 2013; Jara et al., 2013; Méndez, 2014).

Similarly, there are molecular markers, such as the heat shock protein gene (*HSP70*) and the mannose phosphate isomerase gene (*MPI*), which, by amplification using conventional PCR and Sanger sequencing, make it possible to barcode the species or the etiological agents causing leishmaniasis (Córdova et al., 2011; Ramírez et al., 2016; Patiño et al., 2017). The previous to analyze the dynamics between the infecting species, vector, prognosis of the disease, and pharmacological treatments given to patients, as well as other clinical-epidemiological variables.

In Colombia, the pharmacological treatment of CL follows the guidelines of the Ministry of Health and Social Protection through the National Institute of Health (NIH). These guidelines suggest meglumine antimoniate (N-methyl glucamine) as a first-line drug, which is administered intramuscularly or intravenously in doses of 20 mg/kg/day. In case of the therapeutic failure of pentavalent antimonials, miltefosine should be administered orally as a second line of treatment in doses of 1.5-2.5 mg/kg/day for 28 days or 4 to 10 intramuscular or endovenous pentamidine isethionate doses of 3-4 mg/kg/day on alternate days. If therapeutic failure persists, 2-3 mg/kg/day doses of liposomal amphotericin B should be applied intravenously (Durán et al., 2018).

This study is the first in military health to perform a detailed tracking or monitoring of the treatment of patients in the leishmaniasis program, by using qPCR to quantify the number of parasite DNA equivalents at the start, during, and end of the treatment. Only one previous study has validated and standardized the qPCR test, amplifying the *kDNA* and *G6PD* molecular markers as an assertive diagnostic technique (Méndez, 2014), to reduce logistical, administrative costs concerning not only patient post-operation recovery time but also the treatment of leishmaniasis within the Military.

The objectives of this study were 1) to estimate the parasitic load of *Leishmania* spp. in active military personnel of the Colombian National Army with cutaneous leishmaniasis being treated with meglumine antimoniate and pentamidine isethionate, 2) to determine the success or failure of the current pharmacological treatment by relating the parasitic load with different clinical-epidemiological data, and 3) to molecularly identify the infecting species by determining its distribution and abundance in possible infection sites.

Theoretical framework

Leishmaniasis is a growing global public health issue. The situation in Colombia has become concerning because of the increase in CL cases registered in the last years and the change in the epidemiological pattern of new outbreaks. The emergent processes of domiciliation and urbanization, affecting the transmission cycle, is an issue that especially affects the Military Forces because of the missions they carry out in areas endemic for this zoonosis. In Colombia, meglumine antimoniate is the drug of choice for the treatment of cutaneous leishmaniasis; it is supplied by the Ministry of Health and Social Protection. Based on the experience of Army dermatologists, it is essential to carry out clinical and laboratory monitoring of patients treated with meglumine antimoniate and other leishmanicidal drugs in order to determine the therapeutic performance according to the species and the susceptibility pattern shown by the etiological agents of the disease. Globally, clinical resistance or therapeutic failure to antimonials has increased proportionally to the epidemic. Failure rates of

treatments using pentavalent antimonials, according to the scheme recommended by the World Health Organization (WHO), range from 10-23% (Expert Committee on the Control of the Leishmaniases & World Health Organization, 2010).

The use of real-time PCR helps determine whether or not the etiological agent of leishmaniasis is present in the processed sample. Moreover, it allows detecting and quantifying the parasitic load as a diagnostic measure that meets the current needs of the Operational Health Unit of the Colombian National Army and the WHO. With this information, it is possible to determine the initial parasitic load, the response to treatment, and the prognosis on the evolution of the disease can be made to predict future adverse events such as MCL.

There are few studies in Colombia related to the quantification of the parasitic load of cutaneous leishmaniasis. One of them (Méndez, 2014) standardized a real-time PCR from the molecular targets *G6PD* and *kDNA*, to quantify the parasitic load of *Leishmania* spp.; it was normalized with the human single-copy gene, *ERV3*. One of the most relevant findings of this study was the significant decrease of the parasitic load with the evolution time of the disease, which led to the suggestion of using this technique to evaluate treatment efficacy and disease resolution prognosis. Another Colombian study (Romero et al., 2010) focused on using RT-qPCR to determine parasite viability at extra-lesional sites by transcriptions of the molecular marker *7SLRNA*, as determining whether or not the parasite is viable in monocytes may alert about future CL and MCL. In other countries of the region, such as Peru, the parasitic load of *Leishmania* spp. in skin and mucosal lesions was detected and quantified. In this case, *k-DNA* qPCR proved to be highly sensitive and accurate for the detection and quantification of parasites from biopsies; no differences were found in parasite load in relation to parasite species, nor patient age, number, or area of the lesions (Jara et al., 2013). Another study in Peru, quantitatively compared parasite loads between different skin lesion sites, as well as sampling methods using qPCR to provide an idea of the likely distribution of the *Leishmania* spp. amastigote in the ulcer, thus improving the diagnosis and prognosis of CL (Suárez et al., 2015). Finally, a study in Brazil estimated the parasitic load of *SSU rRNA* DNA using a qPCR. In this study, some children with VL were monitored before, during, and at the end of the drug treatment, and the identification of risk factors was performed to establish the appropriate prognosis (Mourão et al., 2014).

Methods

Ethical considerations

According to the classification outlined in Article 11 of Resolution 8430 of 1993 of the Ministry of Health, this study is a minimum risk investigation. It was approved

under Register No. 2043 of March 22, 2017, pursuant to the Committee on Ethics in Research of the *Hospital Militar Central* in Bogota.

The handling of the patient's data, clinical-epidemiological records, and results was carried out under the strictest rules of confidentiality, with the previous authorization of each patient through the use of informed consent. The informed consent was used following Colombian Resolution 2378 of 2008 on Best Clinical Practices.

Type of study

This study was defined as longitudinal, descriptive, and observational. It involved the monitoring of a group of patients being treated for cutaneous leishmaniasis. The study included sampling before, during, and at the end of the treatment; this was accompanied by clinical evaluation.

Sampling size

The sample size was estimated by considering the average number of patients treated during one year in Bonza, Boyacá (first-time treatment with meglumine antimoniate), as well as the patients treated in the Health Battalion (Basan) in Bogotá (pentamidine isethionate as a second choice treatment). This number was adjusted by including the average annual number of patients recurring treatment (19% to 50%), excluding patients with facial lesions, according to the data available from the Operational Health Department of the Army and Sivigila. The sample size of n = 139 patients (Bonza = 86, and Basan = 53 patients) was estimated using Epi Info v7.2.2.6 (https://www.cdc.gov/epiinfo/index.html), yielding a CI of 95% and an expected sample error of 5%. The analyses of this study were performed by correlating the parasitic loads with the clinical-epidemiological data, as well as the spatial-temporal analysis of the circulating *Leishmania* spp. species in a military population of only n = 23 patients; this corresponds to the samples with high parasitic load and considered at the end of the treatment.

The study included male military patients over 18 years old, with a microscopic diagnosis of cutaneous leishmaniasis with a lesion over 1 cm in diameter and without clinical evidence of bacterial or fungal superinfection of the ulcer at the beginning of the study. These patients participated voluntarily in the study, with prior informed consent.

Sampling

The smear samples were obtained by manual ischemia. Using the thumb and index fingers, the base and center of the ulcer were scraped with a sterile lancet (Suárez et al., 2015). The sample obtained was placed in a screw-cap vial with 250 µL of sterile saline solution. It was then stored at -70 °C until processing. Additionally, a peripheral blood sample of approximately 3 mL was collected in a tube with EDTA and stored at 4 ºC before processing.

Peripheral blood mononuclear cell (PBMC) collection

In a 15 ml tube, 3 ml of peripheral blood was added to 7 ml of 0.85% sterile saline. They were mixed by inversion and vortex for better homogenization. Subsequently, 3 ml of ficoll were added to another 15 ml tube. Seven milliliters of the initial mixture of peripheral blood and saline were taken and transferred through the walls of the tube to avoid mixing the sample with the reagent. The mixture was centrifuged for 30 min at 4 ºC and 2500 r/min. After centrifugation, the opaque interface containing the PBMC was aspirated, deposited in a 1.5 ml vial, and stored at -20 ºC for further processing.

DNA extraction and quantification by BioDrop

The DNA extraction from smears, biopsies, PBMC, and *Leishmania braziliensis* culture samples from the LRI-DISAN-EJC (MHOM/DR/75/M2904) was performed by adsorption chromatography, according to the kit manufacturer's instructions. The final elution of the extract was performed in 60 µL of EL-buffer. Following this, all the DNA extracts were quantified using a BioDrop uLite Spectrophotometer (Biochrom US, Holliston, MA). The values accepted were equal to or greater than 4 ng/µL with ratios of 260/230 (nucleic acids/chaotropic salts, phenols, or carbohydrates) > 1.0 and < 3.0 while 260/280 (nucleic acids/aromatic compounds such as phenols, proteins, and RNA) > 1.6 and < 3.0.

Real-time PCR for treatment monitoring

The *kDNA* minicircle was amplified using kDNAr (5' GAA CGGGT TTC TGT ATG C 3') and kDNAr (5' TAC TCC CCG ACA TGC 3') *primers* (Jara et al., 2013). Meanwhile, the *ERV3* gene served as a normalizer, as it is a single-copy in the human genome. The *primers* for *ERV3* were: PHP10F (5' CAT GGG AAG CAA GGG AAC TAA TG 3') and PHP10R (5'CCC AGC GAG CAA TAC AGA ATT 3') (Yuan et al., 2001; Adaui et al., 2006).

The qPCR reaction was run in a LightCycler® 96 Roche equipment. The mixture was previously prepared according to the recommendations of the commercial Luna Universal qPCR Master Mix kit by New England. The mix consisted of 0.2 µM of each primer, 12.6 µL of DNase and RNase-Free Water, 0.5 X of Master Mix, and 2 µL of DNA for a final volume of 20 µL. The thermocycling conditions for *kDNA* and *ERV3* were as follows: pre-incubation at 95 °C for 10 min, followed by 36 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Fluorescence emission was measured at the end of the elongation step using the SYBR-Green system. After the amplification, a melting curve of 95 °C for 60 s, 60 °C for 60 s, and continuous heating at 1 °C/s up to 95 °C was generated (Jara et al., 2013; Méndez, 2014).

Standard curve, parasitic load quantification, and qPCR validation

The MHOM/DR/75/M2904 genomic DNA of *L. braziliensis* was used to design the standard curves of the *kDNA* assays. It was considered that 96.3 fg corresponded to nuclear DNA and kinetoplast DNA (Peacock et al., 2007; Shapiro & Englund, 1995). Eight serial dilutions were performed from 10^5 (4.815 ng/µL) to 10^{-2} (4.815 × 10-7 ng/µL) based on an initial 185.5 ng/ µL genomic DNA concentration of *L. braziliensis*.

The normalization of the parasitic load was achieved by quantifying the number of human cells in parallel using the single-copy *ERV-3* gene. To this end, a standard curve was constructed with points from 10^5 (165 ng/µL) to 10^0 (1.65×10^{-3} ng/µL) using human DNA with an initial concentration of 207 ng/µL. The mass of the human haploid genome of 3300 fg was considered. Finally, the equation was applied to obtain the parasitic load or number of parasites/10^6 human cells (Jara et al., 2013). Duplicate sensitivity assays were performed to validate the test, using 5 points on the curve of pure *L. braziliensis* DNA vs. another *L. braziliensis* DNA contaminated with 10 ng human gDNA. On the other hand, for the reproducibility tests, intra assay and interassay *kDNA* curve assemblies were performed in triplicate, checking, in all cases, the efficiency and R^2 of the curves.

PCR purification and sequencing

The amplification by conventional PCR of the *HSP70* and *MPI* markers in n = 23 from smear extracts was carried out following previously established protocols (Córdova et al., 2011; Ramírez et al., 2016). All the products were tested using 1% agarose gel, stained with Gel Red™ 1000X, and run for 40 minutes at 80 V in a horizontal electrophoresis system with TAE buffer 1X. The PCR products were then purified and sequenced by the terminal dideoxy method in an Applied Biosystem AB3730 automatic sequencer from Macrogen Korea.

Statistical and bioinformatic analysis

All descriptive, univariate, and multivariate statistical analyses for patients reporting high parasitic loads or to be considered at the end of treatment were developed using IBM SPSS Statistics v25. Nonparametric tests were used when samples did not follow Gaussian distribution, according to Shapiro Wilk's test. Variables were analyzed according to their categorical or quantitative nature using the Mann-Whitney U, Kruskal Wallis, and Spearman correlation tests. The variables analyzed in this study were parasitic load in smear/biopsy and PBMC, treatment monitoring (before, during, and after), infecting species (*L. braziliensis* and *L. panamensis*), type of treatment (meglumine antimoniate and pentamidine isethionate), area of internal and external lesions, number of lesions, disease evolution, wound area, as well as department (place) of

possible infection; preventive measures, such as the use of awnings or repellent; age; military-grade; and treatment success or failure. All the tests were two-tailed; the results were considered significant (*) when $p < 0.05$, and highly significant (**) when $p < 0.01$. A principal component analysis (PCA) was conducted for the data of the 23 patients who were found to have high parasite loads or to be considered at the end of treatment. This was done using the varimax rotation matrix correlation method to maximize the variance of each factor, as well as the Anti-Image correlation matrix. The PCA (Principal Component Analysis) was validated using the Kaiser-Meyer-Olkin and Bartlett's Sphericity tests; therefore, several discrete and continuous variables were grouped into three main components that explained more than 60% of the variance of the data. Those with correlations < - 0.5 or > 0.5 were accepted as variables with a high contribution to the components.

The bioinformatics analyses to molecularly identify the *Leishmania* spp. infecting species were performed in BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), looking for similarity with a percentage of identity of > 98% a high score and e-value close to 0.0 between the sequences obtained from this study versus the database of available *Leishmania* spp. sequences in GenBank.

Results

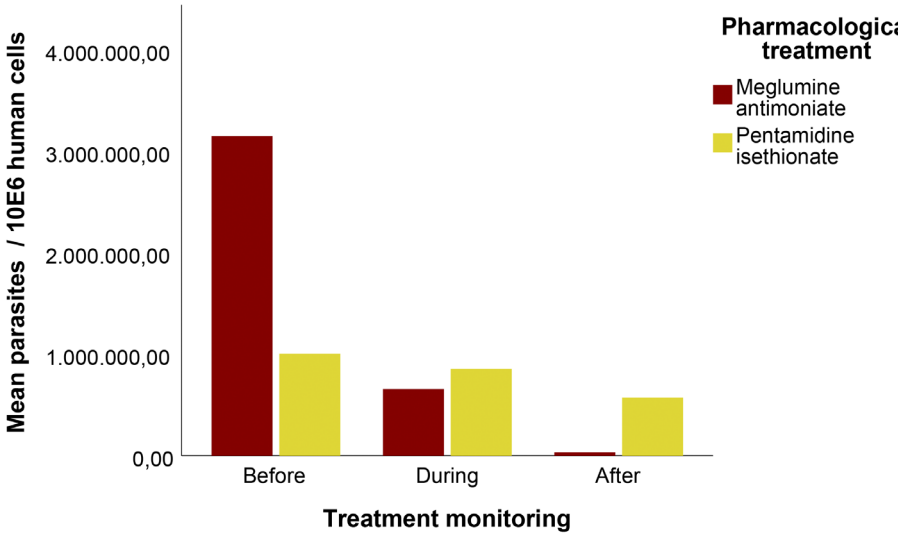
Quantification of parasitic load

The parasitic load of *Leishmania* spp. in 23 men of the active military personnel of the Colombian National Army with cutaneous leishmaniasis was estimated from PBMC samples and smears/biopsy analyzed by qPCR. For the latter, it was determined that only 8.82% (12/136) of the patients initially sampled in the study presented a number of > 10,000 parasites/10^6 human cells at the end of the study. Figure 1A shows the effectiveness of the treatment using meglumine antimoniate and pentamidine isethionate, before, during, and after the treatment of the 23 patients that presented a high parasitic load in the smear/biopsy at the end of the study.

Regarding the number of parasites/10^6 PBMC, only 9.56% (13/136) showed parasite loads > 1.0 at the end of the treatment. Figure 1B shows the tracking of the effect of treatment (before, during, and after) and the type of treatment (meglumine antimoniate and pentamidine isethionate) on the parasitic load to be considered in the PBMC of the 23 patients analyzed.

The parasitic loads of the total number of patients sampled (n = 136) were not reported, only the data of the 23 patients with high parasitic loads of importance for molecular diagnosis in leishmaniasis were analyzed and reported in this study.

A



B

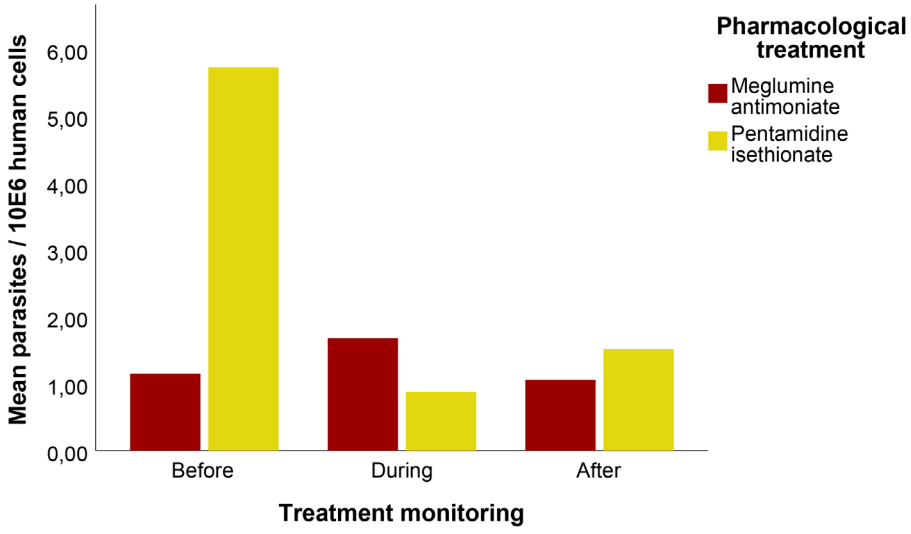


Figure 1. Mean parasitic load during tracking of treatments for cutaneous leishmaniasis with meglumine antimoniate and pentamidine isethionate, using the *kDNA* molecular marker in 23 patients presenting high parasitic load or considered at the end of treatment. (A) Parasitic load estimated from smear samples and biopsies of lesions. (B) Parasite load estimated from peripheral blood mononuclear cells (PBMC).
Source: Created by the author.

Relationship between parasitic loads and clinical-epidemiological data

Among the quantitative clinical-epidemiological variables that were analyzed were age, number of lesions, disease evolution, internal and external area of the lesion (Table 1). The variables that, together with the parasitic loads in smears/biopsy and PBMC, were evaluated by descriptive statistics for the subgroup of 23 patients presenting high parasitic loads or to be considered at the end of the drug treatment are shown in Table 2. We also determined the effect of the type of treatment, as well as the follow-up treatment, using the smear/biopsy and PBMC parasitic loads (Figure 1).

Table 1. Summary of descriptive statistics of clinical-epidemiological data for the 23 patients in the study.

		Age n = 23		Number of lesions n = 23		Disease evolution (months)	
						MA n = 14	PI n = 5
Maximum		36.0		13.0		3.00	7.00
Mean		24.78		2.30		1.39	4.30
Median		24		1.0		1.25	4.00
Variance		15.27		10.68		0.47	6.20
Minimum		19.0		1.0		0.50	0.50
		Before		During		After	
		MA n = 17	PI n = 6	MA n = 16	PI n = 6	MA n = 11	PI n = 6
Area of internal lesion (cm^2)	Maximum	24.00	22.50	32.50	7.50	26.5	6.96
	Mean	4.24	4.32	3.62	1.74	3.44	1.60
	Median	0.90	0.68	0.85	0.76	0.64	0.56
	Variance	56.36	79.67	63.82	8.12	59.78	0.33
	Minimum	0.18	0.12	0.07	0.09	0.09	0.09
		n = 13	n = 5	n = 17	n = 6	n = 11	n = 6
Area of external lesion (cm^2)	Maximum	75.00	42.00	48.00	41.25	33.00	24.00
	Mean	10.91	13.50	6.42	10.47	5.95	7.44
	Median	4.40	3.84	2.60	5.64	2.88	4.44
	Variance	420.20	319.62	137.54	239.25	92.16	75.31
	Minimum	0.80	0.42	0.25	0.30	0.25	1.00

MA = meglumine antimoniate
PI = pentamidine isethionate

Source: Created by the author.

Table 2. Summary of descriptive statistics of parasitic loads in smear/biopsy and PBMC for the 23 patients in the study.

		Before		During		After	
		MA n = 17	PI n = 6	MA n = 17	PI n = 6	MA n = 17	PI n = 6
Parasitic load in smear/biopsy (number of parasites / 10^6 human cells)	Maximum	1.27×10^7	2.80×10^6	9.37×10^6	4.25×10^6	2.68×10^5	3.39×10^6
	Mean	3.28×10^6	1.01×10^6	6.58×10^5	8.57×10^5	3.23×10^4	5.72×10^5
	Median	2.12×10^6	2.01×10^5	3.87×10^4	6.55×10^4	1.04×10^4	8.51×10^3
	Variance	1.70×10^{13}	1.94×10^{12}	5.08×10^{12}	2.85×10^{12}	4.20×10^9	1.91×10^{12}
	Minimum	7.01×10^3	2.21×10^3	7.37×10^1	3.13×10^1	1.89×10^0	4.30×10^2
Parasitic load in PBMC (number of parasites / 10^6 human cells)	Maximum	2.58×10^0	3.13×10^1	9.57×10^0	1.20×10^0	2.40×10^0	5.55×10^0
	Mean	1.15×10^0	5.73×10^0	1.68×10^0	8.80×10^{-1}	1.06×10^0	1.52×10^0
	Median	1.05×10^0	6.47×10^{-1}	6.69×10^{-1}	1.06×10^0	1.12×10^0	7.16×10^{-1}
	Variance	5.54×10^{-1}	1.57×10^2	6.19×10^0	2.03×10^{-1}	6.37×10^{-1}	4.14×10^0
	Minimum	0.0	2.00×10^{-2}	0.00	0.00	0.00	2.00×10^{-1}

MA = meglumine antimoniate
PI = pentamidine isethionate

Source: Created by the author

On the other hand, according to the Shapiro-Wilk normality tests performed on the quantitative variables of the study for n = 23, the following was determined. The parasitic loads in smears/biopsy (S-W = 0.495, $p = 0.00$) and PBMC (S-W = 0.641, $p = 0.00$) do not have a normal distribution, as well as the areas of internal (S-W = 0.487, $p = 0.000$), external (S-W = 0.621, $p = 0.000$) lesions, the number of lesions (S-W = 0.446, $p = 0.000$), and the disease evolution (S-W = 0.641, $p = 0.000$). The only quantitative demographic variable evaluated that indicates normal distribution is the age of the patients (S-W = 0.945, $p = 0.699$).

A non-parametric test or 1-way ANOVA was carried out using Kruskal Wallis for all the variables of non-normal distribution. It was determined that there were significant differences ($p = 0.000$) between smear/biopsy parasitic loads according to the stage in the treatment monitoring (before, during, and after). The type of treatment also affected the disease evolution ($p = 0.00$). In contrast, there were no significant differences in the parasitic loads in PBMC ($p = 0.784$), the area of internal lesion ($p = 0.949$), and external lesion ($p = 0.708$) depending to the stage of the treatment monitoring. There were also no significant differences in the parasitic loads in smear/biopsy and PBMC concerning the infecting species and type of drug treatment. We found no significant effect regarding the patient military-rank, locations of skin lesions, department (place) of possible infection, or preventive measures, such as using

an anti-insect netting or repellent, on the parasitic load, nor the area of internal or external lesions, disease evolution, number of lesions, and treatment success or failure.

A highly significant negative correlation was found for the parasitic loads at the end of smear treatment versus PBMC, according to Spearman's Rho coefficient: -0.560** (*p = 0.005*). Parasitic loads in *L. braziliensis* show a higher dispersion compared to the parasitic loads in *L. panamensis* (Figure 2). Similarly, there was a significant negative correlation between the clinical follow-up of the outside area of the lesion and monitoring of the parasitic load on smears, meaning that when the parasitic smear loads decreased during follow-up, the area of the external lesion in these patients increased (Figure 3). In contrast, there is a highly significant positive correlation between the follow-up of the parasitic load in PBMC versus the follow-up on the external area of the lesion (Rho Spearman: 0.489** *p = 0.000*) and the follow-up on the internal area of the lesion (Rho Spearman: 0.335** *p = 0.004*). Internal versus external lesions were highly positively correlated throughout treatment monitoring (Rho Spearman: 0.686** *p = 0.000*).

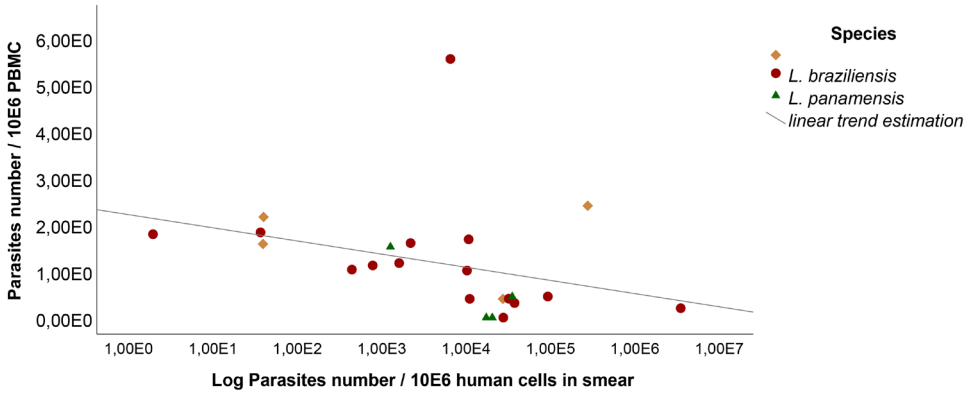


Figure 2. Spearman's correlation between the parasitic load of *Leishmania* spp. in PBMC versus smear/biopsy at the end of drug treatment. n = 23. Spearman's Rho: -0.560** (*p = 0.005*). Diamonds indicate samples that were not molecularly identified with the etiological agent.
Source: Created by the author

Concerning the non-parametric U Mann Whitney test, it was determined that the parasitic loads of smears (U M-W = 126.5**) and the disease evolution (U M-W = 76.5*) were different for those with resolved versus those with unresolved leishmaniasis. Conversely, there were no significant differences for parasitic loads in PBMC, areas of internal and external lesions, and the number of lesions in patients with resolved

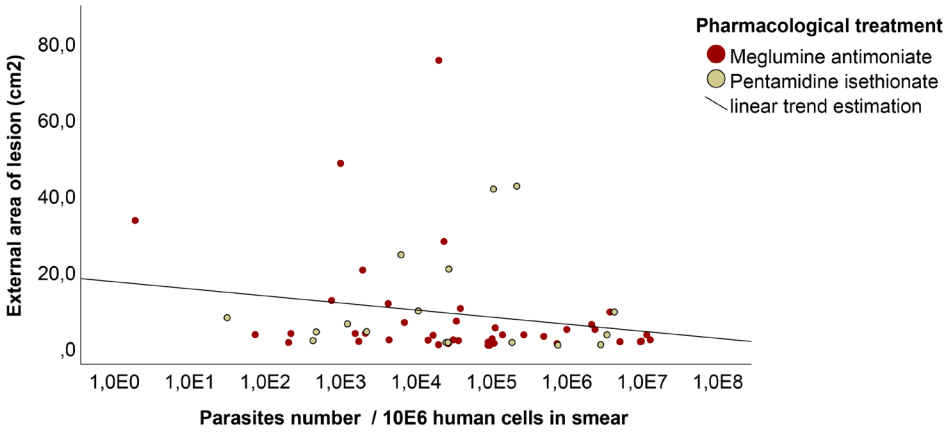


Figure 3. Spearman's correlation between the parasitic load in smears/biopsy and the external lesion area based on drug treatment before, during, and at the end of 20 days.
n = 23. Rho Spearman: -0,264* *p = 0,044.*
Source: Created by the author.

leishmaniasis versus those without. We also found that the infecting species did not influence any non-parametric quantitative variables in the study. The type of treatment affects the differences reported for the disease evolution (U M-W = 114.0**).

Age was the only the only variable with a normal distribution variable; therefore, it was determined by t-Student that there were no significant differences between their mean concerning the infectious species, treatment received, and resolution according to medical criteria.

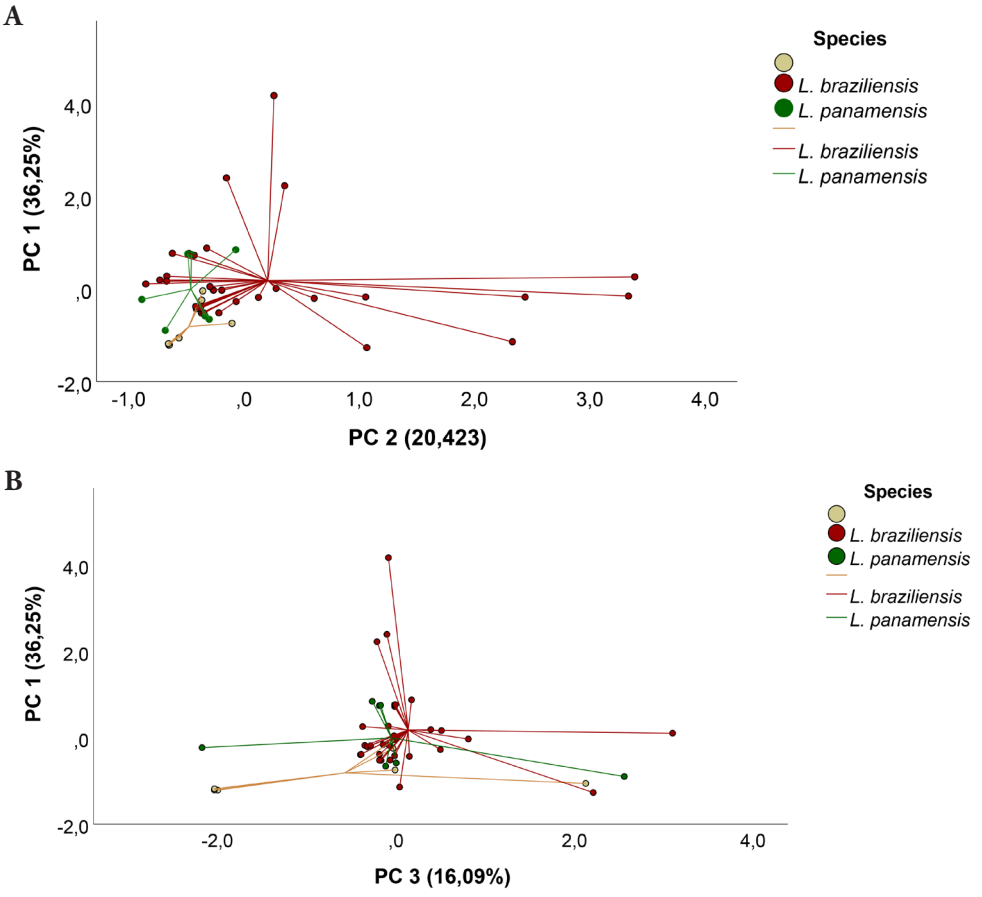
Multivariate analyses were performed, given that not all univariate analyses yielded meaningful data for all the variables. Seven variables were grouped into three principal components (PC), which explained 72.66% of the data variance. The PCA was validated by a Kaiser-Meyer-Olkin measure of sampling adequacy (KMO = 0.604), which was above the suggested minimum value of 0.6. Additionally, Bartlett's sphericity test estimated a $\chi 2 = 83.023^{**}$, meaning that there is a high correlation between the variables.

The PC 1 yielded the following variables with correlations > 0.5: age, disease evolution, and parasitic load in PBMC. The PC 2 included the variables with correlations > 0.5, which were the area inside the lesion and outside the lesion. The PC 3 collected the variables with correlations > 0.5; these were parasitic load in smears/biopsy and the number of lesions. Therefore, it was concluded that the internal and external areas of lesions grouped in the PC 2 explain the differences at the infecting species level (Figure 4). In contrast, neither age, disease evolution, parasitic load in

smears/biopsy and PBMC, and the number of lesions grouped in PC 1 and PC 3 explain the differences between *Leishmania* spp.

All of the interactions of the highly correlated variables grouped in PC 1, PC 2, and PC 3 explain the differences between the drug treatments given to the 23 evaluated patients (Figure 5). Otherwise, the parasitic load in smears/biopsy and the number of lesions grouped in PC 3 explain the differences between the stage before drug administration and the monitoring during and after 20 days (Figure 6).

Whether or not the disease was resolved in the patient was determined with the support of the medical dermatological staff of the leishmaniasis program. For this variable, the PCA concluded that the age of the patient, the disease evolution, and the parasitic load in PBMC, grouped in PC 1, together with the parasitic load in the smears/biopsy and the number of lesions grouped in PC 3, explain the differences between whether or not there is resolution of leishmaniasis according to medical criteria (Figure 7).



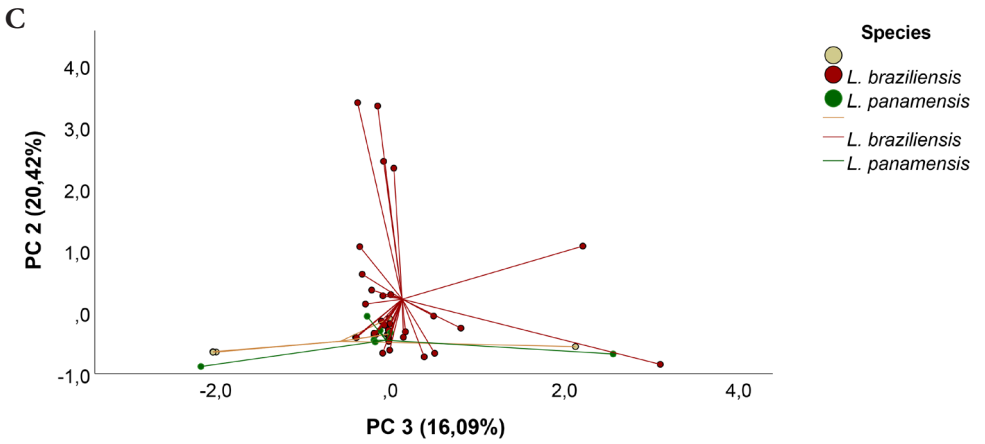
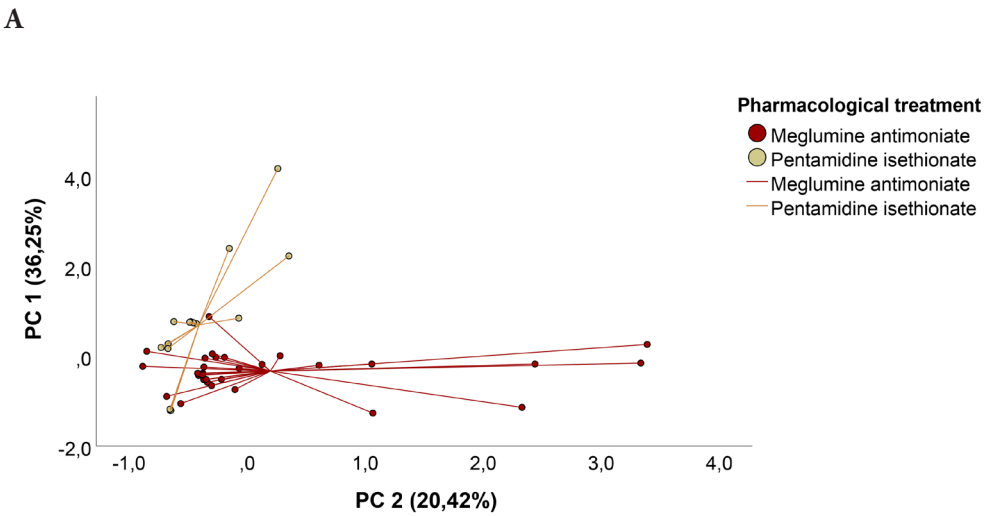


Figure 4. Principal Component Analysis (PCA) for *Leishmania* spp. species before, during, and at the end of 20 days of drug treatment. The main component 1 (PC 1) explains 36.15% of the variance in the data, PC 2 explains 20.42% of the variance, and PC 3 explains 16.09%. Figure 4A: The PCA of PC 1 and PC 2 explains 56.57 % of the model. Figure 4B: The PCA from PC 1 and PC 3 explains 52.34% of the model. Substantial differences in the centroids of the infecting species were reported according to both components. Figure 4C: The PCA of PC 2 and PC 3 explains 36.51% of the model. Differences in the centroids of the infecting species are presented based on PC 2.
Source: Created by the author.



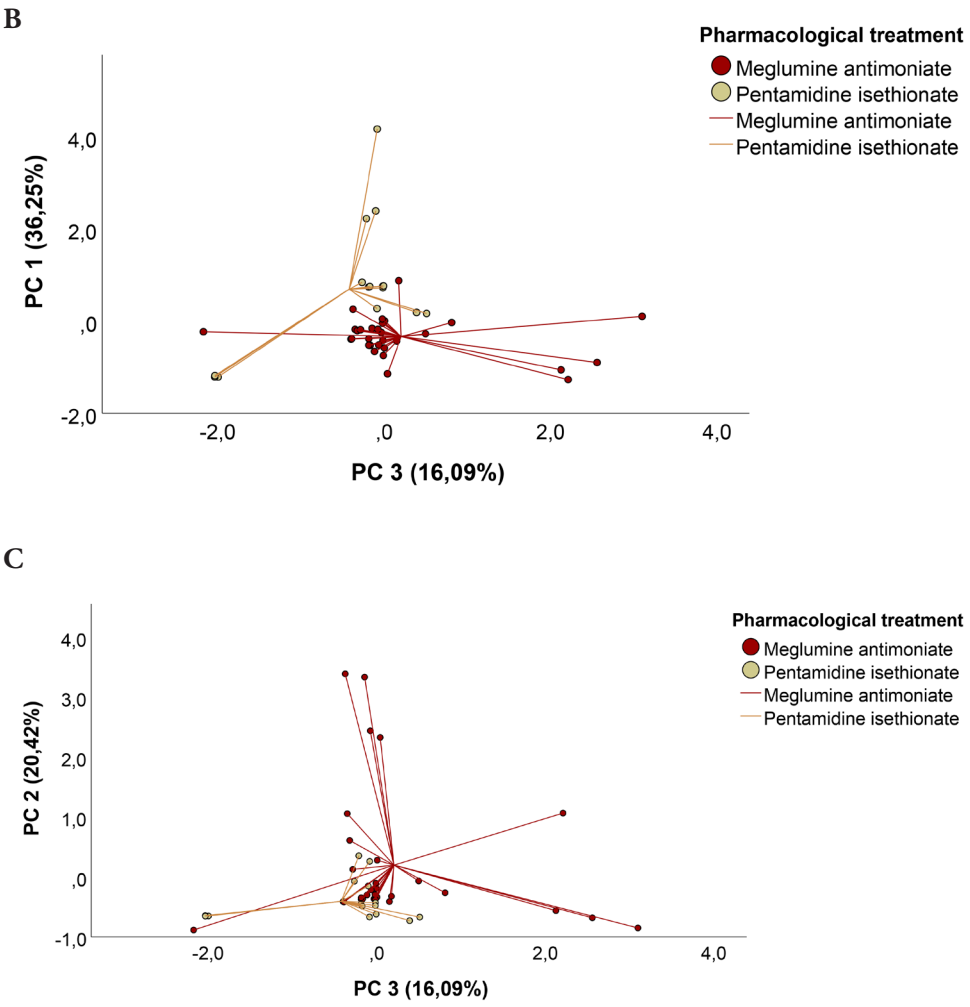


Figure 5. Principal Component Analysis (PCA) in relation to drug treatment provided before, during, and at the end of 20 days. Figure 5A: Differences in the centroids of drug treatments according to PC 1 and PC 2 are represented. Figure 5B: Substantial differences in the centroids of drug treatments according to PC 1 and PC 3 are reported. Figure 5C: Differences in the centroids of drug treatments according to PC 2 and PC 3 are presented.
Source: Created by the author.

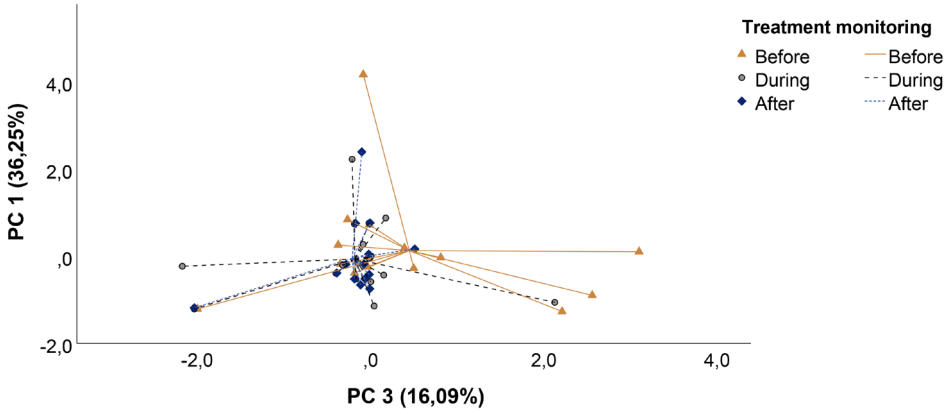
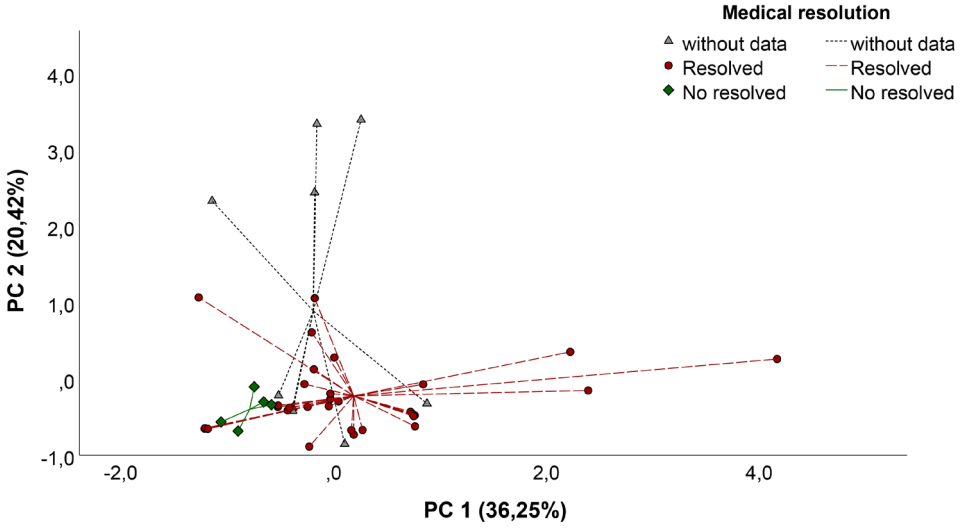


Figure 6. Principal Component Analysis (PCA) in relation to treatment tracking. PC 3 explains the differences between before versus during and after sampling, while PC 1 does not explain any difference between the three follow-ups to the drug treatment. PC 1 and PC 2 do not explain any difference between monitoring stages and drug treatment.
Source: Created by the author.

A



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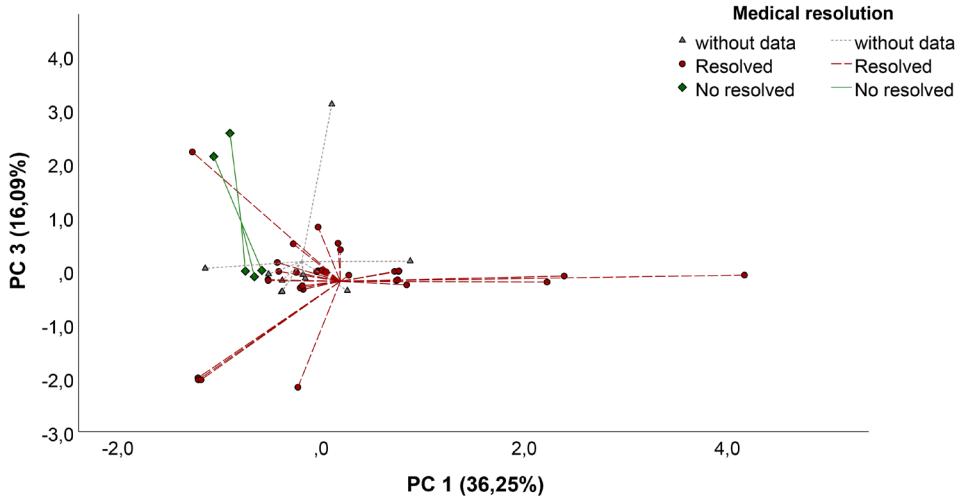

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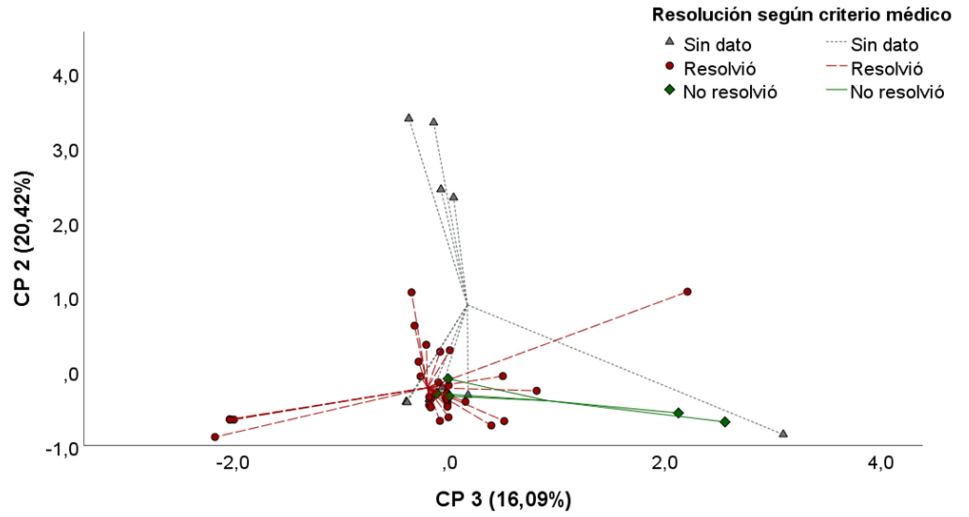

Figure 7. Principal Component Analysis (PCA) in relation to resolution according to medical criteria before, during, and at the end of 20 days of drug treatment. Figure 7A: Differences in centroids of whether or not it was resolved only by PC 1 are represented. Figure 7B: Substantial differences in centroids of whether or not it was resolved according to PC 1 and PC 3 are reported. Figure 7C: Differences in centroids of whether or not it was resolved based on PC 3 are presented. Source: Created by the author.

The patients that represented 8.82% (n = 12) in smears/biopsy and 9.56% (n = 13) in PBMC with high parasitic load or to be considered at the end of the treatments are shown in Table 3. Of the 23 patients with high parasitic load or to be considered at the end of the treatment, 26.1% were those with previous therapeutic failure using meglumine antimoniate, receiving a second line of treatment with pentamidine isethionate in this study. Sixty-nine point six percent (69.6%) were patients infected with *L. braziliensis*, as well as 17.4%, which, according to medical criteria, did not resolve the disease at the final stage (Table 3).

Table 3. Patients with cutaneous leishmaniasis presenting high parasitic load or to be considered at the end of treatment (n = 23)

Number of parasites/10^6 human cells in smear/biopsy > 10000 (n = 12)				
Patient code	**Pharmacological treatment**	**Infecting species**	**† Final load in smear/biopsy**	**Resolution according to medical criteria**
BON 97	Meglumine antimoniate	*L. braziliensis*	10385.77	No data
BAS 126	Pentamidine isethionate	*L. braziliensis*	10676.08	Not resolved
BON 41	Meglumine antimoniate	*L. panamensis*	16806.51	Not resolved
BAS 03	Meglumine antimoniate	*L. braziliensis*	19750.45	Resolved
BAS 26	Pentamidine isethionate	No data	26533.33	Resolved
BON 12	Meglumine antimoniate	*L. braziliensis*	26758.03	Resolved
BON 21	Meglumine antimoniate	*L. braziliensis*	31063.83	Resolved
BON 72	Meglumine antimoniate	*L. panamensis*	34155.60	Resolved
BON 26	Meglumine antimoniate	*L. braziliensis*	36448.89	No data
BON 15	Meglumine antimoniate	*L. braziliensis*	90136.99	Not resolved
BON 90	Meglumine antimoniate	No data	268045.11	Not resolved
BAS112	Pentamidine isethionate	*L. braziliensis*	3390449.44	Resolved
Number of parasites/10^6 human cells in PBMC > 1.00 (n = 13)				
Patient code	**Pharmacological treatment**	**Infecting species**	**† Final load in PBMC***	**Resolution according to medical criteria**
BON 101	Meglumine antimoniate	*L. braziliensis*	1.01	Resolved
BAS 110	Pentamidine isethionate	*L. braziliensis*	1.028	Resolved

Table continues...

Number of parasites/10^6 human cells in PBMC > 1.00 (n = 13)				
Patient code	**Pharmacological treatment**	**Infecting species**	**† Final load in PBMC***	**Resolution according to medical criteria**
BON 77	Meglumine antimoniate	*L. braziliensis*	1.11	Resolved
BON 75	Meglumine antimoniate	*L. braziliensis*	1.17	No data
BAS 130	Pentamidine isethionate	*L. panamensis*	1.52	Resolved
BON 102	Meglumine antimoniate	No data	1.59	No data
BON 49	Meglumine antimoniate	*L. braziliensis*	1.60	Resolved
BON 97	Meglumine antimoniate	*L. braziliensis*	1.68	No data
BON 06	Meglumine antimoniate	*L. braziliensis*	1.79	No data
BON 96	Meglumine antimoniate	*L. braziliensis*	1.83	Resolved
BON 69	Meglumine antimoniate	No data	2.16	Resolved
BON 90	Meglumine antimoniate	No data	2.40	Not resolved
BAS 128	Pentamidine isethionate	*L. braziliensis*	5.55	Resolved

† The final load can be understood as the number of parasites equivalent to the number of real-time PCR-amplified copies of *kDNA* in *Leishmania* spp. relative to 10^6 human cells, according to the real-time PCR-amplification of the *ERV3* standardizer gene.

* Peripheral blood mononuclear cells (PBMC).
BON 90 and BON 97 patients are highlighted in grey; they showed a parasite quantity equivalent to a high *kDNA* in both smear/biopsy and peripheral blood mononuclear cells at the end of treatment.

Source: Created by the author.

Two patients (BON 90 and BON 97) coincided in high loads and were considered to be in both smear/biopsy and PBMC (Figure 8). According to medical criteria, CL in the BON 90 patient was not successfully resolved; therefore, he was referred to internal medicine. Likewise, BON 15, BON 41, and BAS 126 patients were referred to second and third-line treatments with pentamidine isethionate and liposomal amphotericin B, respectively (Figure 9). Additionally, was administered intralesional meglumine antimoniate and thermotherapy as a conditioned therapeutic alternative to BAS 126 patient (Durán et al., 2018). Patients BAS 112 and BAS 128 who have stood out due to the highest parasitic loads at the end of the treatment monitoring in smears and PBMC, respectively (Figure 10).

Figure 8. Lesions in patients with high parasitic loads or to be considered in both smears and PBMC. BON 90: a 20-year-old regular soldier, reportedly infected in Campoalegre, Guainía, with an ulcer on his left arm. BON 97: a 30-year-old professional soldier, with an ulcer on his left forearm, possibly infected in San José del Guaviare, Guaviare. From the treatment monitoring, it is evident that the lesions were activated during the treatment.
Source: Created by the author.

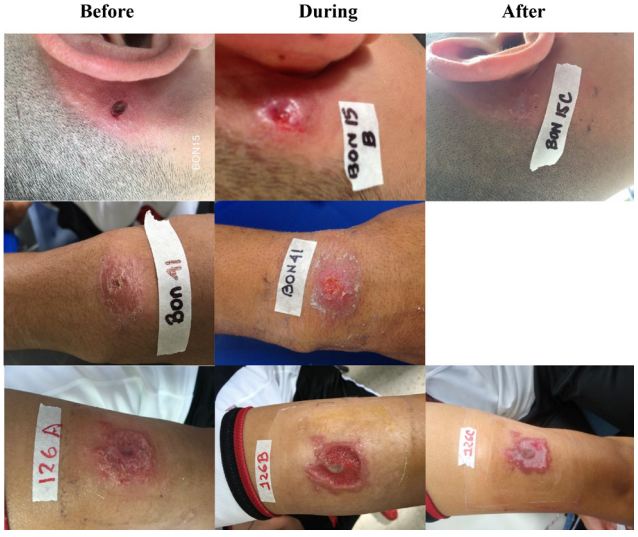

Figure 9. Lesions in patients that were not resolved, according to medical criteria at the end of treatment.
BON 15: a 25-year-old second-lieutenant with an ulcerated lesion located in the mastoid region, possibly infected in La Macarena, Meta. BON 41: a 19-year-old professional soldier with an ulcer on his right hand; patient reports having been infected in Puerto Caicedo, Putumayo. BON 126: a 30-year-old corporal first with an ulcer on the left forearm, possibly infected in San José del Guaviare, Guaviare. None of the lesions in these three patients decreased in size sufficiently to be considered a medical cure.
Source: Created by the author.

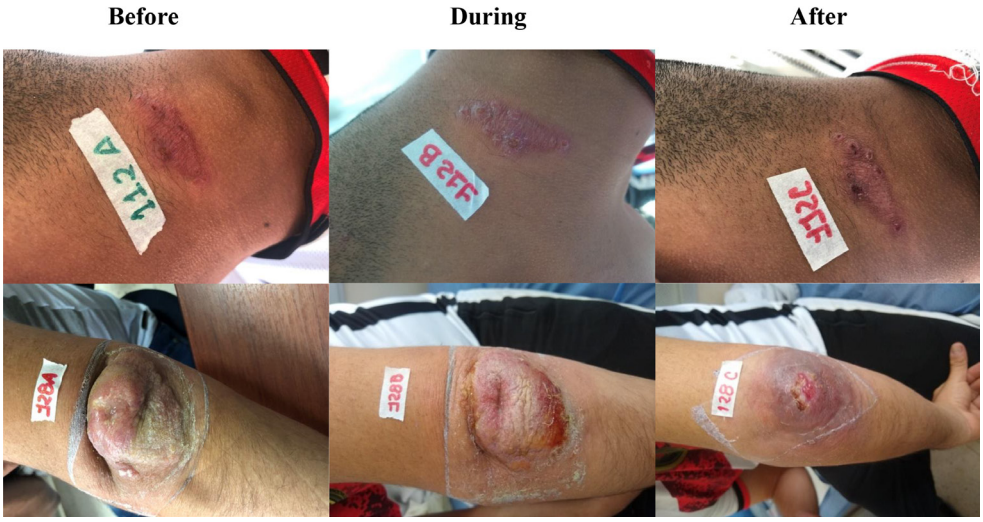

Figure 10. Lesions in patients presenting the highest parasitic loads in smears (BAS 112) and PBMC (BAS 128) at the end of the treatment monitoring.
BAS 112: a professional 24-year old soldier, with a plaque lesion on the neck, possibly infected in San José del Guaviare. The appearance of the lesion changed during treatment to a warty plaque.
BAS 128: a 36-year old Second Sergeant, with an ulcerated elbow lesion with an inflammatory reaction, which subsided during treatment. He reports having been infected at El Retorno, Guaviare, with no change in the area of the lesion at the end of treatment (approximately 42 mm^2).
Source: Created by the author.

Molecular identification and spatial-temporal analysis of *Leishmania* spp. in Colombia

82.6% (n = 19) of the 23 patients in the study were amplified and appropriately sequenced by *HSP70* and *MPI*. The molecular identification of the *Leishmania* spp. infecting species circulating in this particular military population, treated under the leishmaniasis program in Bonza, Boyacá, and Basan, Bogotá in 2017 and 2019, was completed. The sample of interest presented a spatial-relative abundance of 84.21% for *L. braziliensis* and 15.78% for *L. panamensis*. Concerning the temporal analysis, only 14 samples were available from 2017 and five from 2019. For 2017, a relative abundance of 85,71% (n = 12) was obtained for *L. braziliensis*, while for 2019, it was 80% (n = 4). *L. braziliensis* was reported from three departments of Colombia with exclusive distribution in the Orinoco and Amazon regions. *L. panamensis* had a reported distribution in three departments in the Pacific and Amazon regions. There was no report of the two Leishmania species circulating in the same department of this sampling (Figure 11).

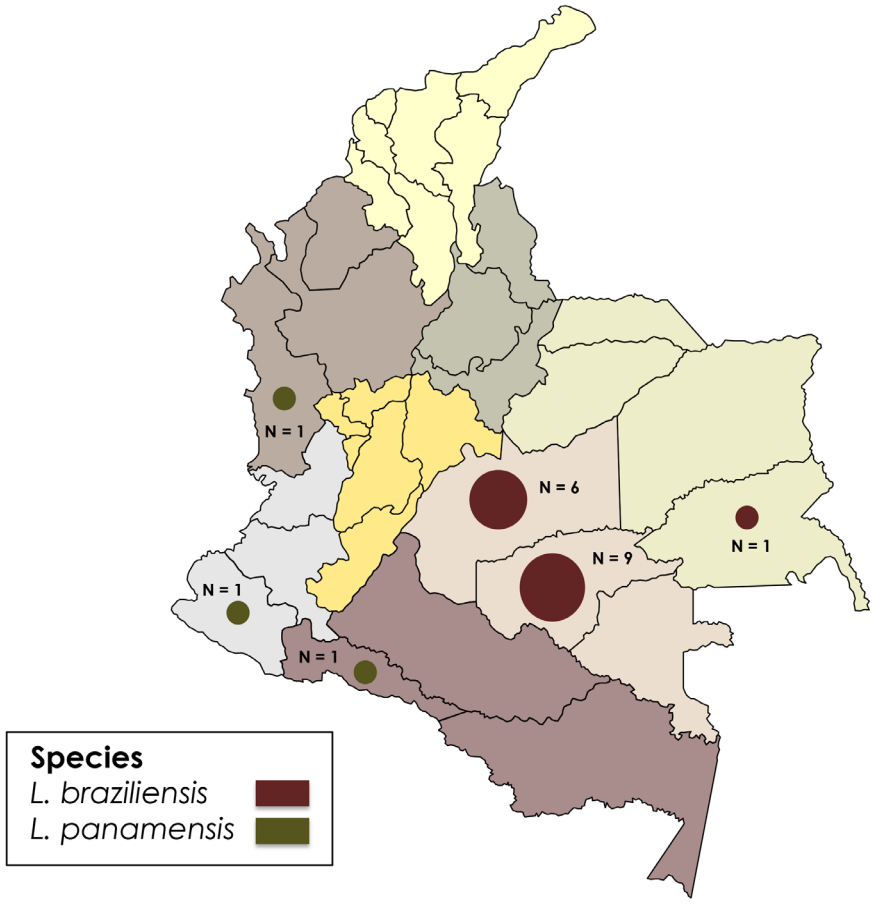

Figure 11. Spatial distribution and abundance of *Leishmania* spp. species in 19 men of the Colombian National Army with high parasitic loads and to be considered at the end of the pharmacological treatment. The molecular identification of the parasite species was performed using BLASTn with a % identity greater than 98%, from *HSP70* and *MPI* sequences obtained by PCR amplification and terminal dideoxy sequencing of the smears in the study.
Source: Created by author.

Discussion

Quantification of the parasitic load

It should be noted that patients initially treated with meglumine antimoniate have, on average, high parasitic loads at the skin lesions, but very low into PBMC. The opposite occurs in patients treated with pentamidine isethionate that start with lower parasitic loads at skin ulcers, but significant average blood loads (Figure 1). This could suggest

hematogenous or lymphatic spread and, therefore, the circulation of *Leishmania* spp. in the blood once there is a therapeutic failure, and the patient is referred to a second treatment scheme (Romero et al., 2010).

The parasitic loads at the end of treatment on the smears of the patients were between 10^4 and 10^6. Therefore, the 3,390,449.44 parasites/1,000,000.00 human cells in patient BAS 112 (Table 3) was considered a very high value. Depending on the virulence of the infecting species, the patient's immune system, among many other pathogens/host factors, could mean a risk of future mucocutaneous leishmaniasis. This form occurs in a large number of cases due to the reactivation of parasites circulating in the blood under the previously mentioned conditions (Machado et al., 1992; Conceição-Silva et al., 2018). It is worth mentioning that although patient BAS 112 presented a low or insignificant load of 2.02×10^{-1} parasites/10^6 human PBMC cells, this does not mean that the parasites underlying the lesion at the end of the treatment do not spread through the bloodstream (Souza et al., 2017).

In contrast, at the end of the treatment, patient BAS 128 presented a load of 5.55×10^0 parasites/10^6 human PBMC cells and 6.32×10^3 parasites/10^6 smear cells. We suggest that the BAS 128 patient should be followed-up and monitored for the next two years to prevent or treat a later MCL. The significant parasite loads in PBMC and smears should alert health care personnel, considering that according to medical criteria, the patient finished the treatment as "Resolved."

Several studies have suggested that the presence of the *Leishmania RNA virus* (*LRV-1*), an endogenous virus reported among other species of *L. braziliensis*, induces the expression of type 1 interferon, negative regulation of *IFN-γ* receptors by macrophages, and recognition by the *TLR3* receptor. This means that it has an effect on inflammation and immune response processes and has a higher ability to survive in a hostile environment, such as the bloodstream (Ives et al., 2011; Macedo et al., 2016; Rossi & Fasel, 2017). A significant relationship has also been found between therapeutic failure or non-resolution according to medical criteria compared to cases of patients who are positive for *LRV-1* (Bourreau et al., 2016). This opens the possibility of future evaluation of the presence of *LRI-1* in samples of military personnel under "not resolved" outcomes or therapeutic failure for CL, as well as in patients with MCL. Moreover, according to the Army Health Directorate, treatment failures occur in 13% of CL patients.

It should also be considered that the third parasitic load in this study was evaluated at the end of the dose of the pharmacological treatment and not the treatment itself, which, according to the Ministry of Health and Social Protection's guidelines for comprehensive clinical care of leishmaniasis in Colombia is 45 days after the end of treatment in which time it is estimated that the patient will recover completely (Durán et al., 2018). Furthermore, the drug is excreted through the urine in the form

of pentavalent antimoniate but continues to circulate in the patient's body and after ten days. According to pharmacokinetic studies, concentrations of 0.1-0.3 mg/ml are still reached in the blood (Jaramillo-González, 2017), meaning that the parasitic concentrations in smears and blood may continue to decrease somewhat because of the residual effect of the drug remaining in the bloodstream.

It should be noted that, in the Americas, MCL is strongly correlated with *L. braziliensis* (Schwartz et al., 2006; Cincurá et al., 2017). Therefore, it is also suggested to perform clinical and preventive monitoring of the 15 patients that were molecularly identified with this infecting species who exhibited high parasitic loads, or to be considered at the end of the treatment, both in smear/biopsy and in PBMC (Table 3). Nonetheless, the rest of the patients reported here should not be discarded entirely. Previous studies in Colombia have reported cases of MCL caused by *L. panamensis* (Osorio et al., 1998). Other studies in Brazil have observed it in patients infected with *L. amazonensis*, *L. guyanensis*, and *L. peruviana* (Cincurá et al., 2017). Currently, the Tropical Diseases Research Group of the Army (GINETEJ) is doing a viability analysis of the parasite from the amplification of the *7SLRNA* marker by RT-qPCR (Romero et al., 2010) to corroborate whether the parasites circulating in the blood of these patients are alive, which could be the cause of a reactivation of CL or MCL in the future. In the meantime, a report was presented to the medical dermatological staff regarding the 23 patients with high parasitic loads to fulfill the purpose of the research project and the commitment with the participants.

The 23 samples analyzed in this study had parasitic loads of between 10^5 and 10^7 before of start the treatment. These values are within the normal reporting range according to previous studies for the *kDNA* mini-circle in *Leishmania* spp. (Jara et al., 2013; Méndez, 2014). Furthermore, the quantification of the parasitic load by the *kDNA* mini-circle as an estimate of the number of parasites equivalent to DNA supports the diagnosis and the favorable monitoring of the treatment (Moreira et al., 2018).

The significant negative correlation of parasitic loads in smears and PBMC at the end of treatment may be explained by the fact that parasites decrease in the area of skin lesion once they spread in the bloodstream; while they are exacerbated in the lesion, they have not yet migrated into the bloodstream. Regarding the significant negative correlation between the area of the external lesion and the parasitic load in smears, it could be suggested that lesions with large areas have less parasitic load due to the concomitant microbiota in the skin lesion, which can negatively affect the abundance of the infecting species. This sign on the skin is more related to the patient's immune response than to the number of infecting parasites (Antonelli et al., 2005; Kip et al., 2015).

Several authors have highlighted that the infectivity of *Leishmania* spp. can be multifactorial. In this study, it was reported that there are differences in the type of infecting species, explained by the internal and external area of skin lesions; this is

consistent with intra- and interspecific variations in relation to clinical manifestations (Conceição-Silva et al., 2018). Regarding the pharmacological treatment, differences between meglumine antimoniate and pentamidine isethionate were explained, given the set of seven variables such as age, disease evolution, parasitic load in smear/biopsy, parasitic load in PBMC, the internal and external area of lesions, the area outside the lesion, and the number of lesions. This corroborates, for example, that the parasitic loads were highly differentiated, given that the patients subjected to pentamidine isethionate came from previous exposure to meglumine antimoniate; therefore, their parasite totals in smears/biopsy and PBMC was significantly lower.

Differences in treatment monitoring (before, during, and after) may be explained by the effectiveness of the drugs to treat leishmaniasis. However, according to the PCAs, there was no difference between monitoring during and after; this may be because the 23 patients analyzed were patients that, according to the quantification of the parasitic load, had not resolved the disease. Finally, regarding the resolution according to medical criteria, there are differences between the patients who were resolved versus those were not; this may be explained by the differences between the areas of their lesions, the parasitic loads, and the number of lesions.

Molecular identification and spatial-temporal analysis of *Leishmania* spp. in Colombia

As reported by previous studies, the infecting species with the highest relative abundance in military populations is *L. braziliensis* (84.21%), while in civilian populations, it is *L. panamensis* (Patiño et al., 2017). In this study by Patiño et al. (2017), in the military population, an increase of 23.11% in the relative abundance of *L. braziliensis* and a decrease in richness was observed, as *L. guyanensis*, *L. Mexicana*, and *L. lainsoni* were not reported in this study. These three species, unreported in our study, represented a relative abundance of 5.4% in Patiño et al. (2017). It is worth noting that in our study, the sample size was significantly smaller than in the mentioned study. Similarly, for the department of Meta, only six patients out of the total 19 were reported to have identified the *Leishmania* species. In Patiño et al. (2017), there were 131 patients reported with probable infection in Meta out of a total of 272 patients sampled for Colombia.

Conclusions

In conclusion, we estimated the parasitic load of *Leishmania* spp. in 23 Colombian military men subjected to two different treatment schemes. Through significant differences found during the monitoring of the treatment, it was determined that these schemes are very effective in treating cutaneous leishmaniasis in the National Army.

The success of the pharmacological treatment was defined in terms of significant differences between those who were resolved of the disease and those who were not. The previous was explained in a multifactorial manner by age, the disease evolution, parasitic load in PBMC, the internal area of the lesion, the external area of the lesion, parasitic load in smear/biopsy, and the number of lesions. It was determined that the most abundant species and, therefore, the most related to high parasitic loads and worthy of consideration is *L. braziliensis*. The importance of molecular identification of the species for the patient's prognosis and choice of treatment is noted, as well as the importance of establishing a medical follow-up and control for these 23 patients to prevent a reactivation of the CL or to treat an MCL in time, which is part of the authors' commitment to the patients participating in this study.

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