UNIVERSIDADE NOVA DE LISBOA

INSTITUTO DE HIGIENE E MEDICINA TROPICAL

Leishmaniasis in Portugal:

Prevalence of asymptomatic infection, knowledge of blood donors and healthcare students and professionals, and characterization of clinical cases from 2010 to 2020

Rafael Amorim Rocha

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE DOUTOR EM MEDICINA TROPICAL

(OUTUBRO, 2024)

UNIVERSIDADE NOVA DE LISBOA

INSTITUTO DE HIGIENE E MEDICINA TROPICAL

Leishmaniasis in Portugal:

Prevalence of asymptomatic infection, knowledge of blood donors and healthcare students and professionals, and characterization of clinical cases from 2010 to 2020

Autor: Rafael Amorim Rocha

Orientadora: Carla Alexandra Soares Maia

Coorientadora: Maria Cláudia Gomes dos Santos Rodrigues da Conceição

Coorientadora: Luzia Augusta Pires Gonçalves

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Medicina Tropical

Apoio financeiro da Fundação para a Ciência e a Tecnologia, através da bolsa de doutoramento UI/BD/151067/2021

Bibliographic elements

Published articles in peer-reviewed international scientific journals directly related to the research work presented in this thesis:

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. Prevalence of asymptomatic *Leishmania* infection and knowledge, perceptions, and practices in blood donors in mainland Portugal. Parasites & Vectors 16, 357 (2023). https://doi.org/10.1186/s13071-023-05980-1

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. Knowledge, perceptions and practices of health students and professionals regarding leishmaniasis in Portugal: a cross-sectional study. Parasites & Vectors 16, 381 (2023). https://doi.org/10.1186/s13071-023-05982-z

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. Knowledge and practices regarding leishmaniasis in Portugal - Comparison between blood donors and health students/professionals. One Health. 2024 Mar 2;18:100697. doi: 10.1016/j.onehlt.2024.100697. PMID: 38463613; PMCID: PMC10924195.

Rocha, R., Conceição, C., Gonçalves, L., Carvalho, AC., Maia, A., Martins, A., Carujo, A., Maio, A., Forra, C., Melita, C., Couto, D., Fernandes, D., Pereira, D., Leal, E., Sarmento, H., Sousa, I., Gonçalves, JP., Marinho, J., Vasconcelos, J., Cunha, J., Rodrigues, J., Silva, JM., Caley, L., Malheiro, L., Santos, L., Garcia, M., Cunha, M., Lima, M., Andrade, MM., Marques, M., Alpalhão, M., Silva, M., Ferraz, R., Soares, R., Fernandes, S., Llobet, S., Cruz, S., Guimarães, T., Branco, T., Robalo-Nunes, T., Almeida, V., Maia, C. Epidemiological and clinical aspects of cutaneous and mucosal leishmaniases in Portugal: retrospective analysis of cases diagnosed in public hospitals and reported in the literature between 2010 and 2020. Microorganisms. 2024 Apr 18;12(4):819. doi: 10.3390/microorganisms12040819. PMID: 38674763; PMCID: PMC11052487.

Rocha, R., Conceição, C., Gonçalves, L., LeishPT group, Maia, C. Epidemiological and clinical trends of visceral leishmaniasis in Portugal: retrospective analysis of cases diagnosed in public hospitals between 2010 and 2020. Infectious Diseases of Poverty 13, 41 (2024). https://doi.org/10.1186/s40249-024-01204-5

Published articles in peer-reviewed international scientific journals partially related to the research work presented in this thesis:

Berriatua, E., Maia, C., Conceição, C., Özbel, Y., Töz, S., Baneth, G., Pérez-Cutillas, P., Ortuño, M., Muñoz, C., Jumakanova, Z., Pereira, A., Rocha, R., Monge-Maillo, B., Gasimov, E., Van der Stede, Y., Torres, G., Gossner, CM. Leishmaniases in the European Union and Neighboring Countries. Emerging Infectious Diseases. 2021 Jun;27(6):1723–7. doi: 10.3201/eid2706.210239. PMID: 34013857; PMCID: PMC8153892.

Rocha, R., Pereira, A., Maia, C. Non-endemic leishmaniases reported globally in humans between 2000 and 2021- A comprehensive review. Pathogens. 2022 Aug 16;11(8):921. doi: 10.3390/pathogens11080921. PMID: 36015042; PMCID: PMC9415673.

Rocha, R., Pereira, A., Maia, C. A global perspective on non-autochthonous canine and feline *Leishmania* infection and leishmaniosis in the 21st century. Acta Trop. 2023 Jan;237:106710. doi: 10.1016/j.actatropica.2022.106710. Epub 2022 Oct 3. PMID: 36198329.

Maia, C., Conceição, C., Pereira, A., Rocha, R., Ortuño, M., Muñoz, C., Jumakanova, Z., Pérez-Cutillas, P., Özbel, Y., Töz, S., Baneth, G., Monge-Maillo, B., Gasimov, E., Van der Stede, Y., Torres, G., Gossner, CM., Berriatua, E. The estimated distribution of autochthonous leishmaniasis by *Leishmania infantum* in Europe in 2005-2020. PLoS Negl Trop Dis. 2023 Jul 19;17(7):e0011497. doi: 10.1371/journal.pntd.0011497. PMID: 37467280; PMCID: PMC10389729.

Communications in scientific meetings directly related with the research work presented in this thesis:

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. (2021) Prevalência da infeção assintomática por *Leishmania* em dadores de sangue em Portugal Continental. XII Jornadas Científicas do Instituto de Higiene e Medicina Tropical. December 10, Lisbon, Portugal. Poster presentation.

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. (2022) Prevalência da infeção assintomática por *Leishmania* em dadores de sangue em Portugal Continental. XIII Jornadas Científicas do Instituto de Higiene e Medicina Tropical. December 9, Lisbon, Portugal. Oral presentation.

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. (2023). Prevalence of asymptomatic *Leishmania* infection and Knowledge, Perceptions and Practices in blood donors in mainland Portugal. 17th Symposium of the CVBD World Forum. September 24-25, Lisbon, Portugal. Oral presentation.

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. (2023). Knowledge, perceptions and practices of health students and professionals regarding leishmaniasis in Portugal – a cross-sectional study. 17th Symposium of the CVBD World Forum. September 24-25, Lisbon, Portugal. Oral presentation.

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. (2023). Conversas saudáveis entre técnicos de saúde ambiental: leishmaniose. Associação Portuguesa de Saúde Ambiental. November 28. Online. Oral presentation.

Rocha, R., Conceição, C., Gonçalves, L., LeishPT group, Maia, C. (2024). Leishmaniose visceral em idade pediátrica. Reunião da Unidade de Infeciologia do Hospital Dona

Estefânia, Unidade Local de Saúde de São José. March 27. Lisboa, Portugal. Oral presentation.

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. (2024). Knowledge and practices regarding leishmaniosis in Portugal – comparison between blood donors and health students/professionals. *2nd Animal Leishmaniosis International Veterinary Event (ALIVE - 2024)*. 18-20 April, Nice, France. Abstract book pages 61-62. Oral presentation.

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. (2024). Prévalence de l'infection asymptomatique à *Leishmania* et connaissances, perceptions et pratiques chez les donneurs de sang au Portugal continental. Congrès Société Francophone de Médecine Tropicale et Santé Tropical · Santé en Méditerranée. May 22-24, Saint-Raphaël, France. Poster presentation.

Rocha, R., Conceição, C., Gonçalves, L., LeishPT, Maia, C. (2024). Tendances épidémiologiques et cliniques des leishmanioses viscérales et cutanées au Portugal: analyse rétrospective des cas diagnostiqués dans les hôpitaux publics entre 2010 et 2020. Congrès Société Francophone de Médecine Tropicale et Santé Tropical · Santé en Méditerranée. May 22-24, Saint-Raphaël, France. Poster presentation.

Rocha, R., Conceição, C., Gonçalves, L., LeishPT group, Maia, C. (2024). Aspectos epidemiológicos y clínicos de las leishmaniasis en Portugal entre 2010 y 2020: Análisis Retrospectivo de Casos Diagnosticados en Hospitales Públicos. Reunião dos Serviços de Doenças Infeciosas e Microbiologia Clínica do Hospital Universitário Vírgen del Rocío. May 27. Seville, Spain. Oral presentation.

Rocha, R., Conceição, C., Gonçalves, L., LeishPT group, Maia, C. (2024). Leishmaniose visceral em idade pediátrica. Reunião do Serviço de Pediatria do Hospital Fernando Fonseca, Unidade Local de Saúde Amadora/Sintra. June 5. Amadora, Portugal. Oral presentation.

To all the individuals affected by leishmaniasis

To the professionals committed to ending disease and neglect

Acknowledgements

The research documented in this doctoral thesis owes its completion to the collaborative efforts of numerous individuals.

I wish to extend my sincere gratitude to my supervisor, Professor Carla Maia, for her invaluable friendship, unwavering belief in me from the outset, and for serving as one of the foremost pillars of my scientific journey. Her enthusiastic encouragement, support, and guidance have been instrumental and have been decisive for the conclusion of this work and for laying out the foundations of future collaborations.

I am also indebted to my co-supervisor, Professor Cláudia Conceição, for her friendship and the sharing of her unique expertise, especially regarding ethical conduct in research. I am also grateful for her invaluable and transparent professional advice. I am also thankful to my co-supervisor, Professor Luzia Gonçalves for her important input and suggestions that significantly contributed to the enhancement of this work.

I would like to acknowledge Professor Jorge Seixas, director of the Tropical Medicine PhD program, for welcoming me to the Instituto de Higiene e Medicina Tropical during my first contact with the Institute and for introducing me to Tropical Medicine in Portugal.

Special appreciation goes to Professor António Sarmento and Professor Jean-Pierre Gonçalves, members of the tutorial commission, for their availability, guidance, enthusiastic assistance, and encouragement. Also, to Professor António Sarmento, Head of the Department of Infectious Diseases of the Centro Hospitalar Universitário de São João, I would like to express my gratitude for believing in the importance of clinical research and for providing me an opportunity to conciliate this work with my medical residency.

To Dr. Susana Ferreira, a warm thank you for recognizing my potential and motivating me to enroll in a doctoral program.

My sincere thanks also go to the researchers, and graduate students with whom I had the privilege to interact and learn from. Among these, I would like to highlight: Mr. José Cristóvão from the Leishmaniasis Laboratory Group for his friendship and invaluable assistance in the laboratory techniques; Patrícia Andrade, Regina Loesch and Marta Varandas for their contributions in the laboratory techniques and the insertion of data into the database.

I express my gratitude to the all the professionals of the Instituto Português do Sangue e da Transplantação and of the hospitals of the Alentejo and Algarve who have tirelessly contributed to obtaining the biological samples for this study. Acknowledgment is due to the clinicians of the public hospitals who contributed to identifying human cases of leishmaniasis and to extracting their clinical information.

Finally, I extend my deepest gratitude to my family. To my parents, Isabel e Jorge, for providing me with opportunities to pursue my dreams. To my sister, Rita, for her unwavering support. To José Sotero, for his constant encouragement, unwavering support, love, and sacrifices, which enabled me to achieve this professional milestone.

To all mentioned, I offer my heartfelt thanks.

Resumo

Leishmania infantum, um parasita zoonótico transmitido por vetores, é endémico na região do Mediterrâneo. A infeção assintomática é o desfecho mais comum da exposição ao parasita. Na região do Mediterrâneo, estudos sobre a prevalência da infeção assintomática frequentemente baseados em testes serológicos em dadores de sangue sugeriram seroprevalências regionais entre 1 e 8%. Quando sintomática, a infeção por *L. infantum* manifesta-se principalmente como leishmaniose visceral (LV), mas também como leishmaniose tegumentar (cutânea – LC - e leishmaniose mucosa - LM). Em Portugal, a notificação às autoridades de saúde pública é obrigatória apenas para os casos de LV e é provável uma subnotificação significativa. Neste contexto, este projeto de doutoramento teve como objetivo esclarecer a situação epidemiológica atual e o impacto da leishmaniose em Portugal, por meio de: (i) avaliação da prevalência de infeção assintomática em dadores de sangue em Portugal Continental; (ii) caracterização dos conhecimentos, perceções e práticas (CPP) de estudantes de saúde, profissionais e dadores de sangue em relação à leishmaniose; e (iii) análise dos aspetos sociodemográficos e clínicos dos casos de LV, LC e LM diagnosticados em hospitais do Serviço Nacional de Saúde em Portugal Continental, entre 2010 e 2020.

Para tal, foram realizados três estudos, baseados em: deteção de anticorpos contra *Leishmania* no sangue e aplicação de um questionário sociodemográfico e de CPP em dadores de sangue; aplicação de um questionário de CPP a estudantes e profissionais de saúde; revisão de dados clínicos de episódios de leishmaniose em processos hospitalares.

A seroprevalência verdadeira nacional estimada em dadores de sangue foi de 4,8% (intervalo de confiança a 95%: 4.1-5.5%). Sexo masculino, idade superior a 25 anos e residência na região Centro foram fatores significativamente associados a seropositividade mais elevada, na análise multivariada. No geral, 72,3% dos dadores de sangue já tinham ouvido falar da leishmaniose. Entre estudantes e profissionais de saúde, a leishmaniose animal foi considerada diagnosticada em Portugal por 87% dos participantes, e a humana por apenas 69%. As pontuações médias de conhecimento e perceção foram maiores entre os profissionais de saúde animal. Em hospitais públicos, foram identificados 221 casos de LV, 43 de LC e 7 de LM entre 2010 e 2020. Foi observado um aumento na incidência nacional estimada nos anos após 2016. A LV foi predominantemente diagnosticada em pessoas que vivem com VIH e em crianças, mas o desfecho clínico geralmente foi pior em pessoas com imunossupressão não associada a VIH. Apresentações atípicas foram observadas em 8,5% dos casos de LV. Apenas 49,7% dos novos casos de LV foram notificados. Na LC, indivíduos imunossuprimidos constituíram uma proporção significativa dos casos (48%) e, neste grupo, LC disseminada (22%) e LV simultânea (54%) foram comuns. A abordagem ao tratamento foi muito heterogénea. Os casos de LM foram todos autóctones, diagnosticados principalmente em indivíduos mais idosos, imunossuprimidos, e geralmente foram tratados com anfotericina B lipossomal.

Pela interceção de dados de infeção assintomática, casos sintomáticos e CPP obtidos durante este projeto de doutoramento, pode concluir-se que a infeção por *Leishmania* representa uma ameaça persistente em Portugal. Mesmo assim, o reconhecimento da doença como autóctone em humanos é insuficiente entre a comunidade de estudantes e profissionais de saúde. Os aumentos regionais recentes na incidência e as seroprevalências, inesperadamente altas, devem ser monitorizados de perto para permitir intervenções rápidas. Os programas para controlar a doença devem concentrar-se em fornecer ferramentas para um diagnóstico mais precoce, em reduzir a subnotificação e promover uma vigilância integrada de doença humana e animal, seguindo uma abordagem de Saúde Única.

Palavras-chave: *Leishmania*, leishmaniose, Portugal, assintomática, prevalência

Abstract

Leishmania infantum, a zoonotic vector-borne parasite, is endemic in the Mediterranean region. Asymptomatic infection is the most common outcome of exposure to *this* parasite. In the Mediterranean region, studies on the prevalence of asymptomatic infection often relied on serological testing in blood donors have suggested regional seroprevalences between 1 and 8%. When symptomatic, infection with *L. infantum* presents mostly as visceral (VL), but also as tegumentary leishmaniasis (cutaneous – CL - and mucosal leishmaniasis - ML). In Portugal, reporting to public health authorities is mandatory only for VL cases and significant underreporting is likely. In this context, this doctoral project aimed to clarify the current epidemiological situation and burden of leishmaniasis in Portugal, by: (i) assessing the prevalence of asymptomatic infection in blood donors in mainland Portugal; (ii) characterizing the knowledge, perceptions and practices (KPP) of health students, professionals and blood donors regarding leishmaniasis; and (iii) analyzing the sociodemographic and clinical aspects of the VL, CL and ML cases diagnosed in hospitals of the National Health Service in mainland Portugal, between 2010 and 2020.

For this purpose, three studies were conducted, based on: detection of antibodies against *Leishmania* in blood and the application of a sociodemographic and KPP questionnaire to blood donors; application of a KPP questionnaire to healthcare students and professionals; review of clinical data from cases of leishmaniasis in hospital records.

The estimated national true seroprevalence in blood donors was 4.8% (95% confidence interval: 4.1-5.5%). Male sex, age over 25 years old and living in the Centro region were factors significantly associated with higher seropositivity in multivariate analysis. Overall, 72.3% of blood donors had previously heard of leishmaniasis. Among health students and professionals, animal leishmaniasis was considered to be diagnosed in Portugal by 87% of participants, and human leishmaniasis by only 69%. Median knowledge and perception scores were higher among professionals in the animal health field. In public hospitals, 221 VL, 43 CL and 7 ML cases were identified between 2010 and 2020. An increase in estimated national incidence was seen in the years after 2016. VL was predominantly diagnosed in people living with HIV and in children, but the clinical outcome was generally poorer in non-HIV patients with associated immunosuppression. Atypical presentations were seen in 8.5% of VL cases. Only 49.7% of new VL cases were reported. In CL, immunosuppressed individuals constituted a significant proportion of patients (48%) and, in this group, disseminated CL (22%) and simultaneous VL (54%) were common. Approach to treatment was very heterogeneous. ML cases were all autochthonous, diagnosed primarily in older immunosuppressed individuals and were generally treated with liposomal amphotericin B.

By interception of data from asymptomatic infection, symptomatic cases and KPP obtained during this doctoral project, it can be concluded that *Leishmania* infection presents a continuing threat in Portugal. Even so, recognition of the disease as autochthonous in humans is insufficient among the health student and professional community. Recent regional increases in incidence and unexpectedly high seroprevalences should be closely monitored to allow prompt interventions. Programs to control the disease should focus on providing tools for earlier diagnosis and on reducing underreporting and promoting an integrated surveillance of human and animal disease, following a One Health approach.

Keywords: *Leishmania*, leishmaniasis, Portugal, asymptomatic, prevalence

Table of contents

List of figures

List of tables

Chapter 1

Chapter 2

Chapter 3

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
AML	Lisbon Metropolitan Area (Área Metropolitana de Lisboa)
AMP	Oporto Metropolitan Area (Área Metropolitana do Porto)
ANOVA	Analysis of variance
aOR	Adjusted odds ratio
APSAi	Associação Portuguesa de Saúde Ambiental
BSE	Beiras e Serra da Estrela
CanL	Canine leishmaniasis
CCDR	Comissão de Coordenação e Desenvolvimento Regional
CI	Confidence interval
CL	Cutaneous leishmaniasis
CR	Can't remember
CRP	C-reactive protein
CST	Pearson Chi-Square test
CSV	Comma-separated values
CU5	Children 5 years of age or younger
DAT	Direct agglutination test
df	Degrees of freedom
DGES	Directorate-General for Higher Education
DGS	Directorate-General for Health (Direção-Geral da Saúde)
DK	Don't know
DNA	Desoxyribonucleic acid
E.	*Endotrypanum*
EHS	Environmental Health students
EHT	Environmental Health technicians
ELISA	Enzyme-linked immunosorbent assay
ESCO	European Skills, Competences, Qualifications and Occupations
FET	Fisher's exact test

HIV	Human immunodeficiency virus
HLH	Hemophagocytic lymphohistiocytosis
ICD	International Classification of Diseases
ID	Infectious Diseases
IFAT	Immunofluorescent antibody test
IHD	Immunohemotherapy department
IHMT	Instituto de Higiene e Medicina Tropical
IL	Intralesional
IM	Intramuscular
IQI	Interquartile interval
IQR	Interquartile range
ISA	non-HIV infected immunosuppressed adults
IV	Intravenous
K	Knowledge
KAP	Knowledge, attitudes and practices
kDNA	Kinetoplast DNA
KPP	Knowledge, perceptions and practices
KWT	Kruskal-Wallis test
L.	*Leishmania*
LAmB	Liposomal amphotericin B
Lu.	*Lutzomyia*
LVT	Lisboa e Vale do Tejo
MA	Meglumine antimoniate
MCL	Mucocutaneous leishmaniasis
med	Medicine
ML	Mucosal leishmaniasis
MSc	Master of Science
MWT	Mann-Whitney U test
NA	Not applicable
NAAT	Nucleic acid amplification test
NHS	National Health Service

NISA	Non-immunosuppressed adults and children over 5 years old
NLM	National Library of Medicine
NUTS	Nomenclature of Territorial Units for Statistics
nyp	Number of years of professional
OM	Ordem dos Médicos
OMV	Ordem dos Médicos Veterinários
OR	Odds ratio
p	p-value
P.	*Phlebotomus*
PCR	Polymerase chain reaction
Per	Perceptions
PhD	Doctor of Phylosophy
PLWH	People living with HIV
Pra	Practices
profs	Professionals
qPCR	Quantitative PCR
REVIVE	Rede de Vigilância de Vetores
RFLP	Restriction Fragment Length Polymorphism
rK39	Recombinant K39
S.	*Sergentomyia*
s.l.	Sensu lato
s.s.	Sensu stricto
SD	Standard deviation
SINAVE	National Epidemiologic Surveillance System (Sistema Nacional de Vigilância Epidemiológica)
sp.	Species (singular)
spp.	Species (plural)
syn.	synonym
TNF	Tumor Necrosis Factor
TP	True prevalence
TV	Television

USA	United States of America
vet	Veterinary
VL	Visceral leishmaniasis
WHO	World Health Organization
y	Year
yo	Years old

CHAPTER 1

General introduction

1. The pathogen: *Leishmania*

Leishmaniases are a group of diseases caused by protozoan parasites of the genus *Leishmania*. These eukaryotic single-celled parasites present two primary morphological forms: the promastigote in the invertebrate host and the amastigote in the vertebrate host (1). The promastigote form is characterized by an elongated body (15 to 20 μm in length and 1.5 to 3.5 μm in width), an anterior projecting free flagellum of variable size, and a centrally located oval nucleus. Amastigotes present a round or ovoid shape (diameter of about 2–6 μm), a rudimentary flagellum, and a typically eccentric, large nucleus (1). Both forms present a modified mitochondrion, called the kinetoplast; this structure appears anterior to the nucleus, as a dense disk-shaped structure, that is directly associated with the basal body, from which the flagellum emerges through the flagellar pocket (1,2).

Some genomic features of trypanosomatids, compared to other eukaryotes, include presence of a kinetoplast, genes without introns, a nucleus with polycistronic transcripts and small chromosomes with high gene density (3). *Leishmania* parasites present two genomic pools, the nucleus and the kinetoplast: the nuclear genome is organized in 34 to 38 chromosomes, depending on the species (4); the kinetoplast genome comprises a network of catenated circular deoxyribonucleic acid (DNA) molecules, known as kinetoplast DNA (kDNA), present in several thousand copies. *Leishmania* reproduction is primarily asexual via clonal propagation (3), but there is evidence for the occurrence of genetic exchanges, within and between different *Leishmania* spp., during cyclical development in the phlebotomine sand fly vector (5).

The genus *Leishmania* Ross, 1903, according to current taxonomical classification (6), belongs to the Kingdom Protista Heackel, 1996; Phylum Euglenozoa Cavalier-Smith, 1993; Class Kinetoplastea Honigberg, 1963; Subclass Metakinetoplastina Vickerman, 2004; Order Trypanosomatida Kent, 1880; Family Trypanosomatidae Döflein, 1901; and Subfamily Leishmaniinae Makloves and Lukeš, 2012. The genus *Leishmania* is further divided into the Euleishmania and Paraleishmania sections (7). The Euleishmania section includes all species classified within the subgenera *Leishmania*, *Mundinia*, *Sauroleishmania* and *Viannia*, while the Paraleishmania section comprises several *Leishmania* spp., some of which, following recent phylogenetic analysis, have been suggested to be classified under the *Endotrypanum* and *Porcisia* genus (6,8). Over 50 species of *Leishmania* are currently described in the literature (Table 1) (6), although some represent cases of synonymy. Using multi locus enzyme electrophoresis, *Leishmania* strains of each species have been classically classified in zymodemes (strains that share the same isozyme profile). More recently, different molecular approaches have been used including multilocus sequence typing (9) and comparative DNA sequencing of nuclear and mitochondrial targets (4).

Table 1. Revised taxonomy of the genus *Leishmania* (adapted from (6,8,10–15)). Human pathogenic species are highlighted in bold.

Genus	***Leishmania***				
Section	Euleishmania				
Subgenus	*Leishmania*				*Mundinia*
Complex	*L. donovani* complex	*L. major* complex	*L. mexicana* complex	*L. tropica* complex	*L. enrietti* complex
Species	***L. donovani***	*L. arabica*	***L. amazonensis***	***L. aethiopica***	*L. enrietti*
	L. infantum	***L. major***	*L. aristidesi*	***L. tropica***	***L. chancei***
		L. gerbilli	***L. ellisi***		*L. macropodum*
		L. turanica	*L. forattinii*		***L. martiniquensis***
			L. mexicana		***L. orientalis***
			L. venezuelensis		*L. procaviensis*
			L. waltoni		

Genus	***Leishmania***				
Section	Euleishmania				Paraleishmania
Subgenus	*Sauroleishmania*	*Viannia*			
Complex		*L. braziliensis* complex	*L. guyanensis* complex		
Species	*L. adleri*	***L. braziliensis***	***L. guyanensis***	***L. lainsoni***	***L. colombiensis****
	L. agamae	***L. peruviana***	***L. panamensis***	***L. lindenbergi***	*L. equatorensis**
	L. chameleonis		***L. shawi***	***L. naiffi***	*L. herreri**
	L. hemidactyli			*L. utigensis*	*L. monterogeii**
	L. helioscopi				*L. schaudinni**
	L. ceramodactyli				*L. deanei***
	L. davidi				*L. hertigi***
	L. gulikae				
	L. gymnodactyli				
	L. henrici				
	L. hoogstraali				
	L. nicollei				
	L. phrynocephali				
	L. platycephala				
	L. senegalensis				
	L. sofieffi				
	L. tarentolae				
	L. zmeevi				
	L. zuckermani				
	L. sp. *I*				
	L. sp. *II*				

[1] syn. *L. archibaldi*; [2] syn. *L. chagasi*; [3] syn. *L. garnhami;* [4] syn. *L. pifanoi*; [5] syn. *L. killicki*
*suggested as genus *Endotrypanum*; **suggested as genus *Porcisia* by Espinosa (2018)

2. The hosts

2.1. Invertebrate hosts

Leishmania parasites are naturally transmitted through the bite of phlebotomine sand flies (Diptera; Psychodidae; Phlebotominae). These arthropods belong mostly to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (16). Only around 10% of the over 1000 species/subspecies of sand flies that have been validated/described are proven or suspected vectors of *Leishmania* (17). Most sand fly species do not support the development of the parasite and are considered refractory. Some sand fly species are considered specific or restrictive vectors, displaying strong specificity for certain *Leishmania* species, while others support development of multiple *Leishmania* species and are termed permissive vectors (17).

Although they prefer warm, humid, tropical climates and semi-desert vegetation habitats, sand flies are distributed from latitude 50° N to latitude 40° S, from below sea level to over 3,000 m in altitude (17).

Phlebotomine sand flies are small (body length 1.5-3.5 mm), hairy and have long, stilt-like legs. Their hairy wings extend at a 40° angle over the body at rest. Sand flies are weak fliers (short hopping flight, horizontally near ground level) and usually disperse only a few hundred meters from their breeding sites (18,19). Although some sand fly species are common all year-round in tropical areas, in temperate climate zones adult sand flies are only present during the warmer months (in Europe, the active season spans from April to November, depending on the latitude) (20).

Sand flies' life cycle is exclusively terrestrial and comprises complete metamorphosis (holometabolous development) through four developmental stages: egg, larva, pupa and adult (18,19). Preferred breeding sites are high humidity microhabitats, such as cracks and holes in the ground or buildings, animal burrows and among tree roots or holes (21). Eggs hatch after 4-20 days. Larvae are mainly scavengers (feeding on organic matter); their development is completed in 20-30 days and involves four instars. After a pupal stage lasting 6-13 days, the adult sand flies emerge (18,19). Both sexes feed on natural sources of sugars (such as plant sap and nectar). However, hematophagy in females is required to successfully produce eggs; they naturally feed on blood from a wide range of mammalian, reptilian, amphibian and avian host species and mostly feed at dusk and during the night. Many species are opportunistic (feeding on the animals most easily accessible) (17).

2.2. Vertebrate hosts

Parasites of the genus *Leishmania* can infect a wide range of vertebrate species, most of which are mammals. Vertebrates can be divided epidemiologically into primary reservoir hosts, secondary reservoir hosts, or accidental hosts (22).

A primary reservoir host serves as the main source for maintaining the parasite in nature over time, even in the absence of other host species, typically without displaying noticeable signs of infection. Conversely, a secondary reservoir host can also act as a source of infection for vectors, enhancing the parasite's ability to spread, but cannot sustain parasite transmission independently of the primary reservoir host. Lastly, an accidental host, while susceptible to infection, typically does not transmit the parasite to vectors and thus does not play a crucial role in the ecological system where the parasite persists indefinitely in nature (22,23). Vector-borne transmission of leishmaniasis to humans can either be anthroponotic (from human to human) or zoonotic (from a non-human vertebrate reservoir to humans) (24).

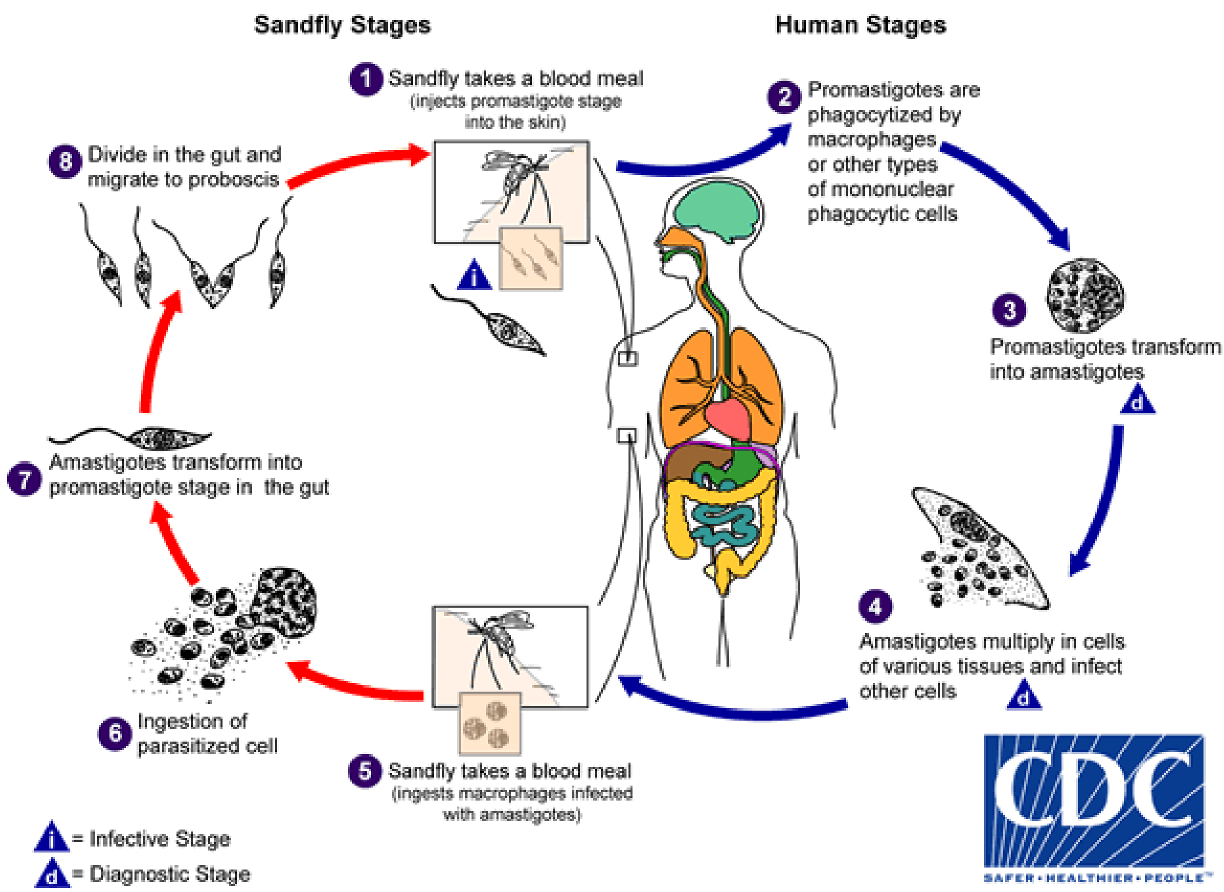
3. Life cycle in nature

Leishmania parasites undergo a digenetic life cycle, transitioning between a vertebrate host and an insect vector (Figure 1). Upon feeding on an infected host, a female phlebotomine sand fly acquires *Leishmania* amastigotes located in phagocytes. The shift in environmental conditions, from the mammalian host to the sand fly midgut (characterized by a decrease in temperature and an increase in pH), prompts the development of the parasites within the vector (25). Initially, the amastigotes differentiate into procyclic promastigotes, exhibiting limited motility, and undergo rapid multiplication through binary fission (16,26). Within 2-3 days, they further differentiate into elongated, highly motile nectomonad promastigotes (27). These promastigotes migrate towards the thoracic portion of the midgut, where they subsequently differentiate into leptomonad promastigotes, shorter forms capable of resuming replication (25,27). A subset of leptomonad promastigotes differentiate into metacyclic promastigotes, flagellated infective forms, characterized by their small size and heightened motility, well-suited for survival within the vertebrate host (27). Upon the sand fly's subsequent blood meal, metacyclic promastigotes, along with insect saliva and midgut content, are directly inoculated into the bite wound (16). In the skin, metacyclic promastigotes are engulfed by mononuclear phagocytes and undergo differentiation within parasitophorous vacuoles to form amastigotes (28). These amastigotes actively multiply within macrophages (main host cells). Following proliferation, the macrophage membrane ruptures, releasing the parasites. Free amastigotes in circulation can then infect other phagocytes in the skin or disseminate through the circulatory system to various internal organs, including the bone marrow, liver, and spleen (28). The cycle is completed when amastigotes are ingested by a competent vector.

While sand fly bites are the primary mode of visceral leishmaniasis (VL) transmission, other routes of transmission have been documented, including: blood transfusion (29), occupational percutaneous exposure (30), intravenous drug use (31), solid organ transplantation (32) and vertical transmission (33).

Although sexual transmission has been clearly demonstrated in canids (34), only one suspected case of human sexual transmission is documented in the literature (35). On the other hand, while sporadic cases of transmission by wounds or bites have been reported in dogs (which may be particularly relevant in non-endemic settings), no cases in the literature solidly support this route of transmission in humans (36).

Figure 1. Schematic life cycle of *Leishmania* species (image source: Leishmaniasis: life cycle; Centers for Disease Control and Prevention; 2024. Licensed under CC BY 4.0 (37)).



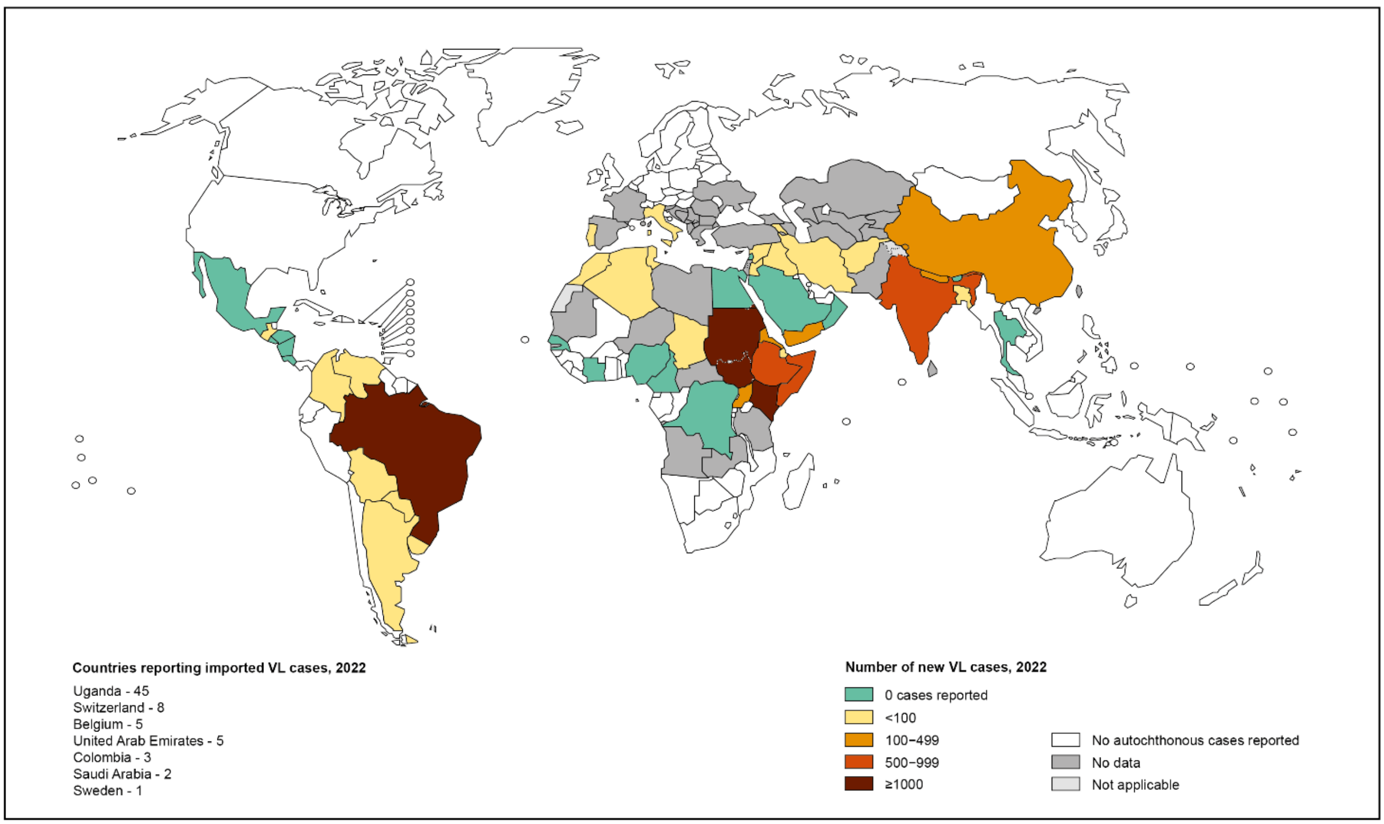
4. Epidemiology of human leishmaniasis

4.1. Global perspective

According to Alvar et. al, in 2012, endemic areas for human leishmaniasis were distributed in a total of 98 countries and three territories, encompassing all continents, except Antarctica (38). Formally included in the list of Neglected Tropical Diseases by the World Health Organization (WHO) (39), leishmaniasis is still one of the world's most neglected diseases, primarily affecting impoverished individuals in low-income countries and in conflict-stricken regions. It is intricately linked with factors such as malnutrition, inadequate housing, population displacement, compromised immune systems, and limited financial resources (40). Globally, the population potentially at risk of developing leishmaniasis exceeds 1 billion people (41).

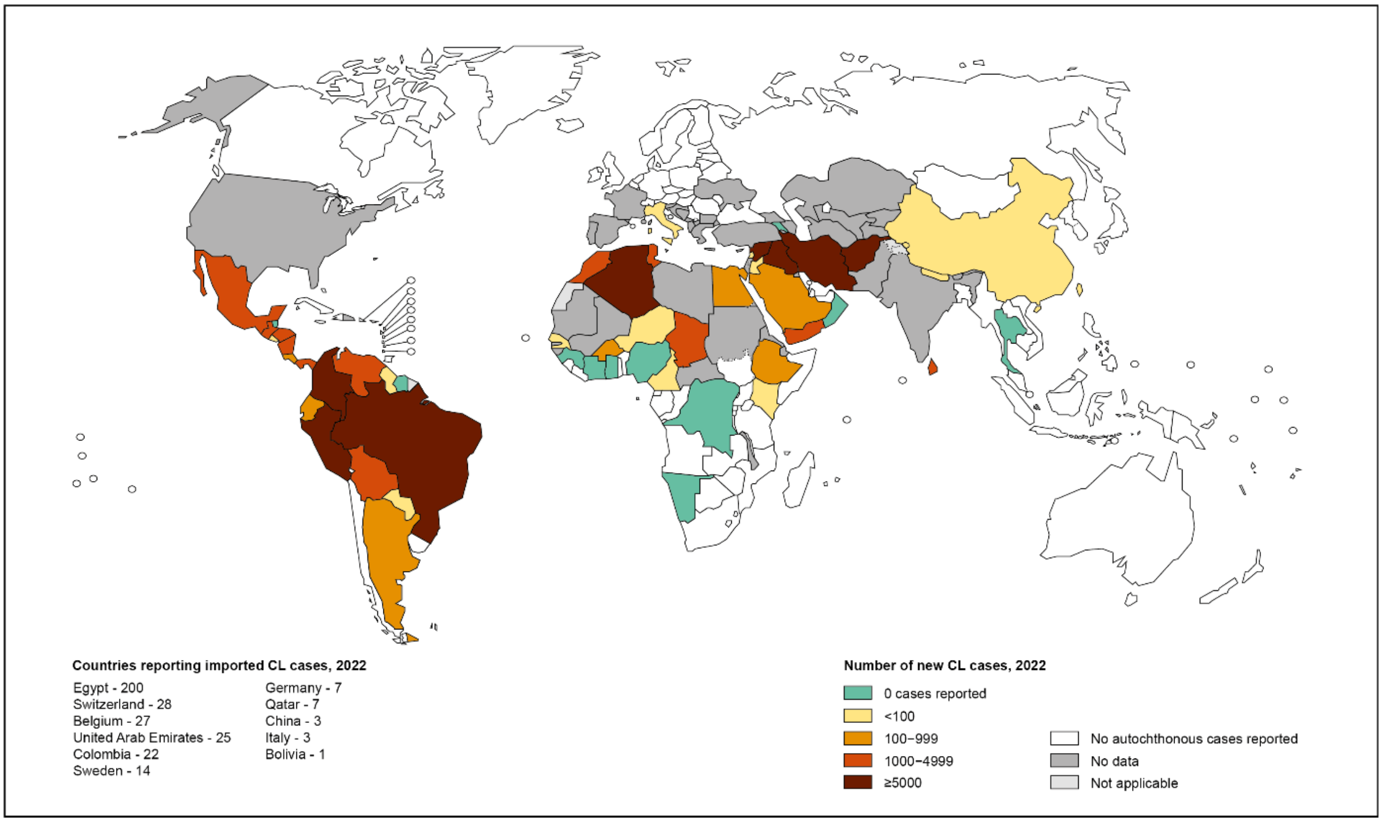
In 2017, the WHO estimated the annual incidence of VL to range between 50,000-90,000 new cases globally (41). In 2022, seven countries (i.e., Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan) accounted for approximately 85 % of the worldwide cases of VL (Figure 2) (42). Nevertheless, VL remains endemic in approximately 80 countries, including Portugal (43).

Figure 2. Status of endemicity of human visceral leishmaniasis (VL) worldwide in 2022 (image source: Status of endemicity of visceral leishmaniasis: 2022; World Health Organization; 2023. Licensed under CC BY-NC-SA 3.0 IGO (43)).



Annually, 600,000-1,000,000 cases of cutaneous leishmaniasis (CL) were estimated to occur, in 2022; approximately 85 % of these cases occurred, in 2022, in Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq, Pakistan, Peru, Syria (Figure 3) (44). Forced migration resulting from armed conflicts seems to be a significant factor influencing the incidence of CL, with imported cases frequently observed in non-endemic countries (45).

Figure 3. Status of endemicity of human cutaneous leishmaniasis (CL) worldwide in 2022 (image source: Status of endemicity of cutaneous leishmaniasis: 2022; World Health Organization; 2023. Licensed under CC BY-NC-SA 3.0 IGO (46)).



4.2. Regional perspective – Europe

Both clinical forms of human leishmaniasis, VL and CL, are endemic and geographically widespread in the WHO European Region. In this region, *L. infantum* is the etiologic species of most autochthonous human leishmaniasis cases, although sporadic cases of localized transmission of anthroponotic cutaneous leishmaniasis caused by *L. tropica* in Greece (Crete) and VL and CL caused by *L. donovani* in Cyprus have been described (47). Additionally, *L. major* has been detected in sand flies in Portugal (48) and *L. major/L.*

infantum hybrids have been documented in humans (in immunosuppressed patients) (49) and in cats (50).

In European regions where *L. infantum* is endemic, dogs are considered to be the main reservoir host for human infection. Increasing evidence suggests that cats may also play a relevant epidemiological role, as reservoir hosts, especially in areas where they are present in high numbers, with limited availability of effective ectoparasiticides, and where most dogs are well-protected against sand fly bites (51). Wild animals such as canids (wolves, foxes) and leporids (hares, rabbits) have also shown to be infected by *Leishmania* parasites and may connect the wild, rural and urban environments (52). In the largest community outbreak of leishmaniasis documented in Europe (in Madrid, Spain, from 2009 to 2012) hares, and in less extent rabbits, were implied as the potential sylvatic reservoir (53,54).

The estimated distribution of autochthonous leishmaniasis by *L. infantum* in Europe in 2005–2020 has been recently reviewed (55). In this period, 8,367 human cases were reported to the WHO, of which 69% were VL (94% considered autochthonous) and 31% were CL (53% imported). The highest cumulative incidence in this period per 100,000 population of VL was in Albania (2.15 cases), followed by Montenegro, Malta, Greece, Spain and North Macedonia (0.53–0.42), Italy (0.16), Portugal (0.09). However, for several countries, incidence estimates according to hospital discharges were significantly higher than calculated using WHO reported cases. Overall, there was no evidence of widespread increased incidence of autochthonous human leishmaniasis by *L. infantum* in European countries. According to the available data, VL incidence followed a decreasing trend in Albania, Italy and Portugal. Animal leishmaniasis and human CL likely remain highly underreported, since reporting is not mandatory in many countries and, in some countries, mandatory reporting of canine leishmaniasis is restricted to specific regions of the country or contexts (e.g., municipal veterinarians).

4.3. National perspective - Portugal

In Portugal, although human leishmaniasis is considered hypoendemic, the estimated prevalence of canine *Leishmania* infection seems to be high as suggested by a recent nationwide serological survey, conducted in 2021, where national seroprevalence was estimated at 12.5%, with some districts reaching approximately 30% (Portalegre and Castelo Branco) (56). This represented an increase compared to data from a similar survey performed in 2009, in which the global seroprevalence was 6.3% (57). The role of cats in the epidemiology of leishmaniasis in Portugal has also been the subject of study. Antibodies against *Leishmania* have been detected in 0.6-3.7% of cats in the Trás-os-Montes e Alto Douro (58), Lisboa (59,60) and Algarve (61) regions. Detection of *Leishmania* DNA in peripheral blood by molecular testing ranged from 0.3 to 30.4%, in several studies covering the Norte, Centro and South regions of the country (62–65).

Human leishmaniasis in Portugal was classically considered a pediatric disease; however, in the 1980s, the emergence of a new immunosuppressive factor (human immunodeficiency virus – HIV-infection/acquired immunodeficiency syndrome - AIDS) led to an overall increase in cases, with a relative increase in adults. With the introduction of antiretroviral therapy (ART) in Portugal in the second half of the 1990s, the incidence of leishmaniasis and the number of relapses decreased significantly, although at a slower pace than in other Mediterranean countries (66).

In the 20th century, the Alto Douro region was the most active focus of human leishmaniasis, with an incidence of 8.3 cases/100,000 inhabitants/year (67). In the Algarve Region, the Pediatrics department of the district hospital reported 43 cases of VL diagnosed between 1980 and 1988 (68) and the incidence of human leishmaniasis in that region and at that time was estimated at 1.2 cases/100,000 inhabitants/year (67). In the Área Metropolitana de Lisboa (AML) region, where the burden of co-infection with HIV was especially high and infections were more often associated with urban than rural environments, the estimated incidence was 0.2 cases/100,000 inhabitants/year (67).

More recently, the sociodemographic and clinical aspects of cases of VL diagnosed in public hospitals in Mainland Portugal between 1999 and 2009 were reviewed (69). A total of 375 patients were hospitalized in this period (51.1% were people living with HIV – PLWH - and 6.5% presented immunosuppression from another cause). Around 70% of patients were male; bone marrow aspirate or biopsy was performed for diagnosis in almost all cases, with detection of *Leishmania* by microscopic observation (nucleic acid amplification tests –

NAAT - were used in only 25.1% of cases). Liposomal amphotericin B was the preferred choice for treatment (53.5%), followed by meglumine antimoniate (40.8%). Only 38.6% of VL cases were notified to public health authorities, although declaration is mandatory since the 1950s. After 2010, available data from reported cases (up to 2018) showed a decreasing trend in national incidence of VL; the Alto Tâmega, Alto Alentejo, Baixo Alentejo and Beira Baixa Nomenclature of Territorial Units for Statistics (NUTS) 3 regions presented the highest incidences in this period, although an apparent reemergence of disease in the Algarve was noted after 2013 (70,71). Cases of VL published in the literature after 2010 highlight the threat of *Leishmania* in Portugal in the setting of solid organ transplant (72), hematopoietic stem cell transplant (73) and drug-induced immunosuppression for autoimmune and inflammatory disorders (74).

The epidemiology of CL is not as well known in Portugal, probably due to the benign course of the lesions (leading to underdiagnosis) and the fact that it is not a mandatory notifiable disease. Although CL seems to be not as frequent in Portugal as in Spain, where the incidence of this form is some areas matches the incidence of VL (75), it is estimated that about ten new cases are diagnosed annually (76). In the reference laboratory for leishmaniasis of the IHMT, 27 cases of CL were diagnosed between 1999 and 2014 (77). Recent, isolated autochthonous cases of CL in Portugal have also been reported in the literature (78), as well as *L. infantum*-associated mucosal leishmaniasis (ML) (79).

Early studies on sand flies in Portugal have revealed the species *P. perniciosus* and *P. ariasi* as proven vectors of *L. infantum* in the country (80). In the 21st century, additional sand fly surveys have been conducted in the Médio Tejo and Algarve regions of Portugal, with detection of *Leishmania* DNA in specimens of *P. perniciosus* (in all studies) and *P. ariasi* (in one study) and an infection rate of 0.13-0.48% (81–85). Additionally, *Leishmania* DNA has been detected in *Sergentomyia minuta* specimens in the Alentejo (*L. infantum*) (86) and Algarve (*L. major* and *Leishmania* sp.) (83) regions; however, members of this genus, proven vectors of *Sauroleishmania* sp., have not been completely established as competent vectors of human pathogenic *Leishmania* species (87).

5. Clinical presentation and management of human leishmaniasis

At least 22 species of *Leishmania* have been identified as pathogens in humans, and numerous species of phlebotomine sand flies are implicated in their transmission (45) (Tables 2 and 3). Human leishmaniasis is primarily zoonotic, except for *L. donovani* and *L. tropica* in most settings (22).

Table 2. Leishmaniasis in the Old World: etiology, geographical distribution, vectors, reservoir hosts, transmission type and clinical forms (adapted from (11,13,19,22,40,88–92)).

Leishmania species	Geographical distribution	Proven or suspected vector	Proven or suspected reservoir	Transmission	Clinical form*
L. donovani	Bangladesh; Bhutan; Chad; China; Cyprus; Djibouti; Ethiopia; India; Iraq; Israel; Kenya; Nepal; Saudi Arabia; Somalia; Sri Lanka; Sudan; Uganda; Ukraine; Yemen	*P. alexandri*; *P. argentipes*; *P. celiae*; *P. chinensis s.l.; P. longiductus; P. martini; P. orientalis; P. vansomerenae*	Humans; Dogs; Mongooses; Rodents	Anthroponotic	VL; (CL; ML)
L. infantum	Afghanistan; Albania; Algeria; Armenia; Azerbaijan; Bosnia and Herzegovina; Bulgaria; Central African Republic; China; Croatia; Cyprus; Egypt; France; Gambia; Georgia; Greece; Iran; Iraq; Israel; Italy; Jordan; Kazakhstan; Kosovo; Kyrgyzstan; Lebanon; Libya; Macedonia; Malta; Mauritania; Monaco; Montenegro; Morocco; Oman; Pakistan; Palestine; Portugal; Romania; Saudi Arabia; Senegal; Slovenia; Spain; Sudan; Syria; Tunisia; Turkey; Turkmenistan; Ukraine; Uzbekistan; Yemen	*P. alexandri*; *P. ariasi*; *P. balcanicus*; *P. chinensis*; *P. duboscqi*; *P. galileus*; *P. halepensis; P. kandelakii; P. kandelakii; P. langeroni; P. longicuspis; P. longiductus; P. major s.l.; P. neglectus; P. perfiliewi s.l.; P. perniciosus; P. sichuanensis; P. smirnovi; P. syriacus; P. tobbi; P. transcaucasicus; P. turanicus; P. wui*	Canids; Cats; Lagomorphs; Rodents	Zoonotic	VL; CL; (ML)
L. major	Afghanistan; Algeria; Azerbaijan; Burkina Faso; Cameroon; Chad; Egypt; Ethiopia; Georgia; Gambia; Ghana; Guinea; Guinea-Bissau; India; Iran; Iraq; Israel; Jordan; Kazakhstan; Kenya; Kuwait; Libya; Mali; Mauritania; Mongolia; Morocco; Niger; Nigeria; Oman; Pakistan; Palestine; Saudi Arabia; Senegal; Sudan; Syria; Tunisia; Turkmenistan; Uzbekistan; Yemen	*P. ansarii*; *P. bergeroti*; *P. caucasicus*; *P. duboscqi; P. mongolensis; P. papatasi; P. perniciosus; P. salehi; S. darlingi; S. garnhami*	Rodents	Zoonotic	CL; (ML; VL)
L. aethiopica	Ethiopia; Kenya	*P. aculeatus*; *P. longipes*; *P. pedifer*; *P. sergenti*	Hyraxes; Rodents	Zoonotic	CL; (ML)
L. tropica	Afghanistan; Algeria; Azerbaijan; Egypt; Ethiopia; Greece; India; Iran; Iraq; Israel; Jordan; Kenya; Libya; Morocco; Namibia; Pakistan; Palestine; Saudi Arabia; Syria; Tunisia; Turkey; Turkmenistan; Uzbekistan Yemen	*P. aculeatus*; *P. arabicus*; *P. chabaudi*; *P. grovei; P. guggisbergi; P. perniciosus; P. rossi; P. saevus; P. sergenti; P. similis*	Humans; Hyraxes; Rodents; Dogs	Anthroponotic	CL; (ML; VL)
L. chancei	Ghana	Unknown	Unknown	Zoonotic**	CL
L. martiniquensis	Myanmar; Thailand	*S. barraudi*; *S. gemmea;* Biting midges	Rodents	Zoonotic	CL; VL; (ML)
L. orientalis	Thailand	*S. barraudi; S. Iyengari;* Biting midges	Unknown	Zoonotic**	CL; VL

*Clinical forms between brackets have rarely been reported; **Suspected.

Abbreviations: *L., Leishmania; P., Phlebotomus; S., Sergentomyia*; s.l., sensu lato; CL, cutaneous leishmaniasis; ML, mucosal leishmaniasis; VL, visceral leishmaniasis.

Table 3. Leishmaniasis in the New World: etiology, geographical distribution, vectors, reservoir hosts, transmission type and clinical forms (adapted from (19,22,40,88,90,93–99)).

Leishmania species	Geographical distribution	Proven or suspected vector	Proven or suspected reservoir	Transmission	Clinical form*
L. infantum	Argentina; Bolivia; Brazil; Colombia; Costa Rica; El Salvador; Guatemala; Honduras; Mexico; Nicaragua; Paraguay; United States of America; Venezuela	*Lu. almerioi*; *Lu. cruzi*; *Lu. evansi*; *Lu. forattinii*; *Lu. longipalpis s.l.; Lu. migonei; Lu. pseudolongipalpis; Lu. sallesi*	Canids; Cats; Marsupials	Zoonotic	VL; (CL)
L. amazonensis	Argentina; Bolivia; Brazil; Colombia; Costa Rica; Ecuador; French Guiana; Peru; Suriname; Venezuela	*Lu. flaviscutellata*; *Lu. longipalpis*; *Lu. nuneztovari anglesi*; *Lu. olmeca nociva*; *Lu. olmeca reducta*; *Lu. youngi*	Marsupials; Rodents	Zoonotic	CL; (MCL; VL)
L. ellisi	United States of America	Unknown	Unknown	Zoonotic**	CL
L. mexicana	Belize; Colombia; Costa Rica; Ecuador; Guatemala; Mexico; United States of America; Venezuela	*Lu. anthophora*; *Lu. ayacuchensis*; *Lu. columbiana*; *Lu. cruciata; Lu. diabolica; Lu. flaviscutellata; Lu. migonei; Lu. olmeca bicolor; Lu. olmeca olmeca; Lu. ovallesi; Lu. panamensis; Lu. shannoni; Lu. ylephiletor*	Dogs; Marsupials; Rodents	Zoonotic	CL
L. venezuelensis	Venezuela	*Lu. olmeca bicolor*	Cats	Zoonotic	CL
L. waltoni	Dominican Republic	*Lu. christophei*	Unknown	Zoonotic**	CL
L. martiniquensis	Brazil; Grenada; Martinique; United States of America	Unknown	Rodents; Equids	Zoonotic	CL; VL
L. braziliensis	Argentina; Belize; Bolivia; Brazil; Colombia; Costa Rica; Ecuador; French Guiana; Guatemala; Honduras; Mexico; Nicaragua; Panama; Paraguay; Peru; Venezuela	*Lu. ayrozai*; *Lu. carrerai*; *Lu. columbiana*; *Lu. complexa; Lu. cruciata; Lu. edwardsi; Lu. fischeri; Lu. gomezi; Lu. intermedia; Lu. llanosmartinsi; Lu. migonei s.l.; Lu. neivai; Lu. nuneztovari anglesi; Lu. ovallesi; Lu. panamensis; Lu. paraensis; Lu. pescei; Lu. pessoai; Lu. pia; Lu. shawi; Lu. spinicrassa; Lu. tejadai; Lu. townsendi; Lu. trinidadensis; Lu. wellcomei; Lu. whitmani; Lu. ylephiletor; Lu. youngi; Lu. yucumensis*	Dogs; Equids; Marsupials; Rodents	Zoonotic	CL; MCL
L. peruviana	Peru	*Lu. ayacuchensis*; Lu. maranonensis; *Lu. peruensis* s.l.; *Lu. verrucarum s.l.*	Dogs; Marsupials; Rodents	Zoonotic	CL
L. guyanensis	Argentina; Bolivia; Brazil; Colombia; Ecuador; French Guiana; Guyana; Peru; Suriname; Venezuela	*Lu. anduzei*; *Lu. ayacuchensis*; *Lu. longiflocosa*; *Lu. migonei*; *Lu. shawi*; *Lu. umbratilis*; *Lu. whitmani*	Edentats; Marsupials; Rodents	Zoonotic	CL; MCL
L. panamensis	Colombia; Costa Rica; Ecuador; Guatemala; Honduras; Nicaragua; Panama	*Lu. cruciata*; *Lu. edentula*; *Lu. gomezi*; *Lu. hartmanni*; *Lu. panamensis*; *Lu. sanguinaria*; *Lu. trapidoi; Lu. ylephiletor; Lu. yuilli*	Dogs; Edentates; Marsupials; Procyonids; Rodents	Zoonotic	CL; (MCL)
L. shawi	Brazil	*Lu. whitmani*	Edentates; Primates; Procyonids	Zoonotic	CL
L. lainsoni	Bolivia; Brazil; Ecuador; French Guiana; Peru; Suriname	*Lu. auraensis; Lu. nuneztovari anglesi*; *Lu. ubiquitalis*	*Rodents*	Zoonotic	CL
L. naiffi	Brazil; Colombia; Ecuador; French Guiana; Panama; Peru; Suriname	*Lu. amazonensis*; *Lu. ayrozai*; Lu. gomezi; *Lu. paraensis*; *Lu. squamiventris; Lu. tortura; Lu. trapidoi*	Armadillos; Edentates	Zoonotic	CL
L. lindenbergi	Brazil	*Lu. antunesi*	Unknown	Zoonotic**	CL
E. colombiensis	Colombia; Panama; Venezuela	*Lu. hartmanni*; *Lu. gomezi*; *Lu. panamensis; Lu. shannoni*	Edentates	Zoonotic	CL

*Clinical forms between brackets have rarely been reported; **Suspected.

Abbreviations: *L., Leishmania; Lu, Lutzomyia*; s.l., sensu lato; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.

Leishmania infection in humans can be restricted to the skin and/or mucosa, presenting as tegumentary leishmaniasis (cutaneous or mucosal, respectively), or it can disseminate systemically, involving the reticuloendothelial system and causing visceral leishmaniasis (90). These different presentations are mostly related to the infecting *Leishmania* species. However, there is still not a complete understanding of why certain species spread systemically, while others remain localized to the skin/mucosa, although the A2 gene family (present as a functional gene among species causing VL) likely plays a crucial role in visceralization (100).

Additionally, the outcome of infection by a *Leishmania* species is heterogenous and can be seen as a spectrum of manifestations: at one extreme lie polyparasitic infections (e.g., diffuse CL or VL), in which a Th2-type immune response dominates, with relative anergy and heavily parasitized macrophages. At the opposite end are oligoparasitic infections (such as ML and latent VL), in which a Th1-type immune response is predominant, and amastigotes are sparse (101,102).

Concerning viscerotropic species (belonging to the *L. donovani* complex - *L. donovani* and *L. infantum*), the reasons why some individuals mount a protective response, leading to asymptomatic/latent VL, while others develop the disease remain incompletely understood (100). Both vector (103) and host related factors have been suggested as linked to disease progression; host determinants include immunosuppression (such as HIV infection/AIDS) (104), genetic factors (105) and poor nutritional status (106). Progression can occur years after exposure and some individuals may develop subclinical presentations, with few/no symptoms, isolated splenomegaly or isolated cytopenias before advancing to overt disease or spontaneously resolving (107).

5.1. Asymptomatic infection

There is no universally accepted definition of asymptomatic (viscerotropic) *Leishmania* infection (108). However, it is usually considered in people who do not have symptoms associated with VL and who have one or more of the following: detection of antibodies against *Leishmania* in serum; detection of *Leishmania* DNA in blood; positive reaction (>5mm induration) in the leishmanin skin test (in which preparations made from killed promastigotes are injected intradermally, eliciting a delayed hypersensitivity reaction); positive result in the soluble *Leishmania* antigen test (109).

There is growing recognition of asymptomatic infection occurring in endemic regions. Depending on factors like geographical location, testing method, and the definition of asymptomatic infection, prevalence ranges from 0.3% to 37%, with a latent to active VL ratio between 4:1 and 50:1 (110).

In endemic regions in Europe, several studies have looked at asymptomatic infection, mostly at regional level. These studies have been performed in blood donors (111), in otherwise healthy volunteers, as well as in specific groups such as renal transplant recipients (112) and PLWH (113). In general, these studies have shown prevalences between 0 and 10%, although marked regional differences were observed and, in similar populations, distinct values were noted when using different techniques. In Portugal, only two studies have been performed in humans in the AML (dog owners) (114) and the Beiras e Serra da Estrela (blood donors) (115) regions, showing seroprevalences of 5% and 0%, respectively.

Most individuals with asymptomatic infection remain asymptomatic. However, asymptomatic *L. donovani* infection has been associated with increased risk of progression to VL, especially if this status is identified by serology with high titers in initial screening or with seroconversion during follow-up, as has been demonstrated in studies in the Indian subcontinent, where this species is endemic (116,117). In this region, it is estimated that 2 to 29% of individuals with asymptomatic infection progress to VL within up to three years (118). No similar studies have been performed at a similar scale in the Mediterranean region, where *L. infantum* is endemic and the demographic profile of symptomatic cases is different from South Asia, representing mostly children and immunosuppressed patients (as opposed to young healthy adults). However, in Brazil, where *L. infantum* is also endemic, longitudinal studies in children have suggested progression to VL in around 11% of individuals within three years after seroconversion (119).

Presently, there is not a sensitive way to predict which patients will progress to overt disease and at what time, although some recent research, including whole-blood transcriptomic, has been focused on identifying such blood molecular markers (120,121). It should be noted that the biological meaning of a positive serology is diverse: from past, resolved infection; to latent infection; to active, subclinical or oligosymptomatic infection (109). In areas where *L. infantum* is endemic, although immunosuppression has been recognized as an important factor for symptomatic disease, there is currently no consensus on screening of asymptomatic patients for *Leishmania*, for example in the setting of HIV infection/AIDS (122) or prior to starting immunosuppressive therapy (123). When screening is performed and asymptomatic infection is detected, only clinical surveillance is recommended, since there is no evidence based on randomized trials for treatment of asymptomatic infection (124).

Asymptomatic individuals could theoretically represent a threat to other humans and animals as a source of viable parasites. Transmission could occur via blood derived products or via organ or tissue transplantation (29). However, it has been suggested that transmission is unlikely to occur when blood products are leucodepleted (125). As such, routine screening of blood donors is not current practice in blood banks (126). Concerns regarding vectorial transmission have led to xenodiagnostic studies, showing that asymptomatic individuals were not infectious to sand flies, except when they presented some degree of immunosuppression (127); as such, they could represent an additional reservoir host of the parasite in its life cycle, and approach to these patients could have a role in controlling the disease in endemic settings.

5.2. Visceral leishmaniasis

VL is a systemic form that is usually caused by parasites taxonomically classified within the *L. donovani* complex. The typical incubation period ranges from two to six months (128). Symptoms usually manifest sub-acutely, with a gradual onset of malaise, fever, and weight loss over the course of weeks/months. Splenomegaly and hepatomegaly are commonly observed (128). Lymphadenopathy has been more commonly described in East Africa (121)(129). Laboratory findings frequently observed in VL include pancytopenia, elevated liver enzymes, polyclonal hypergammaglobulinemia, and mild renal impairment. Advanced stages of VL are associated with marked cachexia, edema, ascites, bacterial superinfections, and spontaneous bleeding from the skin/mucosae. If left untreated, VL is generally fatal (128). In PLWH, VL is characteristically diagnosed when the CD4 cell count drops below 200 cells/mm^3 (130). Coinfection with VL and HIV is characterized by higher rates of drug toxicity, lower cure rates, and increased relapse and mortality rates compared to VL patients without HIV (131). Clinical manifestations in VL–HIV coinfection typically mirror those of VL, although *Leishmania* parasites may invade various organs, including the gastrointestinal tract and lungs, and patients often develop concurrent cutaneous or mucosal involvement (132).

Diagnosing VL relies on various methods; direct visualization of the amastigote form of the parasite by microscopy is the classical approach and is usually performed on bone marrow or spleen samples obtained via biopsy or aspirate and stained with Giemsa, Wright-Giemsa, or haematoxylin–eosin (128). These techniques demonstrate a global sensitivity ranging from 53% to 86% (133) and 93% to 99% (134), for bone marrow or spleen samples, respectively. Culture involves inoculating appropriate samples into vials containing suitable culture media, such as Novy-McNeal-Nicolle or similar blood–agar-based media and should be monitored weekly for up to four weeks post-inoculation, with a sensitivity of approximately 60% to 85% (128). NAAT, such as Polymerase Chain Reaction (PCR)-based detection of parasite DNA in clinical samples, are notably more sensitive than microscopy or culture (sensitivity above 90%) (135); additionally, high sensitivity has been demonstrated in less invasive samples like peripheral blood (136), especially in immunocompromised patients. Furthermore, use of PCR, combined with other techniques such as Restriction Fragment Length Polymorphism (RFLP) or sequencing, enables species identification. Quantitative assessment of parasite DNA (using qPCR) in peripheral blood can be used to measure the initial parasitic load and monitor treatment responses (rapid clearance of *Leishmania* DNA during effective VL treatment); it can also serve as a predictive marker for relapse risk post-treatment (137,138). Although serological tests to detect specific antileishmanial antibodies exhibit high sensitivity in immunocompetent individuals (139), their effectiveness may be limited in people with immunosuppressive conditions, such as HIV infection/AIDS (140). Serological tests preferred for clinical diagnosis include the immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the immunochromatographic strip test utilizing rK39 antigen, a rapid diagnostic tool. Present management guidelines in Europe (for immunocompetent individuals) recommend initial screening of clinically suspected cases with serological tests, followed by confirmatory parasitology methods (141).

In Europe, the preferred treatment for VL is liposomal amphotericin B (LAmB), recommended for both immunocompetent and immunocompromised patients. For immunocompetent individuals, the typical dosage is 3–5 mg/kg per day administered intravenously over a period of 3–6 days, with a total cumulative dose ranging from 18–21 mg/kg. For immunocompromised patients, the regimen involves a daily dose of 3–5 mg/kg administered intravenously intermittently for 10 doses, totaling approximately 40 mg/kg (141). Pentavalent antimonials, which have been the conventional treatment since the 1940s, have been relegated in Europe to a secondary option by the WHO, due to their high toxicity, in countries where formulations of amphotericin B are available/accessible (141). Clinical resistance to antimonials in *L. infantum* in Europe is uncommon, unlike in *L. donovani* in the Indian subcontinent (141). Miltefosine, the sole oral treatment option, has demonstrated efficacy only in anecdotal reports and case series (142), lacking evidence from randomized clinical trials. Resistance to miltefosine has been documented in certain cases of treatment failure, through genetic markers (143). Other alternatives include parenteral administration of paromomycin or pentamidine (141). In the Americas, where *L. infantum* is also endemic, lipid formulations of amphotericin B are the preferred treatment for VL, according to regional guidelines (144).

In areas where *L. donovani* is endemic, other treatment modalities have been studied and proposed as recommended regimens. These include single dose LAmB (10 mg/kg) in the Indian subcontinent (where *L. donovani* shows higher sensitivity to amphotericin B) (145) and combination treatment with paromomycin and miltefosine in East Africa (where *L. donovani* shows intrinsically higher resistance to antimonials and amphotericin B) (146). Shorter LAmB regimens have not been adequately studied in the European region, although case series suggest comparable outcomes in children (147).

For PLWH, combination therapy holds promise for enhancing treatment efficacy, reducing toxicity, and minimizing the emergence of resistant parasites (148). Nevertheless, there are no published clinical trials evaluating the efficacy of combination therapy in VL–

HIV-coinfected patients in the WHO European Region. In the Indian subcontinent and in East Africa, however, randomized clinical trials showing safety and efficacy of combination treatment with LAmB and miltefosine (149,150) have resulted in the incorporation of this regimen as first line for local treatment by the WHO (151).

Clinical cure is defined by the disappearance of signs/symptoms and the normalization of laboratory parameters. Parasitological cure (complete eradication of parasites) is not an endpoint of treatment; parasitological response, defined as the absence of detectable *Leishmania* parasites through microscopy and/or NAAT of tissue samples, is not routinely assessed in clinical practice, but can be useful in certain situations, such as non-improvement and in immunosuppressed individuals (141). Serological tests are not suitable for immediate assessment of treatment response due to the gradual decline in antibody levels over several months (152).

Individuals with self-resolving infection or those who have undergone successful treatment develop protective immune responses. However, the parasite persists, and disease relapse can happen months-years later if the individual becomes immunocompromised (153). Relapse is defined as the reappearance of clinical manifestations, along with parasitological confirmation (141). Currently, there is insufficient data to establish definitive recommendations for the management of relapsing patients. Treatment options may include using an alternative drug, increasing the dose or duration of the same drug, or using a combination therapy approach (141).

Patients coinfected with HIV are at a higher risk of relapse (154); therefore, secondary prophylaxis and continuous monitoring are recommended until sustained immune reconstitution is achieved (151). In this group, secondary prophylaxis has been proven effective in reducing relapse rates (155) and LAmB (3-5mg/kg/dose every 3-4 weeks) is the currently WHO recommended scheme in Europe and the Indian subcontinent (151), although solid comparative data on different regimens and doses is not available. Discontinuation of prophylaxis may be considered for coinfected patients who show no signs of active *Leishmania* infection and have maintained CD4 cell counts above 200 cells/mm3 for at least six months while on ART (122). There is limited information available regarding prophylactic measures for other immunocompromised patient populations (123).

5.3. Tegumentary leishmaniasis

Tegumentary leishmaniasis is a clinical form restricted to the skin and/or mucosae. Numerous species have been identified as causative agents of tegumentary leishmaniasis, with prominent ones including *L. aethiopica*, *L. major*, and *L. tropica* in the Old World, and *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, and *L. mexicana* in the New World (128).

CL is characterized by the emergence of skin lesions that typically either resolve on their own or respond well to treatment, despite their potential disfiguring nature. The incubation period for CL typically spans two to eight weeks (128). Skin lesions typically evolve from a papular stage (an area of erythema at the sand fly bite site, gradually developing into an inflammatory papule), advancing to a nodular stage (transitioning into an indolent nodule or plaque, with the surface exhibiting fine, papery scales), then to an ulcer stage (a shallow, painless ulcer with raised, indurated margins and central granulation tissue), and eventually culminating in a scar (appearing white or pink and depressed - atrophic) (128). The appearance of lesions can vary depending on the infecting *Leishmania* species. Localized CL induced by *L. infantum* commonly presents as a solitary nodular lesion on the face, although other body parts can be affected, and multiple lesions may emerge. Typically, these lesions do not ulcerate. Clinical variability has been noted, including ulcerative lesions and plaques (156,157).

Unusual forms of CL encompass: disseminated CL, characterized by more than 10 lesions in two or more non-contiguous body parts; diffuse CL, where nodules progressively spread to affect nearly the entire cutaneous surface; and leishmaniasis recidivans (primarily linked with *L. tropica*), marked by the appearance of characteristic papular lesions months to years after clinical cure, typically in or around the scar of the previously healed lesion (128).

Mucocutaneous leishmaniasis (MCL) occurs when parasites spread to the nasal and/or oropharyngeal mucosa from a primary cutaneous lesion. This condition is most frequently observed in Bolivia, Brazil, and Peru, which collectively represent approximately 90% of cases (158). While mucosal lesions may manifest concurrently with the primary skin ulcer, they more commonly become apparent several months to many years after the initial cutaneous lesion has healed (159). Approximately 2% to 10% of individuals infected with *L. braziliensis* develop mucosal involvement (160), although MCL has also been reported in cases of *L. panamensis* (161) and, less frequently, *L. guyanensis* (162) infection. The nasal mucosa is typically the initial site affected, with symptoms including nasal congestion, difficulty breathing through the nose, and occasional bleeding, associated with erythema and edema of the affected nasal mucosa. This progresses to ulceration of the septum, covered with a mucopurulent exudate. In severe cases of MCL, there is often significant destruction of the nasal septum, palate, lips, pharynx, and larynx, with lesions exhibiting a chronic and progressive nature (128). Presently, there is no reliable method to predict the development of mucosal involvement in individuals with a primary CL ulcer, although factors associated with increased risk include large lesions (>4 cm^2), comorbidities and immunological factors (160).

Mucosal leishmaniasis (ML) refers to localized *Leishmania* lesions occurring in the nasal, oral, pharyngeal, or laryngeal mucosa without primary skin involvement (141). ML has been increasingly recognized in individuals undergoing immunosuppressive therapies, or as a result of secondary dissemination of VL in PLWH, although cases have also been documented in immunocompetent individuals. *L. infantum* is the typical causative agent (163,164).

Parasitological diagnosis of tegumentary leishmaniasis relies on the demonstration of amastigotes in smears or promastigotes in cultures, or the detection of *Leishmania* DNA. Sampling techniques include scraping, aspiration, tape stripping, and biopsy. Similar staining techniques and culture media are employed as for VL (128). Sensitivity of microscopy and culture techniques varies (40% to 90%), with PCR demonstrating higher sensitivity (96%) (165). Positive cultures permit typing of the isolated agents, and DNA target sequences from positive PCR specimens can be examined by RFLP or by direct sequencing for identification of the agent at the species level, particularly crucial in cases of imported CL or MCL (165). Serological methods are of limited utility due to low sensitivity and are not recommended for laboratory diagnosis of CL (141).

Treatment selection for CL generally depends on factors such as lesion size, number, location, relevant pre-existing conditions including advanced age, comorbidities and immune status, as well as the parasite species (141). Systemic treatment is usually recommended when at least one of the following criteria are met: lesion size exceeding 4 cm, over four lesions present, lesions located around orifices or close to small joints, presence of underlying immunosuppressive conditions, or failure of previous treatment approaches. Recommended systemic treatment options include pentavalent antimonials, miltefosine, fluconazole, itraconazole, and LAmB. For mild disease, local treatment options include superficial cryotherapy, intralesional antimonials, paromomycin-containing ointment, thermotherapy, or combinations of these strategies (141). For MCL, treatment should be systemic and antimonials are currently considered the first line approach (144).

6. Aim and scope

This doctoral project aimed to provide a wider picture of the current epidemiological situation and burden of *Leishmania* infection in Portugal, through a One Health lens, by: (i) assessing the prevalence of asymptomatic infection in blood donors in mainland Portugal; (ii) characterizing the knowledge, perceptions and practices (KPP) of health students, professionals and blood donors regarding leishmaniasis; and (iii) analyzing the sociodemographic and clinical aspects of the VL, CL and ML cases diagnosed in hospitals of the National Health Service in mainland Portugal, between 2010 and 2020.

For this purpose, three studies were conducted, as detailed on Table 4. The results of these studies are presented and discussed in the following chapters. At the level of human infection, data from symptomatic cases and asymptomatic infections were gathered. At the level of reservoir hosts and arthropod vectors, data reflecting knowledge, perceptions and practices were extracted from questionnaires directed to blood donors and health professionals and students. Overlapping this information from different sources allowed to draw conclusions, which could provide a solid base to define plans for managing leishmaniasis in the following years.

Table 4. Overview of the studies that composed this doctoral project

	Study 1	Study 2	Study 3
Objectives	Asses the prevalence of asymptomatic infection in blood donors in mainland Portugal. . Characterize the KPP of blood donors in mainland Portugal regarding leishmaniasis	Characterize the KPP of health students and professionals in Portugal regarding leishmaniasis	Analyze the sociodemographic and clinical aspects of the VL, CL and ML cases diagnosed in hospitals of the NHS in mainland Portugal, between 2010 and 2020
Type of study	Cross-sectional study	Cross-sectional study	Observational retrospective study
Target population	People who donate blood in mainland Portugal	Health students (Medicine, Veterinary Medicine, Environmental Health) and professionals (physicians, veterinarians, environmental health technicians) in Portugal	Individuals diagnosed with leishmaniasis between 2010 and 2020 in hospitals of the NHS in mainland Portugal
Strategy for sampling	> Proportional distribution by municipality; > Random selection in 347 (mobile and stationary) blood collection points of the IPST and the IHD (Alentejo and Algarve).	> All universities and professional societies were contacted; > In collaborating institutions, students/professionals were reached via institutional email.	> Collaboration was requested to every hospital of the NHS in Mainland Portugal; > Cases were screened through a search of diagnostic discharge codes or by a search of positive laboratory results for *Leishmania* infection; > The DGS was contacted to request access to notified cases of VL between 2010 and 2020.
Timeframe of sampling	February-June 2022	July-December 2022	2021 to 2023
Strategy for data collection	> Self-applied sociodemographic and KPP paper questionnaire; > Detection of antibodies against *Leishmania* in serum (ELISA).	> Self-applied sociodemographic and KPP online questionnaire.	> Retrieval of sociodemographic and clinical data from medical records.
Sample size	3763 individuals	486 individulas (254 students and 232 professionals)	256 individuals (221 VL, 43 CL, 7 ML cases)
Analysis	> Descriptive statistics, hypothesis testing and logistic regression models performed using IBM® SPSS® Statistics version 29.0; > Confidence Intervals for proportions and crude and adjusted odds ratio were calculated; > Geographical representation and analysis of results obtained using QGIS® Version 3.22		
Main results	> Estimated national true seroprevalence of 4.8% (95% CI 4.1–5.5%); > Seropositivity significantly higher in male sex, people older than 25 years, or residing in the Centro region; > Higher Knowledge score associated with age 25–40 years, female sex, ownership of dogs, and higher education.	> 75% reported having heard of both human and animal leishmaniasis; > over 80% reported hearing about the disease during their course work; > Median knowledge and perception scores were higher among professionals in the animal health field and higher in professionals than in students.	> Significant increase in estimated national incidence in the years after 2016; > Only 49.7% of incident VL cases were reported; > Most CL cases autochthonous and around half were immunosuppressed individuals.
Chapters	2; 4	3; 4	5; 6

Abbreviations: CI, confidence interval; CL, cutaneous leishmaniasis; DGS, Direção-Geral da Saúde; ELISA, enzyme-linked immunosorbent assay; IHD, Immuno-hemotherapy departments; IPST, Instituto Português do Sangue e da Transplantação; KPP, knowledge, perceptions and practices; ML, mucosal leishmaniasis; NHS, National Health System; VL, visceral leishmaniasis.

7. References

1. Sunter J, Gull K. Shape, form, function and *Leishmania* pathogenicity: from textbook descriptions to biological understanding. Open Biol. 2017 Sep 13;7(9):170165.

2. Wheeler RJ, Sunter JD, Gull K. Flagellar pocket restructuring through the *Leishmania* life cycle involves a discrete flagellum attachment zone. J Cell Sci. 2016 Jan 1.

3. Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Cannet A, et al. *Leishmania* infections: Molecular targets and diagnosis. Mol Aspects Med. 2017 Oct;57:1–29.

4. Cantacessi C, Dantas-Torres F, Nolan MJ, Otranto D. The past, present, and future of *Leishmania* genomics and transcriptomics. Trends Parasitol. 2015 Mar;31(3):100–8.

5. Inbar E, Shaik J, Iantorno SA, Romano A, Nzelu CO, Owens K, et al. Whole genome sequencing of experimental hybrids supports meiosis-like sexual recombination in *Leishmania*. PLoS Genet. 2019 May 15;15(5):e1008042.

6. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS Negl Trop Dis. 2016 Mar 3;10(3):e0004349.

7. Cupolillo E, Medina-Acosta E, Noyes H, Momen H, Grimaldi G. A Revised Classification for *Leishmania* and *Endotrypanum.* Parasitology Today. 2000 Apr;16(4):142–4.

8. Espinosa OA, Serrano MG, Camargo EP, Teixeira MMG, Shaw JJ. An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. Parasitology. 2018 Apr 15;145(4):430–42.

9. Lauthier JJ, Ruybal P, Barroso PA, Hashiguchi Y, Marco JD, Korenaga M. Development of a Multilocus sequence typing (MLST) scheme for Pan-*Leishmania*. Acta Trop. 2020 Jan;201:105189.

10. Maurício IL. *Leishmania* Taxonomy. In: The leishmaniases: old neglected tropical diseases. Cham: Springer International Publishing; 2018. p. 15–30.

11. Jariyapan N, Daroontum T, Jaiwong K, Chanmol W, Intakhan N, Sor-suwan S, et al. *Leishmania (Mundinia) orientalis* n. sp. (Trypanosomatidae), a parasite from Thailand responsible for localised cutaneous leishmaniasis. Parasit Vectors. 2018 Dec 18;11(1):351.

12. Sapp SGH, Low R, Nine G, Nascimento FS, Qvarnstrom Y, Barratt JLN. Genetic characterization and description of *Leishmania (Leishmania) ellisi* sp. nov.: a new human-infecting species from the USA. Parasitol Res. 2024 Jan 15;123(1):52.

13. Kwakye-Nuako G, Mosore MT, Boakye D, Bates PA. Description, Biology, and Medical Significance of *Leishmania (Mundinia) chancei* n. sp. (Kinetoplastea: Trypanosomatidae) from Ghana and *Leishmania (Mundinia) procaviensis* n. sp. (Kinetoplastea: Trypanosomatidae) from Namibia. Journal of Parasitology. 2023 Feb 27;109(1).

14. Mathison BA, Bradbury RS, Pritt BS. Reply to Mungthin et al., "Taxonomy Revision of *Leishmania* spp. in Thailand." J Clin Microbiol. 2021 Apr 20;59(5).

15. Mathison BA, Bradbury RS, Pritt BS. Medical parasitology taxonomy update, January 2018 to May 2020. J Clin Microbiol. 2021 Jan 21;59(2).

16. Dvorak V, Shaw J, Volf P. Parasite Biology: The Vectors. In: The Leishmaniases: Old Neglected Tropical Diseases. Cham: Springer International Publishing; 2018. p. 31–77. Available from: http://link.springer.com/10.1007/978-3-319-72386-0_3

17. Cecílio P, Cordeiro-da-Silva A, Oliveira F. Sand flies: Basic information on the vectors of leishmaniasis and their interactions with *Leishmania* parasites. Commun Biol. 2022 Apr 4;5(1):305.

18. Killick-Kendrick R. The biology and control of Phlebotomine sand flies. Clin Dermatol. 1999 May 6;17(3):279–89.

19. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med Vet Entomol. 2013 Jun 27;27(2):123–47.

20. Alten B, Maia C, Afonso MO, Campino L, Jiménez M, González E, et al. Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by *Leishmania infantum*. PLoS Negl Trop Dis. 2016 Feb 22;10(2):e0004458.

21. Feliciangeli MD. Natural breeding places of phlebotomine sandflies. Med Vet Entomol. 2004 Mar 3;18(1):71–80.

22. Maia C, Dantas-Torres F, Campino L. Parasite biology: The reservoir hosts. In: The leishmaniases: old neglected tropical diseases. Cham: Springer International Publishing; 2018. p. 79–106.

23. Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009 Dec 16;136(14):1915–34.

24. Courtenay O, Peters NC, Rogers ME, Bern C. Combining epidemiology with basic biology of sand flies, parasites, and hosts to inform leishmaniasis transmission dynamics and control. PLoS Pathog. 2017 Oct 19;13(10):e1006571.

25. Dostálová A, Volf P. *Leishmania* development in sand flies: parasite-vector interactions overview. Parasit Vectors. 2012 Dec 3;5(1):276.

26. Secundino NFC, Eger-Mangrich I, Braga EM, Santoro MM, Pimenta PFP. *Lutzomyia longipalpis* peritrophic matrix: formation, structure, and chemical composition. J Med Entomol. 2005 Nov 1;42(6):928–38.

27. Bates PA. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007 Aug;37(10):1097–106.

28. Serafim TD, Iniguez E, Oliveira F. *Leishmania infantum*. Trends Parasitol. 2020 Jan;36(1):80–1.

29. Dey A, Singh S. Transfusion transmitted leishmaniasis: a case report and review of literature. Indian J Med Microbiol. 2006 Jul;24(3):165–70.

30. Perales-González A, Pérez-Garza DM, Garza-Dávila VF, Ocampo-Candiani J. Cutaneous leishmaniasis by a needlestick injury, an occupational infection? PLoS Negl Trop Dis. 2023 Mar 8;17(3):e0011150.

31. Alvar J, Jiménez M. Could infected drug-users be potential *Leishmania infantum* reservoirs? AIDS. 1994 Jun;8(6):854.

32. Antinori S, Cascio A, Parravicini C, Bianchi R, Corbellino M. Leishmaniasis among organ transplant recipients. Lancet Infect Dis. 2008 Mar;8(3):191–9.

33. Dahal P, Singh-Phulgenda S, Maguire BJ, Harriss E, Ritmeijer K, Alves F, et al. Visceral lçeishmaniasis in pregnancy and vertical transmission: A systematic literature review on the therapeutic orphans. PLoS Negl Trop Dis. 2021 Aug 10;15(8):e0009650.

34. Turchetti AP, Souza TD, Paixão TA, Santos RL. Sexual and vertical transmission of visceral leishmaniasis. The Journal of Infection in Developing Countries. 2014 Apr 15;8(04):403–7.

35. St. C. Symmers W. Leishmaniasis acquired by contagion. The Lancet. 1960 Jan;275(7116):127–32.

36. Naucke TJ, Amelung S, Lorentz S. First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. Parasit Vectors. 2016 Dec 10;9(1):256.

37. Centers for Disease Control and Prevention. DPDx - Laboratory identification of parasites of public health concern. 2024. Leishmaniasis.

38. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012 May 31;7(5):e35671.

39. WHO. Neglected tropical diseases. 2024.

40. WHO. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. 2010.

41. WHO. Unveiling the neglect of leishmaniasis [Internet]. 2017 [cited 2023 Nov 7]. Available from: https://www.who.int/images/default-source/departments/ntd-library/leishmaniasis/unveiling-the-neglect-of-leishmaniasis-infographic.png?sfvrsn=6b662033_1

42. WHO. THE GLOBAL HEALTH OBSERVATORY. 2023 [cited 2023 Mar 24]. Number of cases of visceral leishmaniasis reported. Available from: https://www.who.int/data/gho/data/indicators/indicator-details/GHO/number-of-cases-of-visceral-leishmaniasis-reported

43. World Health Organization. Status of endemicity of visceral leishmaniasis: 2022 [Internet]. 2023 [cited 2023 Oct 8]. Available from: https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html

44. WHO. Number of cases of cutaneous leishmaniasis reported: 2022. 2023.

45. Gradoni L. A brief introduction to leishmaniasis epidemiology. In: The leishmaniases: old neglected tropical diseases. Cham: Springer International Publishing; 2018. p. 1–13.

46. World Health Organisation. Status of endemicity of cutaneous leishmaniasis: 2022 [Internet]. 2023 [cited 2023 Oct 8]. Available from: https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html

47. ECDC. Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. Stockholm; 2022. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/leishmaniasis-surveillance-eu.pdf

48. Campino L, Cortes S, Dionisio L, Neto L, Afonso MO, Maia C. The first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal. Mem Inst Oswaldo Cruz. 2013 Jun;108(4):516–8.

49. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. Int J Parasitol. 2006;

50. Pereira A, Parreira R, Cristóvão JM, Castelli G, Bruno F, Vitale F, et al. Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers. Infection, Genetics and Evolution. 2020 Jan;77:104069. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1567134819302953

51. Asfaram S, Fakhar M, Teshnizi SH. Is the cat an important reservoir host for visceral leishmaniasis? A systematic review with meta-analysis. Journal of Venomous Animals and Toxins including Tropical Diseases. 2019;25.

52. Azami-Conesa I, Gómez-Muñoz MT, Martínez-Díaz RA. A systematic review (1990–2021) of wild animals infected with zoonotic *Leishmania*. Microorganisms. 2021 May 20;9(5):1101.

53. Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet Parasitol. 2012 Nov;190(1–2):268–71.

54. Jiménez M, González E, Martín-Martín I, Hernández S, Molina R. Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? Vet Parasitol. 2014 May;202(3–4):296–300. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0304401714002027

55. Maia C, Conceição C, Pereira A, Rocha R, Ortuño M, Muñoz C, et al. The estimated distribution of autochthonous leishmaniasis by *Leishmania infantum* in Europe in 2005–2020. PLoS Negl Trop Dis. 2023 Jul 19;17(7):e0011497.

56. Almeida M, Maia C, Cristóvão JM, Morgado C, Barbosa I, Ibars RF, et al. Seroprevalence and risk factors associated with *Leishmania* infection in dogs from Portugal. Microorganisms. 2022 Nov 15;10(11):2262.

57. Cortes S, Vaz Y, Neves R, Maia C, Cardoso L, Campino L. Risk factors for canine leishmaniasis in an endemic Mediterranean region. Vet Parasitol. 2012;

58. Cardoso L, Lopes AP, Sherry K, Schallig H, Solano-Gallego L. Low seroprevalence of *Leishmania infantum* infection in cats from northern Portugal based on DAT and ELISA. Vet Parasitol. 2010 Nov;174(1–2):37–42.

59. Duarte A, Castro I, da Fonseca IMP, Almeida V, de Carvalho LMM, Meireles J, et al. Survey of infectious and parasitic diseases in stray cats at the Lisbon Metropolitan Area, Portugal. J Feline Med Surg. 2010 Jun 1;12(6):441–6.

60. Maia C, Ramos C, Coimbra M, Cardoso L, Campino L. Prevalence of *Dirofilaria immitis* antigen and antibodies to *Leishmania infantum* in cats from southern Portugal. Parasitol Int. 2015 Apr;64(2):154–6.

61. Maia C, Ramos C, Coimbra M, Cardoso L, Campino L. Prevalence of *Dirofilaria immitis* antigen and antibodies to *Leishmania infantum* in cats from southern Portugal. Parasitol Int. 2015;

62. Maia C, Nunes M, Campino L. Importance of cats in zoonotic leishmaniasis in Portugal. Vector-Borne and Zoonotic Diseases. 2008 Aug;8(4):555–60.

63. Maia C, Gomes J, Cristóvão J, Nunes M, Martins A, Rebêlo E, et al. Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal. Vet Parasitol. 2010 Dec;174(3–4):336–40.

64. Vilhena H, Martinez-Díaz VL, Cardoso L, Vieira L, Altet L, Francino O, et al. Feline vector-borne pathogens in the north and centre of Portugal. Parasit Vectors. 2013 Dec 15;6(1):99.

65. Maia C, Almeida B, Coimbra M, Fernandes MC, Cristóvão JM, Ramos C, et al. Bacterial and protozoal agents of canine vector-borne diseases in the blood of domestic and stray dogs from southern Portugal. Parasit Vectors. 2015;

66. WHO. Report of the Fifth Consultative Meeting on *Leishmania*/HIV Coinfection. Addis Ababa, Ethiopia; 2007.

67. Campino L, Maia C. Epidemiology of leishmaniases in Portugal | Epidemiologia das leishmanioses em Portugal. Acta Med Port. 2010.

68. Vicente A. Kala-azar infantil. Dados epidemiológicos e incidência do kala-azar no Internamento de Pediatria do Hospital de Faro. Rev Port Doencas Infec. 1990;13:173–82.

69. Serrada E. A Leishmaniose visceral em Portugal Continental: 1999-2009 [Tese Saúde Pública]. Universidade Nova de Lisboa; 2010. Available from: http://hdl.handle.net/10362/5546

70. Gaspar C, Augusto G, Albuquerque M, Nascimento M, Vicêncio P, Nogueira P. Doenças de Declaração Obrigatória 2013-2016, Volume II – Portugal. 2017. Available from: http://hdl.handle.net/10400.26/22529

71. SPMS. Portal da Transparência. 2018 [cited 2023 Feb 22]. Doenças de Declaração Obrigatória. Available from: https://transparencia.sns.gov.pt/explore/dataset/doencas-de-declaracao-obrigatoria/table/?disjunctive.regiao_de_saude_de_notificacao&disjunctive.residencia_nut_iii&disjunctive.doenca_de_declaracao_obrigatoria&disjunctive.sexo&disjunctive.grupo_etario&sort

72. Marques N, Bustorff M, Cordeiro Da Silva A, Pinto AI, Santarém N, Ferreira F, et al. Visceral dissemination of mucocutaneous leishmaniasis in a kidney transplant recipient. Pathogens. 2020 Dec 30;10(1):18. Available from: https://www.mdpi.com/2076-0817/10/1/18

73. Dantas Brito M, Campilho F, Branca R, Pinho Vaz C, Silva C, Sousa T, et al. Visceral leishmaniasis: a differential diagnosis to remember after bone marrow transplantation. Case Rep Hematol. 2014;

74. Santos Silva AF, Figueiredo Dias JPBC, Nuak JMNGS, Rocha Aguiar F, Araújo Pinto JA, Sarmento ACEM. Visceral leishmaniasis in a patient with systemic lupus erythematosus. IDCases. 2015;2(4):102–5.

75. Humanes-Navarro AM, Herrador Z, Redondo L, Cruz I, Fernández-Martínez B. Estimating human leishmaniasis burden in Spain using the capture-recapture method, 2016–2017. Yurchenko V, editor. PLoS One. 2021 Oct 29;16(10):e0259225. Available from: https://dx.plos.org/10.1371/journal.pone.0259225

76. Campino L, Abranches P. Cutaneous leishmaniasis. Unusual disease in Portugal?. Acta Med Port. 2002;15(5):387–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12645224

77. Maia C, Campino L. Leishmaniose em Portugal no início do século XXI. An Inst Hig Med Trop. Lisboa. 2014;

78. Carujo A, Reis J, Santos Silva A, Araújo Abreu M, Ludgero Vasconcelos A. Complicated cutaneous leishmaniasis in a patient under combined immunosuppression. Acta Med Port. 2023 Oct 13;

79. Lopes SB, Aleixo RV, Silva JM, Cruz G, Ferreira E, Rabadão E. Recurrent mucosal leishmaniasis of the epiglottis in an immunosuppressed patient. IDCases. 2023;33:e01860.

80. Pires CA. Phlebotomus of Portugal. I--Natural infestation of *Phlebotomus ariasi* Tonnoir, 1921 and *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania* in the zoonotic focus of Arrábida (Portugal). Ann Parasitol Hum Comp. 1984;59(5):521–4.

81. Maia C, Afonso MO, Neto L, Dionísio L, Campino L. Molecular detection of *Leishmania infantum* in naturally infected *Phlebotomus perniciosus* from Algarve region, Portugal. J Vector Borne Dis. 2009 Dec;46(4):268–72.

82. Maia C, Dionísio L, Afonso MO, Neto L, Cristóvão JM, Campino L. *Leishmania* infection and host-blood feeding preferences of phlebotomine sandflies and canine leishmaniasis in an endemic European area, the Algarve Region in Portugal. Mem Inst Oswaldo Cruz. 2013 Jun;108(4):481–7.

83. Maia C, Parreira R, Cristóvão JM, Freitas FB, Afonso MO, Campino L. Molecular detection of *Leishmania* DNA and identification of blood meals in wild caught phlebotomine sand flies (Diptera: Psychodidae) from southern Portugal. Parasit Vectors. 2015 Dec 23;8(1):173.

84. Branco S, Alves-Pires C, Maia C, Cortes S, Cristovão JMS, Gonçalves L, et al. Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in Torres Novas municipality, Central Region, Portugal. Acta Trop. 2013 Mar;125(3):339–48.

85. Amaro F, Vilares A, Martins S, Reis T, Osório HC, Alves MJ, et al. Co-circulation of *Leishmania* parasites and Phleboviruses in a population of sand flies collected in the south of Portugal. Trop Med Infect Dis. 2023 Dec 20;9(1):3.

86. Pereira S, Pita-Pereira D, Araujo-Pereira T, Britto C, Costa-Rego T, Ferrolho J, et al. First molecular detection of *Leishmania infantum* in *Sergentomyia minuta* (Diptera, Psychodidae) in Alentejo, southern Portugal. Acta Trop. 2017 Oct;174:45–8.

87. Maia C, Depaquit J. Can *Sergentomyia* (Diptera, Psychodidae) play a role in the transmission of mammal-infecting *Leishmania*? Parasite Journal. 2016 Dec 6;23:55. Available from: http://www.parasite-journal.org/10.1051/parasite/2016062

88. Ready PD. Biology of Phlebotomine sand flies as vectors of disease agents. Annu Rev Entomol. 2013 Jan 7;58(1):227–50.

89. Leelayoova S, Siripattanapipong S, Manomat J, Piyaraj P, Tan-ariya P, Bualert L, et al. Leishmaniasis in Thailand: A Review of Causative Agents and Situations. Am J Trop Med Hyg. 2017 Jan 16;16–0604.

90. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet. 2018 Sep;392(10151):951–70.

91. Songumpai N, Promrangsee C, Noopetch P, Siriyasatien P, Preativatanyou K. First evidence of co-circulation of emerging *Leishmania martiniquensis*, *Leishmania orientalis*, and *Crithidia* sp. in *Culicoides* biting midges (Diptera: Ceratopogonidae), the putative vectors for autochthonous transmission in southern Thailand. Trop Med Infect Dis. 2022 Nov 15;7(11):379.

92. Hadermann A, Heeren S, Maes I, Dujardin JC, Domagalska MA, Van den Broeck F. Genome diversity of *Leishmania aethiopica*. Front Cell Infect Microbiol. 2023 Apr 20;13.

93. Pratlong F, Floeter-Winter L, Ishikawa E, El Baidouri F, Ravel C, Dedet JP, et al. Characterization of *Leishmania (Leishmania) waltoni* n.sp. (Kinetoplastida: Trypanosomatidae), the parasite responsible for diffuse cutaneous leishmaniasis in the Dominican Republic. Am J Trop Med Hyg. 2015 Sep 2;93(3):552–8.

94. Hashiguchi Y, Gomez L. EA, Cáceres AG, Velez LN, Villegas N V., Hashiguchi K, et al. Andean cutaneous leishmaniasis (Andean-CL, uta) in Peru and Ecuador: the vector *Lutzomyia* sand flies and reservoir mammals. Acta Trop. 2018 Feb;178:264–75.

95. Cantanhêde LM, Cupolillo E. *Leishmania (Viannia) naiffi* Lainson & Shaw 1989. Parasit Vectors. 2023 Jun 8;16(1):194.

96. Ducharme O, Simon S, Ginouves M, Prévot G, Couppie P, Demar M, et al. *Leishmania naiffi* and *lainsoni* in French Guiana: Clinical features and phylogenetic variability. PLoS Negl Trop Dis. 2020 Aug 14;14(8):e0008380.

97. de Almeida JV, de Souza CF, Fuzari AA, Joya CA, Valdivia HO, Bartholomeu DC, et al. Diagnosis and identification of *Leishmania* species in patients with cutaneous leishmaniasis in the state of Roraima, Brazil's Amazon Region. Parasit Vectors. 2021 Dec 7;14(1):32.

98. Salgado-Almario J, Hernández CA, Ovalle CE. Geographical distribution of *Leishmania* species in Colombia, 1985-2017. Biomédica. 2019 Jun 15;39(2):278–90.

99. Liautaud B, Kone M, Cabié A, Plumelle Y, Miossec C, Desbois N, et al. First case of visceral leishmaniasis caused by *Leishmania martiniquensis*. Am J Trop Med Hyg. 2015 Feb 4;92(2):317–9.

100. McCall LI, Zhang WW, Matlashewski G. Determinants for the development of visceral leishmaniasis disease. PLoS Pathog. 2013 Jan 3;9(1):e1003053.

101. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nat Rev Immunol. 2016 Sep 18;16(9):581–92.

102. Alexander J, Brombacher F. T Helper1/T helper2 cells and resistance/susceptibility to *Leishmania* infection: is this paradigm still relevant? Front Immunol. 2012;3.

103. Ockenfels B, Michael E, McDowell MA. Meta-analysis of the effects of insect vector saliva on host immune responses and infection of vector-transmitted pathogens: a focus on leishmaniasis. PLoS Negl Trop Dis. 2014 Oct 2;8(10):e3197.

104. Pagliano P, Esposito S. Visceral leishmaniosis in immunocompromised host: an update and literature review. Journal of Chemotherapy. 2017 Sep 3;29(5):261–6.

105. El-Safi S, Kheir MM, Bucheton B, Argiro L, Abel L, Dereure J, et al. Genes and environment in susceptibility to visceral leishmaniasis. C R Biol. 2006 Nov;329(11):863–70.

106. Osorio EY, Uscanga-Palomeque A, Patterson GT, Cordova E, Travi BL, Soong L, et al. Malnutrition-related parasite dissemination from the skin in visceral leishmaniasis is driven by PGE2-mediated amplification of CCR7-related trafficking of infected inflammatory monocytes. PLoS Negl Trop Dis. 2023 Jan 11;17(1):e0011040.

107. Gama MEA, Costa JML, Gomes CMC, Corbett CEP. Subclinical form of the American visceral leishmaniasis. Mem Inst Oswaldo Cruz. 2004 Dec;99(8):889–93.

108. Ibarra-Meneses AV, Corbeil A, Wagner V, Onwuchekwa C, Fernandez-Prada C. Identification of asymptomatic *Leishmania* infections: a scoping review. Parasit Vectors. 2022 Dec 5;15(1):5. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-021-05129-y

109. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic *Leishmania* infection: a new challenge for *Leishmania* control. Clinical Infectious Diseases. 2014 May 15;58(10):1424–9. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1093/cid/ciu102

110. Pederiva MMC, Santos SM Dos, Rivarola LGS, Guerreiro VJ, Lopes KS, Lima Junior MS da C, et al. Asymptomatic *Leishmania* infection in humans: A systematic review. J Infect Public Health. 2023 Feb;16(2):286–94.

111. Aliaga L, Ceballos J, Sampedro A, Cobo F, López-Nevot MÁ, Merino-Espinosa G, et al. Asymptomatic *Leishmania* infection in blood donors from the Southern of Spain. Infection. 2019 Oct 1;47(5):739–47. Available from: https://pubmed.ncbi.nlm.nih.gov/30888587/

112. Elmahallawy EK, Cuadros-Moronta E, Liébana-Martos MC, Rodríguez-Granger JM, Sampedro-Martínez A, Agil A, et al. Seroprevalence of *Leishmania* infection among asymptomatic renal transplant recipients from southern Spain. Transplant Infectious Disease. 2015 Dec 17;17(6):795–9.

113. Ena J, Pasquau F, López-Perezagua M del M, Martinez-Peinado C, Arjona F. Screening for subclinical *Leishmania* infection in HIV-infected patients living in eastern Spain. Pathog Glob Health. 2014 Dec 2;108(8):356–61.

114. Fonseca M. Estudo da seroprevalência de anticorpos anti *Leishmania* spp. numa população que coabita com canídeos com leishmaniose. Universidade de Lisboa; 2009. Available from: https://repositorio.ul.pt/handle/10451/2383

115. Moreira S. Avaliação do grau de exposição de seres humanos da sub-regiao da Cova da Beira ao agente zoonótico *Leishmania infantum*. Universidade da Beira Interior; 2016. Available from: http://hdl.handle.net/10400.6/5299

116. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, et al. Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. PLoS Negl Trop Dis. 2014;

117. Chakravarty J, Hasker E, Kansal S, Singh OP, Malaviya P, Singh AK, et al. Determinants for progression from asymptomatic infection to symptomatic visceral leishmaniasis: A cohort study. PLoS Negl Trop Dis. 2019 Mar 1;13(3). Available from: https://pubmed.ncbi.nlm.nih.gov/30917114/

118. Hirve S, Boelaert M, Matlashewski G, Mondal D, Arana B, Kroeger A, et al. Transmission dynamics of visceral leishmaniasis in the Indian Subcontinent – A systematic literature review. PLoS Negl Trop Dis. 2016 Aug 4;10(8):e0004896.

119. Jeronimo SMB, Teixeira MJ, Sousa A d. Q, Thielking P, Pearson RD, Evans TG. Natural history of *Leishmania (Leishmania) chagasi* infection in Northeastern Brazil: long-term follow-up. Clinical Infectious Diseases. 2000 Mar 1;30(3):608–9.

120. da Matta VLR, Gonçalves AN, Gomes CMC, Chouman IH, Ferreira FM, Campos MB, et al. Gene signatures of symptomatic and asymptomatic clinical-immunological profiles of human infection by *Leishmania (L.) chagasi* in Amazonian Brazil. Microorganisms. 2023 Mar 3;11(3):653.

121. Zijlstra EE, El-Hassan AM. 3. Visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2001 Apr;95:S27–58.

122. Ambrosioni J, Levi L, Alagaratnam J, Van Bremen K, Mastrangelo A, Waalewijn H, Molina JM, Guaraldi G, Winston A, Boesecke C, Cinque P, Bamford A, Calmy A, Marzolini C, Martínez E, Oprea C, Welch S, Koval A, Mendao L, Rockstroh JK; EACS Governing Board. Major revision version 12.0 of the European AIDS Clinical Society guidelines 2023. HIV Med. 2023 Nov;24(11):1126-1136. doi: 10.1111/hiv.13542. Epub 2023 Oct 18. PMID: 37849432.

123. van Griensven J, Carrillo E, López-Vélez R, Lynen L, Moreno J. Leishmaniasis in immunosuppressed individuals. Clinical Microbiology and Infection. 2014 Apr;20(4):286–99. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1198743X14602743

124. Akuffo H, Costa C, van Griensven J, Burza S, Moreno J, Herrero M. New insights into leishmaniasis in the immunosuppressed. Rafati S, editor. PLoS Negl Trop Dis. 2018 May 10;12(5):e0006375. Available from: https://dx.plos.org/10.1371/journal.pntd.0006375

125. Pereira LQ, Tanaka SCSV, Ferreira-Silva MM, Gomes FVBAF, Santana MP, Aguiar PR, et al. Leukoreduction as a control measure in transfusion transmission of visceral leishmaniasis. Transfusion (Paris). 2023 Mar;

126. Mansueto P, Seidita A, Vitale G, Cascio A. Transfusion transmitted leishmaniasis. What to do with blood donors from endemic areas? Travel Medicine and Infectious Disease. 2014.

127. Singh OP, Tiwary P, Kushwaha AK, Singh SK, Singh DK, Lawyer P, et al. Xenodiagnosis to evaluate the infectiousness of humans to sandflies in an area endemic for visceral leishmaniasis in Bihar, India: a transmission-dynamics study. Lancet Microbe. 2021;

128. Farrar J, Hotez PJ, Junghanss T, Kang G, Lalloo D, White NJ, Garcia PJ. Manson's Tropical Diseases, 24rd edition. Elsevier; 2024.

129. Hurlot Q, Fillaux J, Laurent C, Berry A, Hofman P, Marchou B, et al. A case report of isolated lymphadenopathy revealing localized leishmanial lymphadenopathy in an asthenic 25-year-old man. Medicine. 2016 Jul;95(29):e3932.

130. Pintado V, López-Vélez R. HIV-associated visceral leishmaniasis. Clinical Microbiology and Infection. 2001 Jun;7(6):291–300.

131. Lauletta Lindoso JA, Alves Cunha M, Queiroz I, Valente Moreira CH. Leishmaniasis&HIV coinfection: current challenges. HIV/AIDS - Research and Palliative Care. 2016 Oct;Volume 8:147–56.

132. Diro E, van Griensven J, Mohammed R, Colebunders R, Asefa M, Hailu A, et al. Atypical manifestations of visceral leishmaniasis in patients with HIV in north Ethiopia: a gap in guidelines for the management of opportunistic infections in resource poor settings. Lancet Infect Dis. 2015 Jan;15(1):122–9.

133. Costa CHN, Stewart JM, da Silva MRB. Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. Am J Trop Med Hyg. 2005 Jun 1;72(6):811–4.

134. Chulay JD, Bryceson ADM. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. Am J Trop Med Hyg. 1983 May 1;32(3):475–9.

135. Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, et al. Clinical use of Polymerase Chain Reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: A single-center, 8-year experience in Italy and review of the literature. Clinical Infectious Diseases. 2007 Jun 15;44(12):1602–10.

136. da Costa Lima MS, Zorzenon DCR, Dorval MEC, Pontes ERJC, Oshiro ET, Cunha R, et al. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pac J Trop Dis. 2013 Feb;3(1):10–5.

137. Aquino SR de, Diniz LFB, Nunes SLP, Silva RL de O, Gouveia GV, Gouveia JJS, et al. Blood parasite load by qPCR as therapeutic monitoring in visceral leishmaniasis patients in Brazil: a case series study. Rev Soc Bras Med Trop. 2023;56.

138. Wu Y, Tian X, Song N, Huang M, Wu Z, Li S, et al. Application of Quantitative PCR in the diagnosis and evaluating treatment efficacy of leishmaniasis. Front Cell Infect Microbiol. 2020 Oct 7;10.

139. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. BMJ. 2006 Oct 7;333(7571):723.

140. Cota GF, de Sousa MR, Demarqui FN, Rabello A. The Diagnostic Accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. PLoS Negl Trop Dis. 2012 May 29;6(5):e1665.

141. Gradoni, L. López-Vélez R MM. Manual on case management and surveillance of the leishmaniasis in the WHO European Region. World Health Organization. 2017.

142. Sindermann H, Engel KR, Fischer C, Bommer W. Oral miltefosine for leishmaniasis in immunocompromised patients: compassionate use in 39 patients with HIV infection. Clinical Infectious Diseases. 2004 Nov 15;39(10):1520–3.

143. Cojean S, Houzé S, Haouchine D, Huteau F, Lariven S, Hubert V, et al. *Leishmania* resistance to miltefosine associated with genetic marker. Emerg Infect Dis. 2012 Apr;18(4):704–6.

144. Pan American Health Organization. Guideline for the treatment of leishmaniasis in the Americas. Second edition. Washington, DC: PAHO; 2022. Available from: https://doi.org/10.37774/9789275125038.

145. Mondal D, Alvar J, Hasnain MG, Hossain MS, Ghosh D, Huda MM, et al. Efficacy and safety of single-dose liposomal amphotericin B for visceral leishmaniasis in a rural public hospital in Bangladesh: a feasibility study. Lancet Glob Health. 2014 Jan;2(1):e51–7.

146. Musa AM, Mbui J, Mohammed R, Olobo J, Ritmeijer K, Alcoba G, et al. Paromomycin and miltefosine combination as an alternative to treat patients with visceral leishmaniasis in Eastern Africa: A Randomized, Controlled, Multicountry Trial. Clinical Infectious Diseases. 2023 Feb 8;76(3):e1177–85.

147. Dondi A, Manieri E, Gambuti G, Varani S, Campoli C, Zama D, et al. A 10-year retrospective study on pediatric visceral leishmaniasis in a European endemic area: diagnostic and short-course therapeutic strategies. Healthcare. 2023 Dec 21;12(1):23.

148. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M. Combination therapy for visceral leishmaniasis. Lancet Infect Dis. 2010 Mar;10(3):184–94.

149. Burza S, Mahajan R, Kazmi S, Alexander N, Kumar D, Kumar V, et al. AmBisome monotherapy and combination AmBisome–miltefosine therapy for the treatment of visceral leishmaniasis in patients coinfected with Human Immunodeficiency Virus in India: a randomized open-label, parallel-arm, phase 3 trial. Clinical Infectious Diseases. 2022 Oct 12;75(8):1423–32.

150. Diro E, Blesson S, Edwards T, Ritmeijer K, Fikre H, Admassu H, et al. A randomized trial of AmBisome monotherapy and AmBisome and miltefosine combination to treat visceral leishmaniasis in HIV co-infected patients in Ethiopia. PLoS Negl Trop Dis. 2019 Jan 17;13(1):e0006988.

151. World Health Organization. WHO guideline for the treatment of visceral leishmaniasis in HIV co-infected patients in East Africa and South-East Asia. Geneva; 2022.

152. Kip AE, Balasegaram M, Beijnen JH, Schellens JHM, de Vries PJ, Dorlo TPC. Systematic review of biomarkers to monitor therapeutic response in leishmaniasis. Antimicrob Agents Chemother. 2015 Jan;59(1):1–14.

153. Takele Y, Mulaw T, Adem E, Womersley R, Kaforou M, Franssen SU, et al. Recurrent visceral leishmaniasis relapses in HIV co-infected patients are characterized by less efficient immune responses and higher parasite load. iScience. 2023 Feb;26(2):105867.

154. Abongomera C, Diro E, Vogt F, Tsoumanis A, Mekonnen Z, Admassu H, et al. The risk and predictors of visceral leishmaniasis relapse in Human Immunodeficiency Virus-coinfected patients in Ethiopia: a retrospective cohort study. Clinical Infectious Diseases. 2017 Oct 30;65(10):1703–10.

155. Lopez-Velez R. Amphotericin B lipid complex versus no treatment in the secondary prophylaxis of visceral leishmaniasis in HIV-infected patients. Journal of Antimicrobial Chemotherapy. 2004 Feb 12;53(3):540–3.

156. Giavedoni P, Iranzo P, Fuertes I, Estrach T, Alsina Gibert M. Leishmaniasis cutánea. Experiencia de 20 años en un hospital español de tercer nivel. Actas Dermosifiliogr. 2015 May;106(4):310–6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0001731015000022

157. Gaspari V, Gritti T, Ortalli M, Santi A, Galletti G, Rossi A, et al. Tegumentary leishmaniasis in Northeastern Italy from 2017 to 2020: a neglected public health issue. Int J Environ Res Public Health. 2022 Nov 30;19(23):16047. Available from: https://www.mdpi.com/1660-4601/19/23/16047

158. WHO. Leishmaniasis. 2023. Accessed 12 june 2024. Available at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.

159. Lessa MM, Machado PRL, Cincurá C, Glesby MJ, Carvalho EM, Oliveira-Filho J, et al. Mucosal leishmaniasis: a retrospective study of 327 cases from an endemic area of *Leishmania (Viannia) braziliensis*. Am J Trop Med Hyg. 2017 Sep 7;97(3):761–6.

160. Blum J, Lockwood DNJ, Visser L, Harms G, Bailey MS, Caumes E, et al. Local or systemic treatment for New World cutaneous leishmaniasis? Re-evaluating the evidence for the risk of mucosal leishmaniasis. Int Health. 2012 Sep;4(3):153–63.

161. Castillo CM, Osorio LE, Ochoa MT. Mucosal leishmaniasis due to *Leishmania (Viannia) panamensis* in Colombia: clinical characteristics. Am J Trop Med Hyg. 1998 Jul 1;59(1):49–52.

162. Guerra JA de O, Prestes SR, Silveira H, Coelho LI de ARC, Gama P, Moura A, et al. Mucosal leishmaniasis caused by *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis* in the Brazilian Amazon. PLoS Negl Trop Dis. 2011 Mar 8;5(3):e980.

163. Aliaga L, Cobo F, Mediavilla JD, Bravo J, Osuna A, Amador JM, et al. Localized mucosal leishmaniasis due to *Leishmania (Leishmania) infantum*. Medicine. 2003 May;82(3):147–58.

164. Faucher B, Pomares C, Fourcade S, Benyamine A, Marty P, Pratlong L, et al. Mucosal *Leishmania infantum* leishmaniasis: Specific pattern in a multicentre survey and historical cases. Journal of Infection. 2011 Jul;63(1):76–82.

165. de Vries HJC, Schallig HD. Cutaneous leishmaniasis: a 2022 updated narrative review into diagnosis and management developments. Am J Clin Dermatol. 2022 Nov 14;23(6):823–40.

CHAPTER 2

Prevalence of asymptomatic *Leishmania* infection in blood donors in mainland Portugal.

This chapter is a transcription of the research article:

Rocha, R., Gonçalves, L., Conceição, C., Andrade, P., Cristóvão, J., Condeço, J., Delgado, B., Caeiro, C., Kuzmenko, T., Vasconcelos, E., Escoval, M., Rey, C., Guz, M., Norte, C., Aldeia, C., Cruz, D., Maia, C. Prevalence of asymptomatic *Leishmania* infection and knowledge, perceptions, and practices in blood donors in mainland Portugal. Parasites & Vectors 16, 357 (2023). https://doi.org/10.1186/s13071-023-05980-1

Abstract

Background. Asymptomatic infection is the most common outcome of exposure to *Leishmania* parasites. In the Mediterranean region, where *Leishmania* infantum is endemic, studies on the prevalence of asymptomatic infection have often relied on serological testing in blood donors. In Spain, regional studies have shown seroprevalence in blood donors between 1 and 8%; in Portugal, values of 0 and 2% were suggested by two localized studies, in different populations. The purpose of this study was (i) to estimate the prevalence of asymptomatic *Leishmania* infection in blood donors in mainland Portugal, and (ii) to study the association between the detection of antibodies to *Leishmania* and sociodemographic factors, and also the knowledge, perceptions and practices (KPP) of the blood donors regarding leishmaniasis.

Methods. A cross-sectional study targeted the population of people who donated blood in mainland Portugal. Participants, distributed proportionally by municipality and aged between 18 and 65 years, were selected randomly in 347 blood collection points between February and June 2022, and completed a sociodemographic and a KPP questionnaire. Detection of anti-*Leishmania* antibodies in serum was performed using an ELISA commercial kit. Individual KPP scores were calculated by adding grades defined for each question.

Results. Globally, 201/3763 samples were positive. The estimated national true seroprevalence was 4.8% (95% CI 4.1–5.5%). The proportion of positive results was significantly different between NUTS (Nomenclature of Territorial Units for Statistics) regions. Models suggested that seropositivity was significantly higher in male sex, people older than 25 years, or residing in the Centro NUTS2 region, but not in dog owners nor people with lower KPP scores. Overall, 72.3% of participants had previously heard of leishmaniasis and, in multivariate analysis, a higher Knowledge score was associated with age 25–40 years, female sex, ownership of dogs, and higher education.

Conclusions. Global estimated true seroprevalence (4.8%) was similar to previous regional studies in blood donors in neighboring Spain. Higher seroprevalence values in the NUTS2 Centro region were consistent with incidence data from humans and seroprevalence studies in dogs. On the other hand, the low values in the Alentejo and the high values in the northern

subregions may be the result of geographical shifts in parasite circulation due to climate change and should prompt localized and integrated, vector, canine, and human research, following a One Health approach.

Keywords: *Leishmania*, Leishmaniasis, Asymptomatic, Seroprevalence, Blood donor, Knowledge, Perceptions, Practices, Portugal, One Health

1. Background

Leishmaniases are a group of diseases caused by protozoa of the *Leishmania* genus, transmitted by phlebotomine sand fly bites. In the Mediterranean region, the parasites are maintained in a zoonotic cycle where dogs are the most important domestic reservoirs. In this context, the endemic species causing most cases of visceral leishmaniasis (VL) is *Leishmania infantum* [1, 2]. Systematic passive surveillance of VL cases in Portugal showed 6 to 14 cases per year between 2014 and 2018 [3], although this likely represents significant underreporting, as revealed in a previous study where only 38.6% of cases diagnosed in hospitals were notified to central public health authorities [4].

Infection by species of the *Leishmania donovani* complex, including *L. infantum*, is mostly recognized by its symptomatic presentations, of which VL is the most commonly described, although cutaneous and mucosal involvement are increasingly reported, especially in immunosuppressed individuals [5, 6]. However, many studies in *L. donovani* complex endemic areas suggest asymptomatic infection is the most common outcome of exposure to the parasite [7–9]. As for viscerotropic species, no single definition of asymptomatic *Leishmania* infection is universally accepted, different criteria have been used in healthy people (i.e., without signs/symptoms compatible with VL), namely detection of antibodies against *Leishmania*, detection of *Leishmania* DNA in blood, or a reactive leishmanin skin test or soluble *Leishmania* antigen test [10].

A systematic review included epidemiologic studies to identify and characterize asymptomatic infected individuals, as an indicator of areas and social contexts where active circulation and exposure to the parasite occur [11]. In the Mediterranean region, where *L. infantum* is endemic, several of these studies have been conducted not only in humans but also in animals, especially dogs. Serological techniques were often used to identify asymptomatic infection, and, in humans, target populations have frequently been blood donors, due to ease of access to samples and a higher confidence of the asymptomatic status, since blood donation is usually only permitted after strict symptom and behavior triage by health professionals and exclusion of anemia. In Spain, seroprevalence studies among the general population and blood donors have shown values between 1 and 8%, depending on the region [9, 12]. Factors associated with higher asymptomatic prevalence included older age, male sex, dog tutorship, contact with livestock (either at home or in the environment), living in rural communities, and living in a detached house [9, 13].

In Portugal, asymptomatic *Leishmania* infection in humans was only addressed in two studies, using serological testing, at a regional level and with a limited sample size. One of them found no seropositive individuals among 229 blood donors living in the "Beiras e Serra da Estrela" (BSE) NUTS3 (Nomenclature of Territorial Units for Statistics, from the French Nomenclature des Unités territoriales statistiques) region [14] while in the other only 2/100 healthy dog owners were seropositive in the "Área Metropolitana de Lisboa" (AML) [15]. Contrastingly, the geographical patterns of *Leishmania* infection in dogs in Portugal have been more extensively investigated. A recent nationwide study, involving 1860 dogs, showed a national estimated true seroprevalence of 12.5%, varying between 0 and 30.5% among districts [16] and revealed higher seroprevalence in some areas partially matching the known classical distribution of most human (and animal) symptomatic cases, including the Douro, Coimbra and Médio Tejo NUTS3 regions.

In spite of the wide distribution and significant burden on endemic populations of VL, knowledge, perceptions, and practices (KPP) regarding this clinical form are not homogeneous among countries and even different regions of the same country or different sectors of the population [17–19]. However, few studies have sought associations between KPP and asymptomatic infection. In Portugal, KPP studies have been directed to animal owners and were not coupled with seroprevalence analysis [20, 21]. In these studies, 83–91%

of the owners had heard of animal leishmaniasis, but only 38.6% of human leishmaniasis. Hearing of leishmaniasis was significantly associated with non-rural areas and academic degree.

Therefore, this study aimed to estimate the national prevalence of asymptomatic *Leishmania* infection in blood donors and search for associations between the presence of anti-*Leishmania* antibodies and several sociodemographic factors in this population, as well as with the KPP of blood donors regarding leishmaniasis.

2. Population, materials, and methods

2.1. Study population and sample size calculation

This cross-sectional national study was carried out between February and June 2022 in blood donors in mainland Portugal, which is in Southwest Europe, bordering Spain and the Atlantic Ocean. According to the 2021 national census, the population of mainland Portugal aged 15 to 64 years was 6,257,752 inhabitants [22]. Mainland Portugal is divided into five NUTS2 regions, 23 NUTS3 regions (Additional file 1: Table S1), 278 municipalities ("municípios"), and 2882 parishes ("freguesias"). To ensure a nationwide coverage of sampling, this study was performed in collaboration with the Portuguese Institute of Blood and Transplantation (IPST) and with the immunohemotherapy departments (IHDs) of five hospital centers in the Alentejo and Algarve regions. The IPST and the IHDs perform regular blood collections in fixed centers as well as in shifting stations in rural and urban areas. In 2021, over 190,000 blood donations were performed in these institutions. Individuals are considered eligible for donation after a strict triage conducted by a trained health professional, to exclude acute disease and several chronic conditions and risk behaviors. Additionally, capillary hemoglobin levels are determined; men with less than 13.5 g/dl and women with less than 12.5 g/dl are automatically excluded from donating.

Sample size was estimated using the Epitools© Epidemiological Calculators [23, 24]. At least 3200 individuals were needed to estimate a 95% confidence interval (CI) for prevalence, considering an expected maximum global (national) seroprevalence of 9%

(based on Spanish regional studies [9, 13, 25]) and a minimum sensitivity and specificity of the serological test used of 85% and 90%, respectively, and considering a desired precision of 0.02 to a 95% CI. Additionally, this sample size would allow the detection of small differences in seroprevalence between NUTS3 regions, with a power of 95%, using a Chi-square test.

Sampling was stratified by municipality: the number of participants enrolled from each municipality was proportional to the fraction of the mainland population (aged 15–64 years) living in that region, according to the most recent census data and assuming a similar distribution for blood donors. For five NUTS3 regions in southern Portugal (Alto Alentejo, Alentejo Central, Baixo Alentejo, Alentejo Litoral, and Algarve), where higher seroprevalence was expected a priori (according to human incidence data derived from VL cases reported to the National Surveillance System [3]), recruitment of additional participants was planned in order to increase the precision of estimates, increasing the total sample size to 3494.

2.2. Eligibility criteria

Individuals enrolled in this study presented to one of the institutions collaborating in the study from February to June 2022 and were considered fit for blood donation. Only individuals aged 18 to 65 years were included. Blood donation must have been completed, including collection of a serum sample for routine serological testing (for the following blood-borne pathogens: hepatitis B virus, hepatitis C virus, human immunodeficiency virus [HIV], Treponema pallidum).

2.3. Data and sample collection

Participant enrollment was performed in non-randomly selected blood collection sessions, to ensure a maximum number of municipalities were surveyed. In some municipalities, more than one session was required to complete the calculated sample sizes, and, in this case, different zones of the municipality were preferably surveyed. Blood donation sessions exclusive to specific professional groups (such as police officers or firefighters) were generally avoided. A fixed number of participants was set for each session

(1 to 8). Blood donors were randomly invited to the study, according to hour of presentation at the blood collection center/station–considering non-consecutive, pre-defined time slots. This procedure differed in one center (in the Lisbon Metropolitan Area), due to logistic reasons, where all donors in each session were invited consecutively, by order of arrival, until the sample size for the municipality was fulfilled.

Recruitment was performed in both fixed donation centers and mobile stations, except in the Algarve region. The participants were informed about the study and signed an informed consent declaration. Each participant completed a self-administered structured paper questionnaire about sociodemographic aspects and KPP regarding leishmaniasis (Additional file 2: Fig. S1). This questionnaire was pretested in a convenience sample of 40 blood donors from the Norte and AML regions. 1.5 ml of serum taken from the peripheral blood sample collected for routine serological testing was sent to the Instituto de Higiene e Medicina Tropical (IHMT) and stored at − 20 ºC for the study.

Categorical variables extracted from the questionnaire were analyzed mostly using the original categories provided as answer options, but regrouping was performed in some cases. Classification of professions was performed using the European Skills, Competences, and Occupations (ESCO) classification of occupations, developed by the European Commission in 2010 [26]. NUTS regions, municipalities, parishes, and unions of parishes were defined according to the most recent organizational definition, published in 2013 and implemented in 2015. The order of presentation of NUTS2 and 3 regions in tables follows the numerical and/or alphabetical order of their respective official codes. Classification of parishes as rural or non-rural followed the Portuguese Rural Development Program 2014–2020 [27]. *Leishmania donovani* complex-endemic travel destinations included countries in Europe where disease has been reported in most or all regions: Albania, Bulgaria, Cyprus, Greece, Italy, Malta, and Spain [2]. And outside Europe, countries where over 200 cases of VL were reported to the World Health Organization (WHO) in 2021 [28] include Brazil, Eritrea, Ethiopia, India, Kenya, Nepal, Somalia, South Sudan, Sudan, and Yemen.

2.4. Serological study

Antileishmanial antibody detection in each serum sample was performed using enzyme-linked immunosorbent assay (ELISA) (*Leishmania* ELISA IgG+IgM, Vircell®, Spain), following the manufacturer's instructions and cut-offs. These kits simultaneously detect immunoglobulin M (IgM) and/or IgG antibodies against *Leishmania*; the wells of the plate are coated with an unspecified *L. infantum* antigen. The sensitivity and specificity of the ELISA, according to the manufacturer, are 97% and 99%, respectively. A single determination was performed for each serum sample. Samples were classified as positive, negative, or borderline (when optical density was less than 10% lower or higher than the average value of the borderline controls). Participants from whom positive samples were taken were considered to have been exposed to *Leishmania*—either past or current asymptomatic infection. Positive or borderline results were tested by a second method (in-house immunofluorescence antibody test [IFAT]) and, if these samples showed a titer of 1:64 or higher, participants were informed of the result (if they had expressed this wish in the consent declaration).

2.5. Statistical analysis

True prevalence was estimated at the regional level considering only positive samples and based on the following formula: true prevalence (TP) = (test prevalence − 1 + specificity)/(sensitivity − 1 + specificity), considering the values provided by the manufacturer. The corresponding 95% CIs were obtained using Wilson's method on Epitools© Epidemiological Calculators [23,29]. For finer detail geographical analysis (at municipality or parish level), however, borderline samples were also considered, since it was assumed they could represent some degree of exposure to the parasite.

Absolute and relative frequencies and hypothesis testing were performed using IBM® SPSS® Statistics Version 29.0. Bar charts were built using Microsoft® Excel®. Geographical representation and analysis of results were obtained using QGIS® Version 3.22. Answers to each KPP question were scored according to the criteria presented in Additional file 3: Table S2. A total score was calculated for knowledge (K score), perceptions (Per score), and practices (Pra score), by adding the individual scores of all the questions in each category. The range of possible values for each score was as follows: K 0–19; Per 0–6; Pra 0–6.

Descriptive statistics were expressed as absolute frequencies and percentages for categorical variables and as means with standard deviations or medians with interquartile ranges (IQRs) for continuous variables (e.g., age, K, Per, and Pra scores). Comparisons between groups were performed using the Pearson Chi-square test for categorical variables (or Fisher's exact in case of failure of the assumptions of the $\chi 2$ test). For continuous variables, after checking the assumptions of normality and homogeneity of the variances, instead of t-test and analysis of variance (ANOVA), the Mann–Whitney U test or the Kruskal–Wallis test were used, for comparing two or more independent groups, respectively. A value of $P < 0.05$ was considered statistically significant.

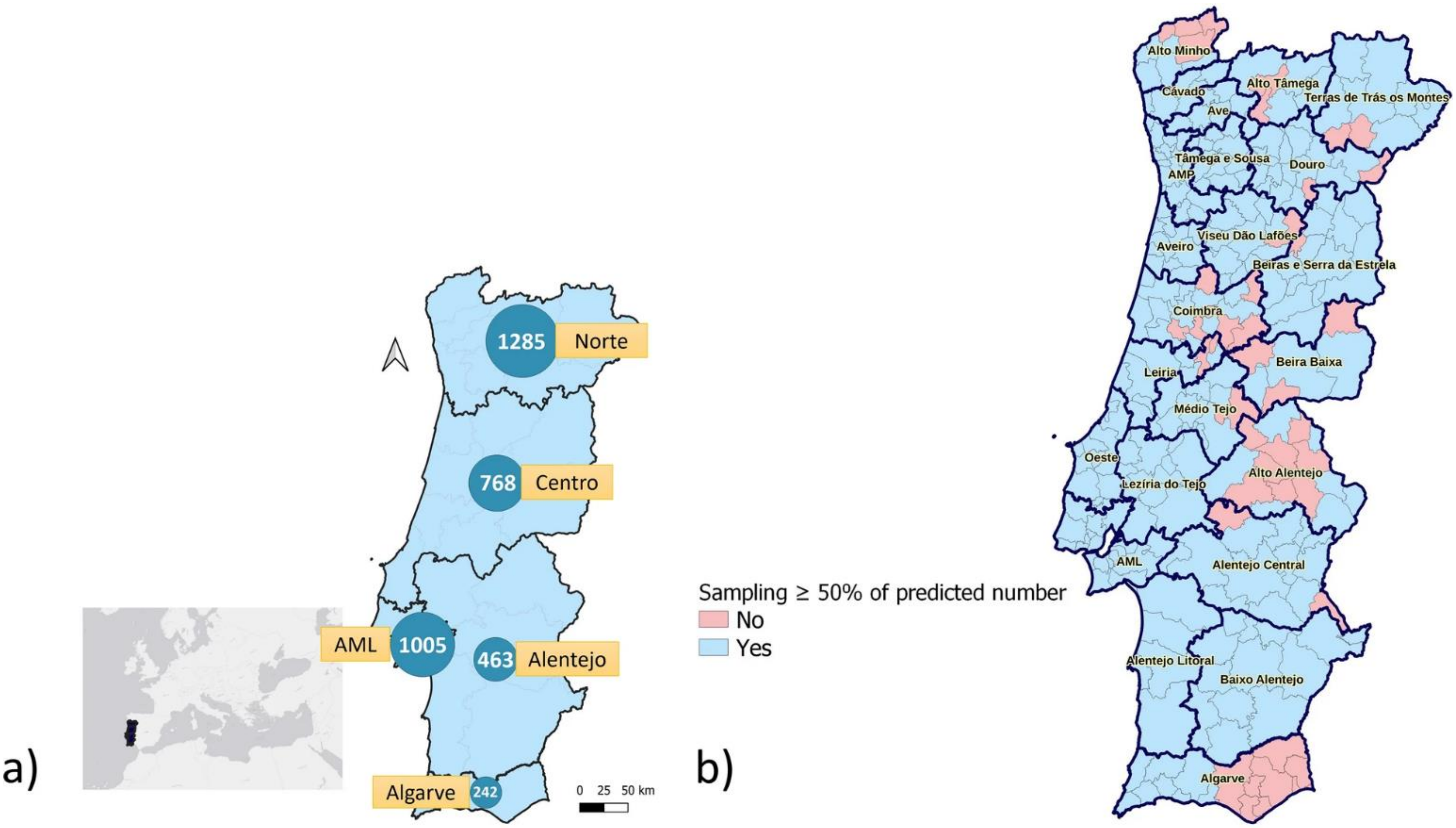
Multivariate analyses were conducted to identify sociodemographic factors and KPP associated with asymptomatic infection, and sociodemographic factors associated with higher K, Per, or Pra scores. These analyses were performed through multiple binary logistic regression models, analyzing variables with statistical meaning in the univariate analysis ($P < 0.05$) and some biologically relevant or potentially confounding variables.

For those variables that remained significant, the crude odds ratio (OR) was updated to adjusted odds ratio (aOR) with 95% CI. The Hosmer–Lemeshow test was used for assessing goodness of fit in each multiple logistic regression model [30]. To determine potential risk factors for positive ELISA results, only two groups were considered: positive and negative samples (borderline samples were excluded). To determine factors associated with higher K, Per, or Pra scores, donors were divided into two groups for each score: above the global median score value, and equal to or below this value (K = 7, Per = 0, Pra = 3.5). The reference categories used for each independent variable are specified in each multivariate analysis results table.

3. Results

In total, 3763 participants were included in this study. Participants were recruited in 636 blood collection sessions, in 347 different collection sites. The number of participants recruited by NUTS2 region is presented in Fig. 1a, and the municipalities where at least 50% of the calculated sample size was achieved are displayed in Fig. 1b. Missing municipalities were concentrated in the eastern Algarve, Alto Alentejo, Coimbra, and Alto Minho NUTS3 regions.

Figure 1. a) Number of participants recruited by NUTS2 region (circle sizes are proportional to the number of participants recruited); b) municipalities where at least 50% of the calculated sample size was achieved



Abbreviations: AML - Área Metropolitana de Lisboa; AMP - Área Metropolitana do Porto

3.1. Sociodemographic characteristics

The median age was 41 years, with significant differences between NUTS2 regions (older in the Alentejo and Algarve) (Table 1; more detailed information by NUTS3 region is presented in the Additional file 4: Table S3). Participants were evenly distributed between sexes globally and in most regions, except for the Alentejo and the Algarve, where male sex was clearly predominant. Education level was significantly higher in the AML (Chi-square test, $\chi 2 = 122.2$, df [degrees of freedom] = 16, $P < 0.001$). Globally, 23.7% of participants mentioned traveling or living abroad in the previous 2 years, mostly (58.6%) to at least one of the *L. donovani* complex endemic countries listed in the "Materials and methods" section.

Table 1. Sociodemographic characteristics of the participants, globally and by NUTS (Nomenclature of Territorial Units for Statistics) 2 region.

	Global	Norte	Centro	AML	Alentejo	Algarve	*p*-value
Total	100 (3763/3763)	34.1 (1285/3763)	20.4 (768/3763)	26.7 (1005/3763)	12.3 (463/3763)	6.4 (242/3763)	
Median age (y) (IQR)	41 (31-48)	39 (30-47)	41 (29-48)	42 (31-50)	43 (34-50)	43 (35-50)	<0.001* (H = 40.7, df = 4)
Male sex (%)	49.8 (1867/3749)	47.6 (609/1280)	48.2 (369/765)	47.7 (478/1003)	59.4 (274/461)	57.1 (137/240)	<0.001* (χ2 = 27.3, df = 4)
Education level [a]							
Basic (1-4)	1.9 (69/3659)	1.6 (20/1240)	2.8 (21/749)	1.2 (12/983)	2.7 (12/451)	1.7 (4/236)	<0.001* (χ2 = 122.2, df = 16)
Basic (5-9)	16.8 (615/3659)	20.7 (257/1240)	16.3 (122/749)	10.8 (106/983)	20.0 (90/451)	16.9 (40/236)	
Secondary (10-12)	44.1 (1612/3659)	43.5 (540/1240)	43.9 (329/749)	39.4 (387/983)	51.9 (234/451)	51.7 (122/236)	
Bachelor's	25.8 (944/3659)	22.9 (284/1240)	27.6 (207/749)	32.3 (318/983)	19.3 (87/451)	20.3 (48/236)	
MSc/PhD	11.5 (419/3659)	11.2 (139/1240)	9.3 (70/749)	16.3 (160/983)	6.2 (28/451)	9.3 (22/236)	
Occupation							
Student	9.9 (295/2993)	8.9 (90/1015)	13.3 (84/630)	11.0 (88/797)	6.9 (25/361)	4.2 (8/190)	
Unemployed	3.4 (101/2993)	4.3 (44/1015)	3.3 (21/630)	3.3 (26/797)	1.1 (4/361)	3.2 (6/190)	
Employed [b]	84.9 (2541/2993)	85.4 (867/1015)	80.6 (508/630)	83.3 (664/797)	88.9 (321/361)	90.0 (171/190)	
Armed forces (0)	2.2 (57/2541)	1.5 (13/867)	2.8 (14/508)	2.1 (14/664)	2.8 (9/321)	4.1 (7/171)	<0.001* (χ2 = 128.9, df = 12)
Managers, professionals and technicians (1-3)	46.0 (1169/2541)	42.7 (370/867)	43.1 (219/508)	59.8 (397/664)	35.5 (114/321)	34.5 (59/171)	
Clerical support, service and sales (4-5)	30.4 (773/2541)	29.3 (254/867)	28.3 (144/508)	25.0 (166/664)	38.6 (124/321)	49.7 (85/171)	
Agriculture, craft, industry and elementary (6-9)	21.3 (542/2541)	26.5 (230/867)	25.8 (131/508)	13.1 (87/664)	23.1 (74/321)	11.7 (20/171)	
Travel abroad							
Yes (previous 2 years)	23.7 (874/3689)	24.9 (310/1247)	22.1 (167/755)	26.8 (267/996)	17.5 (79/452)	21.3 (51/239)	0.001* (χ2 = 17.9, df = 4)
≥1 Endemic country [c]	58.6 (450/768)	52.3 (162/310)	54.4 (81/149)	54.7 (129/236)	76.0 (57/75)	55.3 (21/38)	0.015* (χ2 = 12.4, df = 4)

[a] Numbers in brackets refer to number of years completed of formal school education
[b] Numbers in brackets refer to the numbers of the categories in the classification of European Skills, Competences, and Occupations
[c] Albania, Brazil, Bulgaria, Cyprus, Eritrea, Ethiopia, Greece, India, Italy, Kenya, Malta, Nepal, Somalia, South Sudan, Spain, Sudan, Yemen
[d] According to the results of the 2021 National Census

Abbreviations: AML - Área Metropolitana de Lisboa; y - years; IQR - Interquartile range; MSc - Master of Science; PhD - Doctor of Phylosophy
* Statistically significant

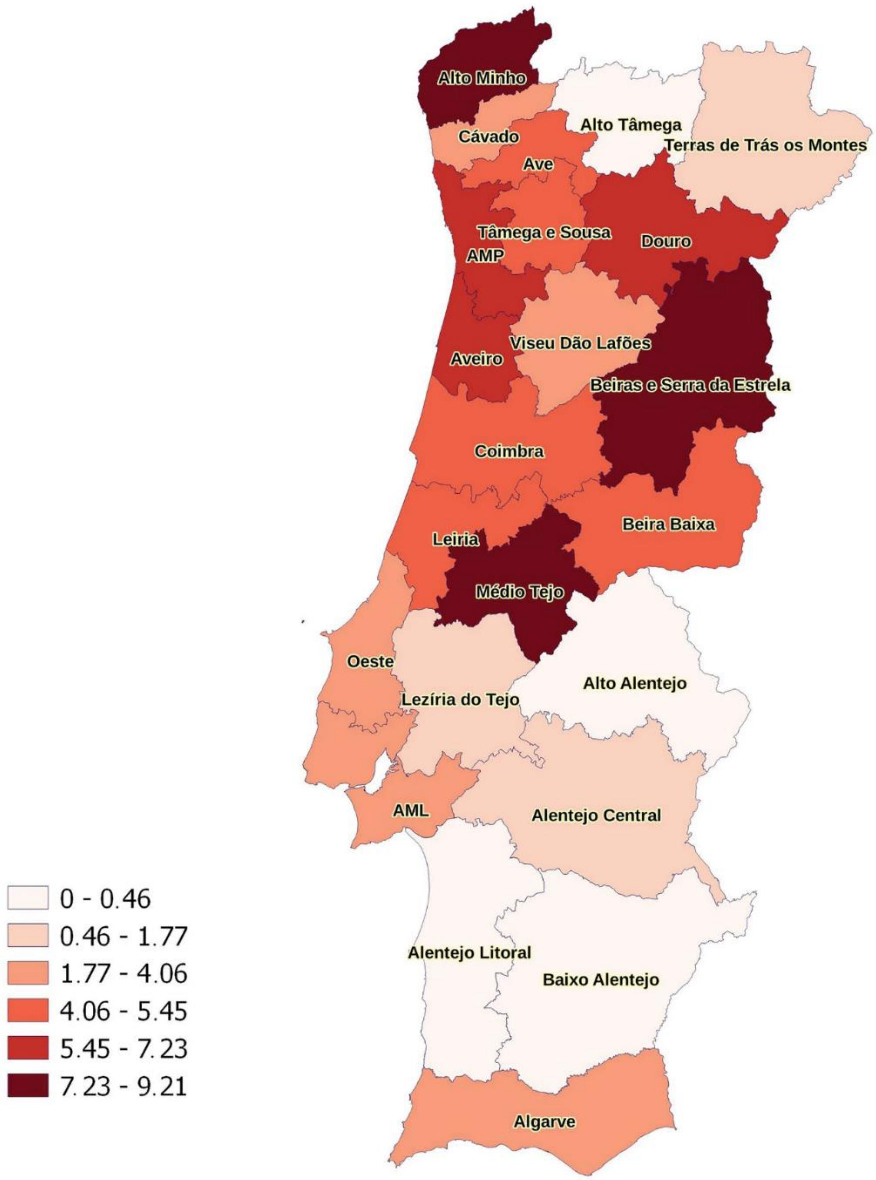
3.2. Serological results

In total, 201 (5.3%) samples were positive by ELISA and 97 (2.6%) were borderline. The distribution of positive results by NUTS2 and 3 regions is represented in Table 2. Adjusted positivity rates considered slight deviations between the expected and the achieved sample size by municipality. Figure 2 shows the distribution of true seroprevalence estimates by NUTS3 region.

Table 2. Table 2 Distribution of positive results by NUTS (Nomenclature of Territorial Units for Statistics) 2 and 3 region and estimated true prevalence.

Region	Sampling sites (*n*)	Samples (*n*)	Positive samples (*n*)	Crude positivity rate (%)	Adjusted positivity rate (%)	True prevalence (%)	95% Confidence Interval
Norte	149	1285	82	6.4	6.3	5.5	4.4-6.9
Alto Minho	12	72	6	8.3	8.5	7.8	3.6-16.0
Cávado	17	160	6	3.8	3.5	2.6	1.0-6.6
Ave	16	155	9	5.8	5.4	4.6	2.3-9.3
Área Metropolitana do Porto	60	591	44	7.4	7.4	6.6	4.9-8.9
Alto Tâmega	4	29	0	0.0	0.0	0.0	0-11.7
Tâmega e Sousa	23	163	9	5.5	5.6	4.8	2.5-9.3
Douro	13	79	6	7.6	7.9	7.2	3.4-14.9
Terras de Trás-os-Montes	4	36	2	5.6	2.0	1.1	0.2-6.0
Centro	119	768	51	6.6	6.5	5.8	4.3-7.6
Oeste	12	145	8	5.5	4.9	4.1	1.9-8.6
Região de Aveiro	19	146	12	8.2	7.7	6.9	3.8-12.3
Região de Coimbra	21	114	7	6.1	6.1	5.3	2.5-11.1
Região de Leiria	14	103	6	5.8	6.2	5.4	2.5-11.4
Viseu Dão-Lafões	15	67	3	4.5	4.2	3.3	0.9-11.4
Beira Baixa	7	33	2	6.1	5.9	5.1	1.4-16.9
Médio Tejo	16	73	6	8.2	9.8	9.2	4.5-17.8
Beiras e Serra da Estrela	15	87	7	8.0	8.8	8.1	4.0-15.7
Área Metropolitana de Lisboa	25	1005	47	4.7	4.9	4.0	3.0-5.4
Alentejo	52	463	9	1.9	1.9	0.9	0.4-2.3
Alentejo Litoral	10	70	1	1.4	1.3	0.3	0.0-5.2
Baixo Alentejo	8	105	1	1.0	0.8	0.0	0.0-3.5
Lezíria do Tejo	10	87	2	2.3	2.3	1.4	0.3-7.5
Alto Alentejo	9	57	1	1.8	1.4	0.5	0.0-6.3
Alentejo Central	15	144	4	2.8	2.7	1.8	0.6-5.1
Algarve	2	242	12	5.0	4.2	3.3	1.7-6.4
Total	347	3763	201	5.3	5.6	4.8	4.1-5.5

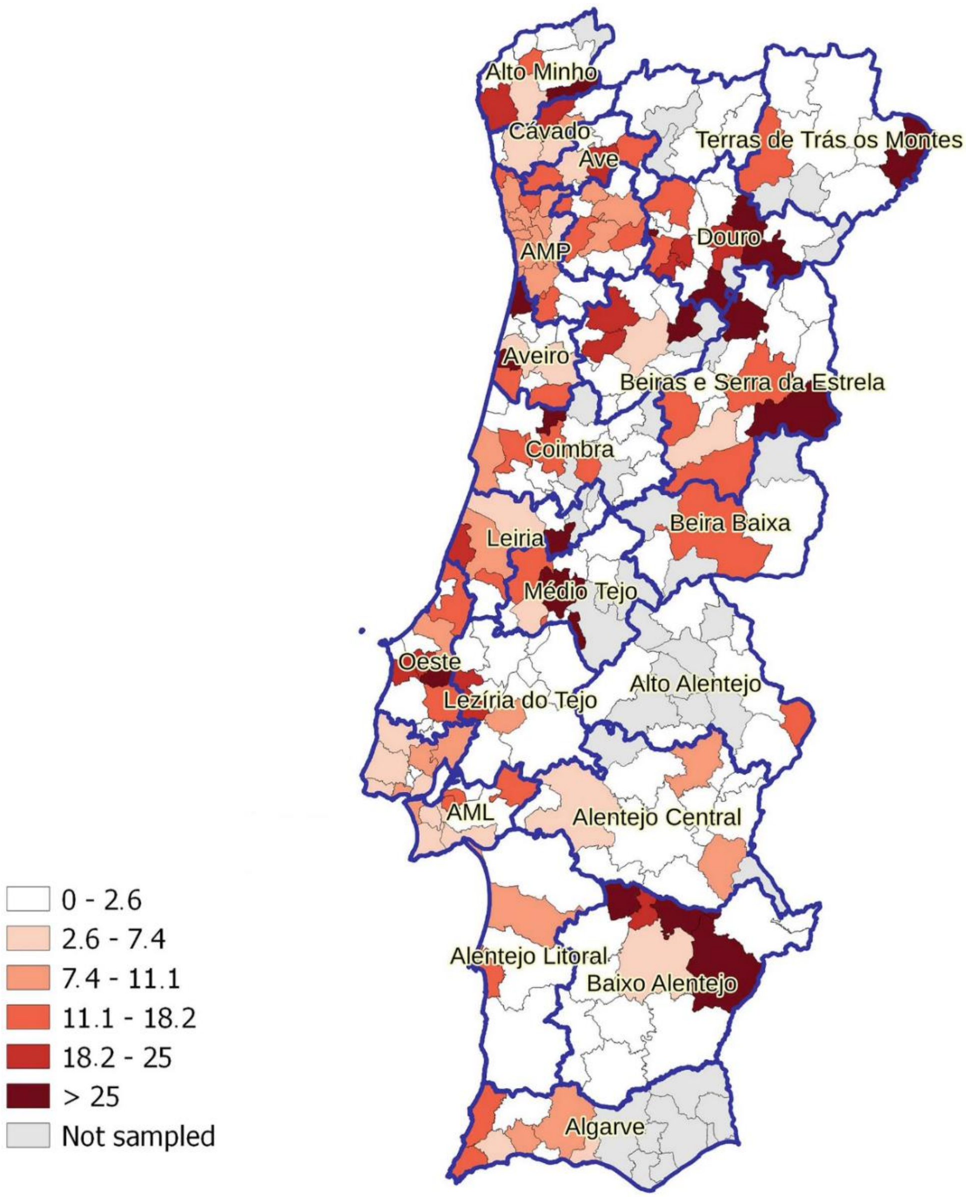
Figure 2. Distribution of estimated true seroprevalence values (%) by NUTS3 region.



Abbreviations: AML - Área Metropolitana de Lisboa; AMP - Área Metropolitana do Porto

Global estimated true seroprevalence was 4.8%. At the NUTS3 level, values ranged from 0.0 to 9.2%, with the highest seroprevalence in the Médio Tejo, Alto Minho, and BSE regions. There was a statistically significant difference in the proportion of positive results between NUTS2 regions (Chi-square test, $\chi 2 = 17.7$, df = 4, $P = 0.001$), but this difference was not significant between NUTS3 regions within the Norte, Centro, and Alentejo NUTS2 regions (P-values of 0.519, 0.949, and 0.896, respectively). To allow a more detailed analysis of the geographical distribution of possible exposure to *Leishmania*, the percentage of positive or borderline samples was calculated by municipality and is presented in Fig. 3. Municipalities where over 20 samples were collected (n = 53) with the highest percentages (> 13%) were (by descending order) Ílhavo, Viana do Castelo, Oliveira de Azeméis, Penafiel, Alcobaça, Alenquer, Moita, Póvoa de Varzim, Vila Real, and Paços de Ferreira.

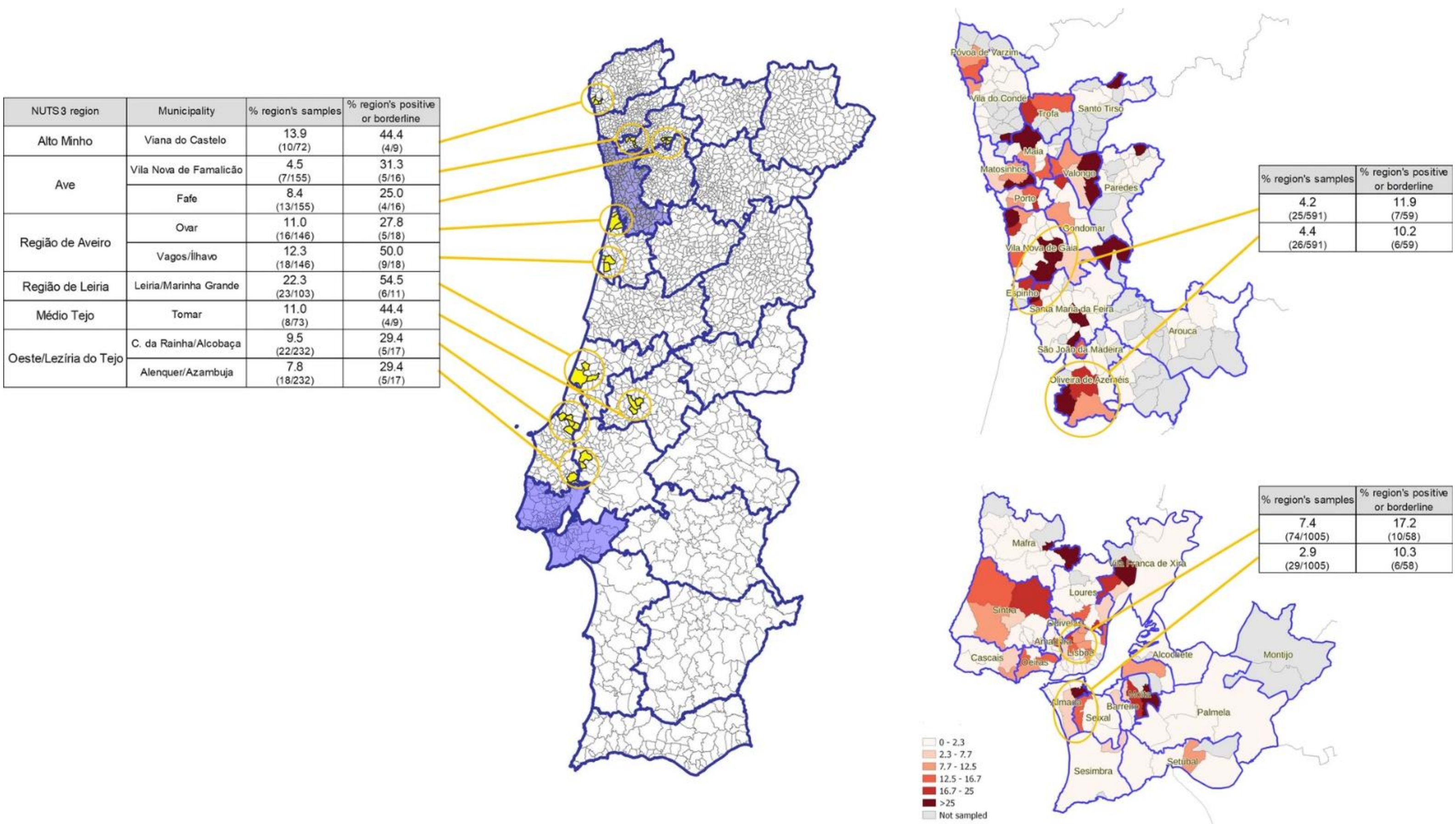
Figure 3. Percentage of positive or borderline samples by municipality (natural breaks were used to define categories).



Abbreviations: AML - Área Metropolitana de Lisboa; AMP - Área Metropolitana do Porto

To understand possible clustering of positive/borderline cases at a more local level, percentage by parish is illustrated for the AML and “Área Metropolitana do Porto” (AMP) NUTS3 region in Fig. 4. For other NUTS3 regions, geographical coverage to such a finer detail was not possible; however, some groups of contiguous parishes represented a high percentage of the total number of positive/borderline samples within their respective NUTS3 region (represented in yellow in Fig. 4).

Figure 4. Percentage of positive or borderline samples, in each parish of the “Área Metropolitana de Lisboa” and “Área Metropolitana do Porto” regions (right) and in some selected groups of parishes in other NUTS3 regions.



NUTS3 region	Municipality	% region's samples	% region's positive or borderline
Alto Minho	Viana do Castelo	13.9 (10/72)	44.4 (4/9)
Ave	Vila Nova de Famalicão	4.5 (7/155)	31.3 (5/16)
	Fafe	8.4 (13/155)	25.0 (4/16)
Região de Aveiro	Ovar	11.0 (16/146)	27.8 (5/18)
	Vagos/Ílhavo	12.3 (18/146)	50.0 (9/18)
Região de Leiria	Leiria/Marinha Grande	22.3 (23/103)	54.5 (6/11)
Médio Tejo	Tomar	11.0 (8/73)	44.4 (4/9)
Oeste/Lezíria do Tejo	C. da Rainha/Alcobaça	9.5 (22/232)	29.4 (5/17)
	Alenquer/Azambuja	7.8 (18/232)	29.4 (5/17)

% region's samples	% region's positive or borderline
4.2 (25/591)	11.9 (7/59)
4.4 (26/591)	10.2 (6/59)

% region's samples	% region's positive or borderline
7.4 (74/1005)	17.2 (10/58)
2.9 (29/1005)	10.3 (6/58)

3.3. Knowledge, perceptions, and practices

Answers to individual KPP questions are summarized in Table 3 (more detailed information by NUTS3 region is presented in the Additional file 5: Table S4).

Table 3. Answers to knowledge, perceptions, and practices questions, globally and by NUTS2 region.

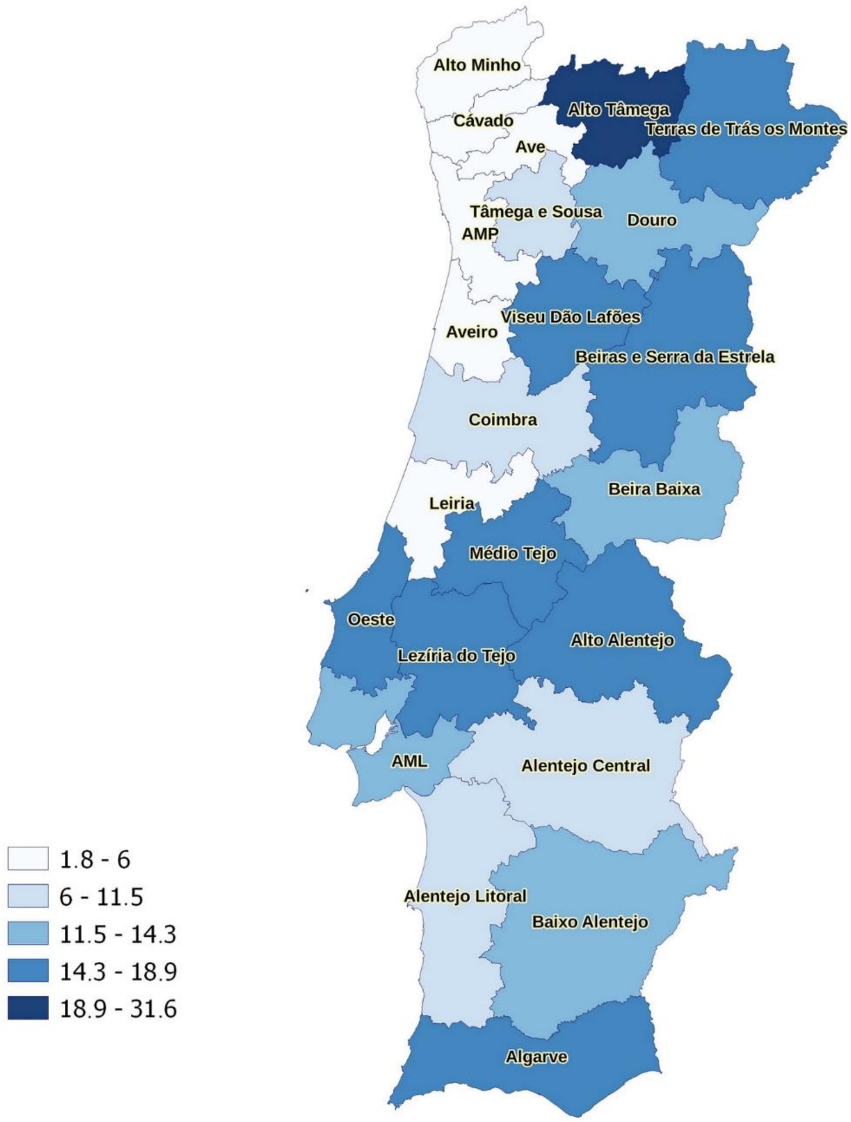
	Global (%)	Norte (%)	Centro (%)	AML (%)	Alentejo (%)	Algarve (%)	*p*-value
Total (*n*)	3763	1284	769	1006	462	242	
Heard of leishmaniasis	72.3 (2704/3740)	61.9 (791/1277)	78.1 (596/763)	77.5 (777/1002)	78.1 (357/457)	75.9 (183/241)	<0.001* ($\chi2$ = 103.9, df =4)
Source of information							
Television	53.2 (1406/2643)	61.0 (469/769)	52.3 (307/587)	51.2 (386/754)	49.7 (175/352)	38.1 (69/181)	
Veterinarian	48.1 (1273/2643)	39.5 (304/769)	48.9 (287/587)	52.0 (392/754)	52.8 (186/352)	57.5 (104/181)	
Cause							
Infectious	68.8 (1817/2640)	66.5 (517/777)	71.8 (416/579)	69.6 (530/761)	67.2 (231/344)	68.7 (123/179)	0.685 ($\chi2$ = 5.1, df =4)
Route of transmission							
DK/CR	19.5 (526/2704)	21.0 (166/791)	18.0 (107/596)	19.3 (150/777)	20.7 (74/357)	15.8 (29/183)	
Knows – Arthropod bite	88.2 (1922/2178)	88.3 (552/625)	89.8 (439/489)	86.8 (544/627)	86.9 (246/283)	91.6 (141/154)	
Knows – Sand fly bite	13.5 (260/1922)	12.7 (70/552)	13.7 (60/439)	15.9 (92/544)	11.8 (29/246)	6.4 (9/141)	
Knows – Contact with animals	19.6 (426/2178)	20.6 (129/625)	19.4 (95/489)	20.1 (126/627)	19.4 (55/283)	13.6 (21/154)	
Affects animals	91.0 (2451/2693)	86.3 (676/783)	93.6 (557/595)	93.2 (722/775)	93.0 (332/357)	89.6 (164/183)	<0.001* ($\chi2$ = 32.4, df =4)
Clinical signs in animals							
DK/CR	46.5 (1139/2451)	49.4 (334/676)	44.2 (246/557)	48.8 (352/722)	43.4 (144/332)	38.4 (63/164)	
Knows – Weight loss	52.3 (686/1312)	43.9 (150/342)	49.8 (155/311)	56.2 (208/370)	64.9 (122/188)	50.5 (51/101)	
Knows – Skin lesions	48.2 (633/1312)	51.5 (176/342)	45.3 (141/311)	51.6 (191/370)	40.4 (76/188)	48.5 (49/101)	
Treatable in animals	62.8 (1397/2226)	63.7 (393/617)	63.3 (317/501)	59.0 (393/666)	64.6 (192/297)	70.3 (102/145)	0.032* ($\chi2$ = 10.4, df =4)
Lethal in animals	72.1 (1487/2063)	62.3 (336/539)	74.5 (350/470)	74.4 (472/634)	75.4 (215/285)	84.3 (113/134)	<0.001* ($\chi2$ = 40.1, df =4)
Preventable in animals	78.0 (1605/2059)	74.8 (415/555)	77.9 (363/466)	78.1 (489/626)	80.1 (221/276)	86.0 (117/136)	0.057 ($\chi2$ = 9.2, df =4)
Present in Portugal	86.1 (2081/2418)	82.2 (544/662)	86.2 (473/549)	87.8 (628/715)	88.1 (289/328)	90.2 (148/164)	0.010* ($\chi2$ = 13.8, df =4)
Species affected							
DK/CR	6.9 (144/2081)	8.1 (44/543)	5.5 (26/473)	7.6 (48/628)	5.5 (16/289)	6.8 (10/148)	
Knows – Dogs	97.4 (1893/1937)	97.6 (487/499)	96.6 (432/447)	97.6 (566/580)	96.7 (264/273)	100 (138/138)	
Knows – Cats	32.6 (632/1937)	43.1 (215/499)	33.3 (149/447)	28.8 (167/580)	26.0 (71/273)	21.7 (30/138)	
Affected animals close to household	11.6 (240/2069)	6.4 (34/534)	12.2 (57/468)	14.0 (88/630)	13.5 (38/281)	15.8 (23/146)	<0.001* ($\chi2$ = 22.4, df =4)
Vaccine available	52.2 (1092/2090)	43.7 (238/545)	53.6 (253/472)	54.6 (347/636)	56.3 (161/286)	62.0 (93/150)	<0.001* ($\chi2$ = 25.3, df =4)
Possible importation	61.6 (1477/2399)	58.9 (387/657)	61.5 (334/543)	64.6 (460/712)	58.5 (189/323)	65.2 (107/164)	0.174 ($\chi2$ = 7.0, df =4)
Affects humans	53.8 (1433/2666)	56.4 (438/776)	51.9 (305/588)	51.6 (396/767)	56.7 (200/353)	51.6 (94/182)	0.333 ($\chi2$ = 6.0, df =4)
Organs affected							
DK/CR	57.1 (818/1433)	55.7 (244/438)	56.4 (172/305)	60.1 (238/396)	58.0 (116/200)	51.1 (48/94)	
Knows – Skin	61.8 (380/615)	69.6 (135/194)	63.9 (85/133)	62.0 (98/158)	47.6 (40/84)	50.0 (23/46)	
Knows – Liver/spleen	37.7 (232/615)	30.9 (60/194)	33.1 (44/133)	48.7 (77/158)	46.4 (39/84)	28.3 (13/46)	

Treatable in humans	55.6 (772/1388)	59.6 (251/421)	56.8 (168/296)	52.2 (206/395)	53.2 (99/186)	53.3 (48/90)	0.652 (χ2 = 5.4, df =4)
Lethal in humans	35.4 (438/1236)	32.3 (118/365)	38.2 (99/259)	32.9 (118/359)	40.6 (69/170)	41.0 (34/83)	0.295 (χ2 = 6.5, df =4)
Preventable in humans	56.2 (705/1254)	58.2 (217/373)	58.1 (151/260)	53.0 (194/366)	55.5 (96/173)	57.3 (47/82)	0.222 (χ2 = 6.7, df =4)
Present in Portugal	78.7 (1135/1442)	77.6 (339/437)	84.6 (259/306)	74.5 (304/408)	78.7 (155/197)	83.0 (78/94)	0.042* (χ2 = 12.1, df =4)
Possible importation	70.2 (1005/1432)	72.1 (313/434)	72.9 (223/306)	68.2 (274/402)	66.8 (131/196)	68.1 (64/94)	0.450 (χ2 = 103.9, df =4)
Regular contact with wild animals	4.3 (144/3387)	2.6 (30/1156)	5.9 (40/673)	3.0 (28/925)	8.1 (34/419)	5.6 (12/214)	<0.001* (χ2 = 32.1, df =4)
Regular contact with domestic animals	70.5 (2524/3578)	70.0 (848/1212)	77.7 (565/727)	63.6 (616/969)	74.1 (326/440)	73.6 (170/231)	<0.001* (χ2 = 44.7, df =4)
Regular nighttime outdoor activities	24.2 (825/3409)	19.7 (229/1162)	28.4 (195/686)	23.1 (214/928)	30.4 (127/418)	27.8 (60/216)	<0.001* (χ2 = 30.2, df =4)
Nets in some/all windows/doors	23.7 (848/3572)	12.9 (157/1213)	25.2 (184/730)	23.0 (221/962)	45.0 (198/440)	38.6 (88/228)	<0.001* (χ2 = 217.0, df =4)
Dog ownership	48.1 (1775/3688)	46.3 (578/1248)	57.6 (433/752)	39.5 (394/997)	55.4 (251/453)	50.2 (119/237)	<0.001* (χ2 = 67.6, df =4)
Dog outdoors during nighttime	63.8 (1055/1653)	60.5 (321/531)	69.2 (277/400)	63.7 (239/375)	62.9 (151/240)	62.0 (67/108)	0.082 (χ2 = 8.3, df =4)
Use of repellents/insecticides	82.2 (1320/1605)	80.7 (413/512)	83.2 (326/392)	86.2 (312/362)	80.9 (190/235)	76.2 (80/105)	0.096 (χ2 = 7.9, df =4)
Spot-on	55.0 (726/1320)	60.0 (248/413)	58.0 (189/326)	51.0 (159/312)	45.8 (87/190)	55.0 (44/80)	
Collar	40.9 (540/1320)	32.0 (132/413)	40.5 (132/326)	49.0 (153/312)	47.9 (91/190)	40.0 (32/80)	
All year round	62.8 (829/1320)	63.2 (261/413)	59.5 (194/326)	66.7 (208/312)	58.9 (112/190)	67.5 (54/80)	0.090 (χ2 = 5.5, df =4)
Regular veterinarian follow-up	90.6 (1472/1625)	89.3 (466/522)	87.9 (348/396)	95.1 (349/367)	88.9 (209/235)	95.3 (101/106)	0.002* (χ2 = 16.8, df =4)
Use of vaccine against canine leishmaniasis every year	21.7 (385/1775)	14.4 (83/578)	20.6 (89/433)	28.2 (111/394)	25.5 (64/251)	31.9 (38/119)	<0.001* (χ2 = 37.9, df =4)

Abbreviations: AML - Área Metropolitana de Lisboa; DK/CR - Don't Know/Can't remember
*Statistically significant

Globally, 72.3% of blood donors said they had previously heard of leishmaniasis, varying significantly among NUTS2 regions (Chi-square test, χ2 = 103.9, df = 4, *P* < 0.001) (lower in the Norte region, also with marked intra-regional variations: from 58.1% in the Ave to 81.0% in the Douro region). The most commonly reported sources of information about leishmaniasis were television (53.2%) and conversation with a veterinarian (48.1%). Television was reported as the predominant source in littoral NUTS3 regions of the Norte and Centro, whereas conversation with a veterinarian clearly dominated in the Algarve. Other sources are summarized in Additional file 7: Fig. S3a. Most people who admitted having previously heard of leishmaniasis (68.8%) recognized the infectious cause of the disease, and approximately 80% reported knowing the route of transmission: arthropod bite was most commonly selected, but direct contact with animals was simultaneously or alternatively selected in a significant proportion of cases (approximately 20%). Mosquitoes were the arthropods most commonly associated with transmission (only 13.5% of respondents selected sand flies). Other routes of transmission are summarized in Additional file 7: Fig. S3b. Understanding of routes of transmission was similar across different NUTS2 and NUTS3 regions. Most donors who admitted having previously heard of leishmaniasis (91.0%) recognized the disease affects animals, but only 53.8% admitted it could be a human disease. Only around 55%/45% of donors admitted knowing signs or symptoms of disease in animals/humans, respectively; in animals, weight loss (52.3%) and skin lesions (48.2%) were the signs most often selected (with skin lesions favored in the Norte and weight loss in the rest of the country); in humans, involvement of the skin was the most commonly recognized presentation of disease (61.8%). Other signs/symptoms of disease reported are represented in Additional file 7: Fig. S3c, d. Leishmaniasis was assumed as a lethal disease much more commonly for animals (72.1%) than for humans (35.4%), although knowledge of lethality in animals differed significantly among regions and was lower in the Norte (Chi-square test, $\chi2 = 40.1$, df = 4, $P < 0.001$). The disease was generally reported as treatable and preventable both in animals and in humans, but more donors admitted it could be prevented in animals (78.0%) than in humans (56.2%). Only around 50% of donors recognized vaccination as a possible prevention strategy in animals; knowledge regarding vaccination was significantly higher in the Algarve and lower in the Norte region (Chi-square test, $\chi2 = 25.3$, df = 4, $P < 0.001$). Disease was considered endemic in Portugal in animals (86.1%) or in humans (78.7%) by most donors who were aware these groups could be affected. In Portugal, dogs were almost universally pointed as the animals affected by leishmaniasis (97.4%), followed by cats (32.6%). Among participants who knew the disease was endemic in animals in Portugal, 11.6% assumed there were or had been diseased animals in the household or nearby. This percentage was significantly different between NUTS3 regions (Chi-square test, $\chi2 = 22.4$, df = 4, $P < 0.001$), ranging from 1.8% (Ave) to 31.6% (Alto Tâmega), as shown in Fig. 5. Only four donors stated they knew people with leishmaniasis nearby: three in the AML and one in the Alto Alentejo.

Figure 5. Percentage of donors reporting knowing animals with leishmaniasis in the household or nearby, by NUTS3 region.



Abbreviations: AML - Área Metropolitana de Lisboa; AMP - Área Metropolitana do Porto

Concerning practices, contact with domestic animals was commonly reported (70.5%) and almost half the donors were dog owners, being both significantly more common in the Centro and Alentejo regions. Regular follow-up of dogs (at least once a year) by a veterinarian was indicated by 90.6% of owners (higher in the Algarve and the AML, Chi-square test, $\chi 2 = 16.8$, df = 4, $P = 0.002$) but only 21.7% reported yearly vaccination of dogs against leishmaniasis. Vaccine uptake differed significantly between regions (higher in the

Algarve and the AML regions, Chi-square test, $\chi 2 = 37.9$, df = 4, $P < 0.001$). Over 80% of owners mentioned applying arthropod repellents or insecticides in the dog(s) and no statistically significant difference was found across NUTS2 regions (Chi-square test, $\chi 2 = 7.9$, df = 4, $P = 0.096$); spot-on was the most commonly identified (globally, 55.0%—and in every NUTS2 region, except for the Alentejo), followed by collar (40.9%); other types of applications are presented in Additional file 7: Fig. S3e. Use of these products all year round was reportedly common practice (62.8%) in all NUTS2 regions (Chi-square test, $\chi 2 = 5.5$, df = 4, $P = 0.090$). Regarding protection measures, 23.7% of donors reported having nets in some or all the windows/doors of their household–this practice was significantly more common in the Alentejo and the Algarve regions (Chi-square test, $\chi 2 = 217.0$, df = 4, $P < 0.001$). Practice of outdoor activities between dusk and dawn, on the other hand, was reported by 24.2% and less common in the Norte and AML regions (Chi-square test, $\chi 2 = 30.2$, df = 4, $P < 0.001$). Regarding perceptions, classification of risk of leishmaniasis for animals was significantly different between donors of different NUTS2 regions (Chi-square test, $\chi 2 = 38.4$, df = 12, $P < 0.001$)—the Algarve was the region where "high" and "medium" risk were selected in the highest percentages, as presented in Fig. 6. Median values for individual K and Pra scores and mean values for individual Per score by NUTS2 and NUTS3 regions are presented in Table 4 and Fig. 7, respectively. The mean value was used to summarize the Per score by region since the median value was 0 in all but four NUTS3 regions.

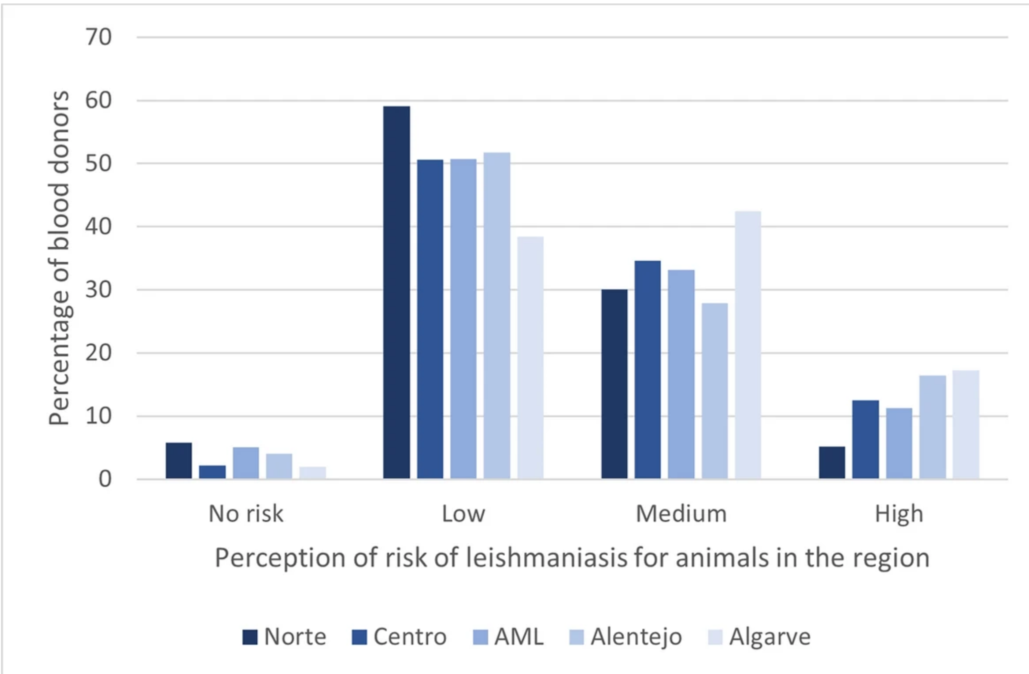
Table 4. Distribution of knowledge (K), perceptions (Per), and practices (Pra) scores globally and by NUTS2 region.

	Global	Norte	Centro	AML	Alentejo	Algarve	*p*-value
Total (*n*)	3763	1285	768	1005	463	242	
K score - median (IQR)	7 (0-10.75)	4.5 (0-9.25)	8 (2-11.25)	8 (1-11)	8 (1-11)	7.6 (1-11.31)	<0.001* (H = 115.5, df = 4)
Per score - mean (±SD)	0.78 (0-1.92)	0.55 (0-1.52)	0.93 (0-2.18)	0.84 (0-1.97)	0.95 (0-2.18)	0.98 (0-2.24)	<0.001* (H = 86.6, df = 4)
Pra score - median (IQR)	3.5 (2.5-4.5)	3.5 (2.5-4.5)	3.25 (2.44-4)	4 (3-5)	3.5 (2.5-4.5)	3.62 (2.75-4.5)	<0.001* (H = 64.2, df = 4)

Abbreviations: AML - Área Metropolitana de Lisboa; K - Knowledge; Per - Perceptions; Pra - Practices; IQR - Interquartile Range; SD - Standard Deviation

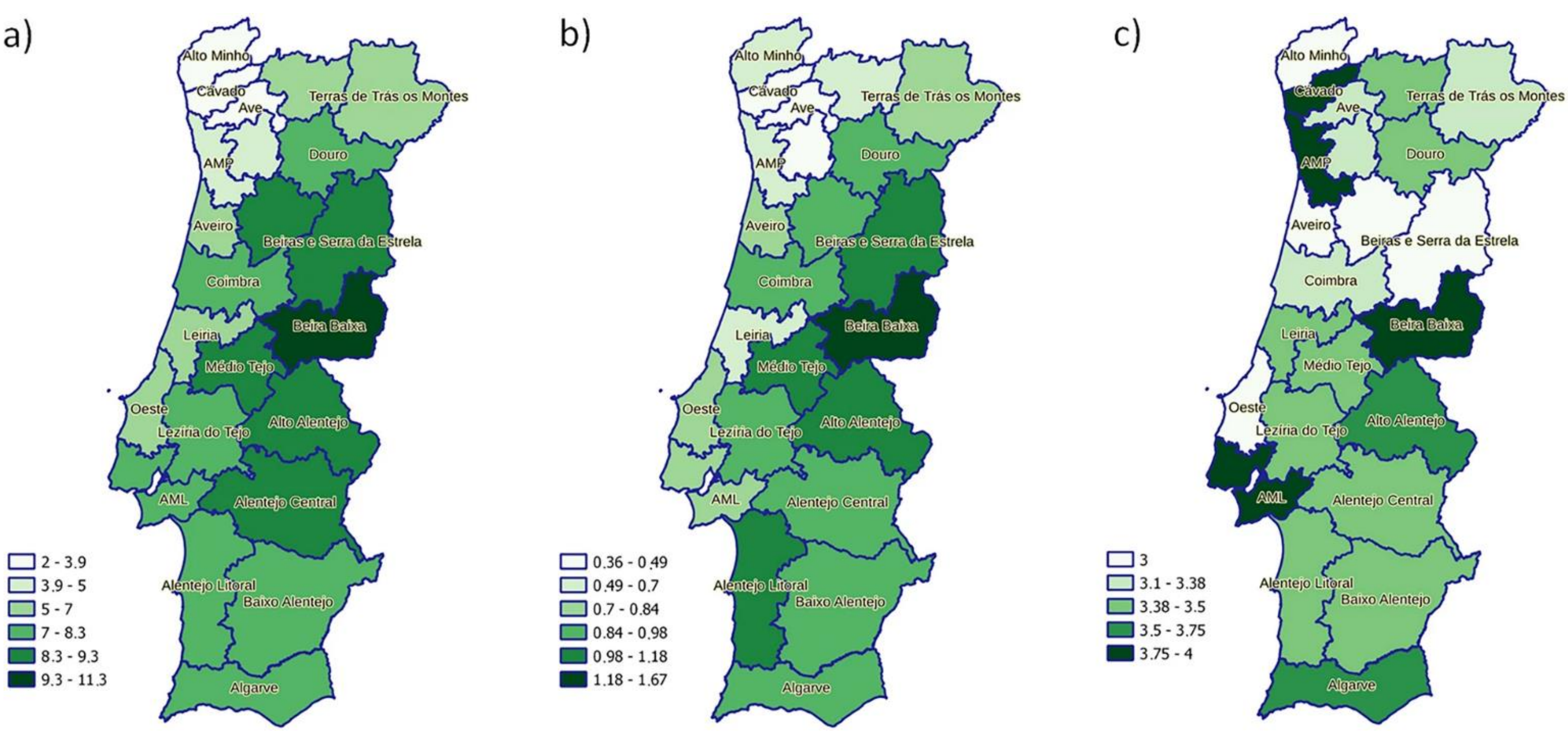
*Statistically significant

Figure 6. Perception of risk of leishmaniasis for animals in the region, according to NUTS2 region of residence.



Abbreviations: AML - Área Metropolitana de Lisboa

Figure 7. Distribution by NUTS3 region of: a) knowledge score (median), b) perceptions score (mean), c) practices score (median) (natural breaks were used to define categories).



The median K score was lowest in the Norte region, especially in the coastal NUTS3 regions. Higher median K scores were obtained in the Centro and Alentejo regions, especially in the following NUTS3 regions (by descending order): Beira Baixa, Alto Alentejo, Médio Tejo, Alentejo Central, BSE, and Viseu Dão-Lafões. Higher mean Per scores largely overlapped with higher median K scores. On the other hand, median Pra scores were highest in the AML, Beira Baixa, and AMP, and lowest in the BSE, Viseu Dão-Lafões, Aveiro, Oeste, and Alto Minho regions. The distribution of individual K, Per, and Pra scores is represented in Additional file 6: Fig. S2.

3.4. Associations between sociodemographic, knowledge, perceptions, and practices variables and asymptomatic infection

In univariate analysis, residing in the Centro region (considering the Alentejo as a reference category), male sex, not having previously heard of leishmaniasis, lower K score, and lower Per score were significantly associated with a positive serological result (Table 5). Higher seropositivity rates were also associated with those aged over 25 years; military, agriculture, and industry workers; and absence of mosquito nets in windows/doors, although these did not reach statistical significance. In multivariate analysis, factors associated with positive serological result were male sex (OR 1.75, 95% CI 1.30–2.38, $P < 0.001$), residing in the Centro region (OR 1.43, 95% CI 1.02–2.00, $P = 0.039$), and age over 25 years (OR 1.79, 95% CI 1.07–2.97, $P = 0.026$) (Table 6).

Table 5. Distribution of participants and positive serological results by category, for sociodemographic and knowledge, perceptions, and practices variables.

Variables	Categories	Samples, % (*n*)	Seropositive donors, % (*n*)	*p* value
Sex	Male	49.8 (1822/3655)	7.1 (129/1822)	**<0.001*** (χ2 = 17.5, df=1)
	Female	50.2 (1833/3665)	3.9 (72/1833)	
Age (y)	18-25	14.4 (513/3567)	3.3 (17/513)	0.127 (χ2 = 7.1, df=4)
	26-35	20.6 (734/3567)	5.6 (41/734)	
	36-45	30.2 (1077/3567)	5.8 (62/1077)	
	46-55	24.6 (876/3567)	6.6 (58/876)	
	56-65	10.3 (367/3567)	6.3 (23/367)	
Level of education [a]	1-4	1.9 (68/3565)	10.3 (7/68)	0.258 (FET = 5.3)
	5-9	16.8 (598/3565)	5.7 (34/598)	
	10-12	44.0 (1570/3565)	5.4 (85/1570)	
	Bachelor's	25.8 (920/3565)	5.9 (54/920)	
	MSc/PhD	11.5 (409/3565)	3.9 (16/409)	
Occupation [b]	Student	9.6 (279/2906)	3.2 (9/279)	
	Retired	1.9 (56/2906)	7.1 (4/56)	
	Unemployed	3.4 (100/2906)	8.0 (8/100)	
	0	1.9 (56/2906)	8.9 (5/56)	0.173 (χ2 = 4.4, df=3)
	1-3	39.4 (1144/2906)	5.2 (60/1144)	
	4-5	25.7 (747/2906)	4.8 (36/747)	
	6-9	18.0 (524/2906)	7.1 (37/524)	
Travel abroad to endemic country [c] (<2y previously)	Yes	58.6 (439/749	5.2 (23/439)	0.739 (χ2 = 0.11, df=1)
	No	41.4 (310/749)	5.8 (18/310)	
Type of parish of residence	Non-rural	58.3 (2133/3661)	5.5 (118/2133)	0.903 (χ2 = 0.02, df=1)
	Rural	41.7 (1528/3661)	5.4 (83/1528)	

Heard of leishmaniasis	Yes	72.4 (2638/3643)	4.9 (130/2638)	**0.037*** ($\chi2$ = 4.3, df=1)
	No/DK/CR	27.6 (1005/3643)	6.7 (67/1005)	
Animals affected by leishmaniasis nearby	Yes	11.7 (235/2017)	4.7 (11/235)	0.878 ($\chi2$ = 0.02, df=1)
	No/DK/CR	88.3 (1782/2017)	4.9 (87/1782)	
Regular contact with domestic animals	Yes	70.5 (2457/3486)	5.2 (127/2457)	0.419 ($\chi2$ = 0.63, df=1)
	No	29.5 (1029/3486)	5.8 (60/1029)	
Regular contact with wild animals	Yes	4.2 (139/3301)	2.9 (4/139)	0.180 ($\chi2$ = 1.7, df=1)
	No	95.8 (3162/3301)	5.4 (172/3162)	
Practice of outdoor activities during nighttime	Yes	24.1 (801/3321)	5.4 (43/801)	0.921 ($\chi2$ = 0.01, df=1)
	No	75.9 (2520/3321)	5.4 (137/2520)	
Use of nets in windows/doors	Yes (all/some)	23.8 (830/3481)	4.3 (36/830)	0.138 ($\chi2$ = 2.2, df=1)
	None	76.2 (2651/3481)	5.7 (150/2651)	
Ownership of dog(s)	Yes	48.1 (1727/3590)	5.5 (95/1727)	0.937 ($\chi2$ = 0.01, df=1)
	No	51.9 (1863/3590)	5.4 (101/1863)	
K score	≤7	50.7 (1858/3664)	6.4 (118/1858)	**0.012*** ($\chi2$ = 6.2, df=1)
	>7	49.3 (1806/3664)	4.5 (81/1806)	
Per score	<1	59.7 (2186/3664)	6.3 (137/2186)	**0.006*** ($\chi2$ = 7.4, df=1)
	≥1	40.3 (1478/3664)	4.2 (62/1478)	
Pra score	≤3,5	52.0 (1901/3664)	5.5 (104/1901)	0.937 ($\chi2$ = 0.01, df=1)
	>3,5	48.0 (1763/3664)	5.4 (95/1763)	

[a] Categories refer to number of years completed of formal school education
[b] Category numbers refer to the numbers of the categories in the classification of European Skills, Competences, and Occupations
[c] Albania, Brazil, Bulgaria, Cyprus, Eritrea, Ethiopia, Greece, India, Italy, Kenya, Malta, Nepal, Somalia, South Sudan, Spain, Sudan, Yemen

Abbreviations: K - Knowledge; Per - Perceptions; Pra - Practices; y - years; MSc - Master of Science; PhD - Doctor of Phylosophy; DK/CR - Don't Know/Can't Remember
*Statistically significant

Table 6. Potential risk factors for asymptomatic *Leishmania* infection, according to logistic regression models to estimate crude and adjusted odds ratio values.

Potential Risk Factor	Univariate			Multivariate		
	% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
Older than 25y	85.6	1.88	1.13-3.12	1.79	1.07-2.97	**0.026***
Male sex	49.9	1.85	1.39-2.50	1.75	1.30-2.38	**<0.001***
Residing in Centro	20.4	1.37	0.99-1.91	1.43	1.02-2.00	**0.039***
Not heard of leishmaniasis	27.6	1.39	1.02-1.89	1.09	0.66-1.82	0.737
K score ≤ 7	50.7	1.39	1.04-1.85	1.14	0.68-1.89	0.621
Per score <1	59.6	1.56	1.09-2.27	1.41	0.93-2.13	0.097
Constant				0.072		<0.001
Hosmer and Lemeshow Test				Sig.=0.936		

Abbreviations: y - years; K - Knowledge; Per - Perceptions; OR - odds ratio; CI - confidence interval
*Statistically significant

3.5. Associations between sociodemographic variables and knowledge, perceptions, and practices

Univariate analysis of sociodemographic variables associated with higher than median K, Per, or Pra score is listed in Additional file 8: Table S5. In multivariate analysis, variables associated with above median K score were female sex (OR 1.23, 95% CI 1.05–1.43, P = 0.010), age between 25 and 40 years (OR 1.23, 95% CI 1.04–1.44, P = 0.013), residing outside the Norte region (OR 1.85, 95% CI 1.56–2.17, P < 0.001), ownership of dogs (OR 2.03, 95% CI 1.74–2.38, P < 0.001), higher education (OR 1.85, 95% CI 1.54–2.24, P < 0.001) and working as a military, manager, professional or technician (OR 1.44, 95% CI 1.20–1.73, P < 0.001). Factors associated with above median Per score were above median K score (OR 25.66, 95% CI 20.57–32.02, P < 0.001), residing outside the Norte region (OR 1.41, 95% CI 1.14–1.72, P = 0.002) and ownership of dogs (OR 1.37, 95% CI 1.12–1.67, P = 0.002). Lastly, even though the multivariate logistic model for Pra score was not statistically satisfactory (HL: P < 0.05), suggested factors associated with above median Pra score as follows: lower than median Per score (OR 1.35, 95% CI 1.16–1.59, P < 0.001), residing outside the Centro region (OR 1.52, 95% CI 1.25–1.85, P < 0.001), in a non-rural parish (OR 1.64, 95% CI 1.41–1.92, P < 0.001), and working as a military, manager, professional or technician (OR 1.29, 95% CI 1.08–1.55, P = 0.005) (Table 7).

Table 7. Potential factors for above median K, Per, or Pra score, according to logistic regression models to estimate crude and adjusted odds ratio values.

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
K >7	Age 25-40y	35.7	1.32	1.15-1.51	1.23	1.04-1.44	**0.013***
	Female sex	50.2	1.27	1.12-1.44	1.23	1.05-1.43	**0.010***
	Residing outside Norte	65.9	1.96	1.69-2.22	1.85	1.56-2.17	**<0.001***
	Ownership of dogs	48.1	1.89	1.66-2.15	2.03	1.74-2.38	**<0.001***
	Higher education [a]	37.3	2.19	1.91-2.51	1.85	1.54-2.24	**<0.001***
	Profession group 0-3 [b]	41.0	1.95	1.68-2.26	1.44	1.20-1.73	**<0.001***
	Constant				0.510		<0.001
	Hosmer and Lemeshow Test					Sig.=0.568	
	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
Per ≥1	Age 25-40	35.7	1.30	1.13-1.49	1.18	0.97-1.45	0.106
	Female sex	50.2	1.22	1.07-1.39	0.98	0.81-1.20	0.865
	Residing outside Norte	65.9	1.89	1.64-2.17	1.41	1.14-1.72	**0.002***
	Ownership of dogs	48.1	1.88	1.65-2.15	1.37	1.12-1.67	**0.002***
	Higher education [a]	37.3	1.80	1.57-2.07	1.13	0.89-1.43	0.328
	Profession group 0-3 [b]	41.0	1.51	1.30-1.75	0.88	0.70-1.12	0.297
	K score >7	49.3	31.34	25.81-38.06	25.66	20.57-32.02	**<0.001***
	Constant				0.093		<0.001
	Hosmer and Lemeshow Test					Sig.=0.379	
	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
Pra >3.5	Age 25-40	35.7	1.03	0.90-1.17	0.89	0.76-1.04	0.153
	Female sex	50.2	1.06	0.93-1.20	1.07	0.92-1.24	0.379
	Residing outside Centro	79.6	1.72	1.47-2.04	1.52	1.25-1.85	**<0.001***
	Non-rural parish	58.0	1.72	1.52-1.96	1.64	1.41-1.92	**<0.001***
	Higher education [a]	37.3	1.40	1.22-1.60	1.17	0.97-1.41	0.094
	Profession group 0-3 [b]	41.0	1.44	1.25-1.67	1.29	1.08-1.55	**0.005***
	Per score <1	59.8	1.23	1.09-1.41	1.35	1.16-1.59	**<0.001***
	Constant				1.244		0.007
	Hosmer and Lemeshow Test					Sig.=0.039	

[a] Completed bachelor's degree or above
[b] Categories 0-3 in the classification of European Skills, Competences, and Occupations: armed forces, managers, professionals, techinicians
Abbreviations: y - years; K - Knowledge; Per - Perceptions; Pra - Practices; OR - odds ratio; CI - confidence interval
*Statistically significant

4. Discussion

This study represents the first nationwide *Leishmania* human seroprevalence study in Portugal. National true seroprevalence was estimated at 4.8%, with regional values ranging from 0.0 to 9.2%. Previous small, regional studies (AML and BSE regions) have shown seroprevalence of 0.0–2.0% in asymptomatic individuals [14, 15]. These studies not only used different serological techniques (IFAT and Direct Agglutination Test [DAT], respectively), but also analyzed different populations (dog owners and outpatients in a tertiary hospital, respectively). In neighboring Spain, regional seroprevalence studies in blood donors have shown values between 1.3 and 7.9% [9, 12, 31–34]. The range of values of true seroprevalence estimated in the present study was similar to these Spanish studies. Studies in the general population of other Mediterranean countries have reported similar findings in Italy [35, 36] and Greece [37], although in some regions of Italy [38] and Croatia [39] seroprevalence exceeded 10%. Of note, studies in which each blood sample was simultaneously tested for the presence of anti-*Leishmania* antibodies and *Leishmania* nucleic acids generally showed low agreement between test results [9]. This raises the question of the biological meaning of the detection of antibodies against *Leishmania*, probably representing very diverse infection status, from previous exposure and elimination of the parasite to latency with undetectability of DNA in peripheral cells and to active replication at low, non-symptom-inducing levels. Large prospective studies in South Asia found no increased risk of progression to disease in asymptomatic seropositive people in general, except when baseline titers were very high or when seroconversion was recent (documented during follow-up) [40]. The absence of clear markers of progression to disease implies a currently limited clinical value of individually testing asymptomatic people, although doing so before programmed start of immunosuppressive therapy could lead to increased clinical and analytical monitoring of positive patients and thus to earlier diagnosis and treatment in case of progression to overt disease. In this sense, the results of the present study could indicate a possible role of screening patients living in areas where exposure is expected to be higher, such as the Médio Tejo and BSE regions. Another question raised is the potential of transmission of *Leishmania* by asymptomatic infected individuals, by blood donation; even though viable parasites and *Leishmania* DNA have been identified in donated blood, the risk of transmission has been considered absent or extremely low, when leucodepletion of donated blood is performed, as is current practice in Portugal [41, 42]. However, no single test has been suggested as a standard or defining the asymptomatic status and some authors would recommend combining serology and polymerase chain reaction (PCR) [10]. Even so, the several serological tests available were developed for diagnosing VL and often produce conflicting results in asymptomatic people [43, 44]. In the present study, only one serological test was used systematically, for all samples, and only one sample was tested per participant;

the 298 positive or borderline samples were tested simultaneously by IFAT and only one was considered positive by both techniques, although 117 had detectable antibodies but bellow cutoff for positivity (titers from 8 to 32), suggesting possible low-level exposure to *Leishmania*. These results were not used for statistical analysis, since no ELISA-negative samples were simultaneously tested by IFAT. It is worth mentioning that it was assumed the specificity of the ELISA kits used was 99% as reported by the manufacturer. Additional comparative studies showed specificity ≥ 98% of this test for diagnosing VL in the Mediterranean setting [45, 46], but it is likely that specificity for asymptomatic infection could not be directly extrapolated. Despite the many caveats for the use of serology for individual determination of asymptomatic infection status, the detection of antibodies to the parasite can be relevant from a public health perspective, especially when comparing the findings between different regions and by crossing the results with distribution of human VL cases and evidence from *Leishmania* in mammal hosts and vectors, following a One Health approach [25]. In Portugal, data on human cases drawn from public health surveillance in the period from 2013 to 2018, inclusively [3, 47], show that the NUTS3 regions with the highest incidence of VL cases (over 0.1/100000/year) were (in decreasing order) Alto Tâmega (0.396), Algarve, Alto Alentejo, Baixo Alentejo, Viseu Dão-Lafões, BSE, and Médio Tejo (0.146). No cases were detected in the following regions: Aveiro, Leiria, Oeste, Ave, Cávado, and Alentejo Litoral. However, data gathered via this passive surveillance system is probably incomplete, as suggested by a study of a previous period (1999–2009), where only 38.6% of cases diagnosed in hospitals were reported to the national surveillance system [4]. Additionally, it is likely that differences in incidence also represent a nonhomogeneous underreporting across regions, especially considering that leishmaniasis is a rare disease in Portugal, and in lesser populated regions, one additional case can represent an important increase in incidence. In the study focusing on 1999–2009, the highest incidence, based on hospital records, was described in the following regions: Douro, Baixo Alentejo, Médio Tejo, AML, BSE, Coimbra, and Viseu Dão-Lafões. All in all, this evidence suggests that Médio Tejo and BSE are among the regions of highest incidence, and these were among the regions with the highest seroprevalence in the present study. In the Algarve region, an increasing trend in incidence is suggested, especially considering most human cases between 2013 and

2018 (70%) were reported to public health surveillance in 2017 and 2018 [3]. In the present study, seroprevalence in the Algarve region was intermediate, but the eastern half of the region was not sampled, and, in the western part, sampling was based exclusively on a single central collection point. Although the suburban areas of Lisboa are classically considered endemic foci of leishmaniasis [48], the present study revealed intermediate seroprevalence in the AML. It is important to note, however, that incidence is related not only to exposure to *Leishmania*, but also to the presence of risk factors for progression to disease, which include immunosuppression, such as HIV infection. The two regions with higher incidence of HIV infection in most years between 2009 and 2018 were the AML and the Algarve [49], which could help explain the discrepancy between incidence of VL and *Leishmania* seroprevalence, compared to other regions of the country.

In the Alentejo region, seroprevalence was low in all NUTS3 regions, in contrast to human incidence data and canine seroprevalence studies for the Alto and Baixo Alentejo [16, 50]. One possible explanation is that in Alto Alentejo, approximately half the municipalities were insufficiently or not at all sampled, possibly including the ones where previous human cases have been detected. In the Baixo Alentejo region, sampling was based mostly on a single central collection point, meaning in some municipalities only a minority of parishes were sampled. On the other hand, in the Alto Minho and Aveiro regions, human VL cases diagnosed have been very scarce [4] and canine seroprevalence is consistently lower [16, 50] but human seroprevalence was among the highest. In these regions, a significant proportion of positive or borderline individuals were residents in one or a few groupings of a small number of contiguous parishes, possibly highlighting the very localized nature of *Leishmania* circulation, related to the limited mobility of the vector. This was probably also the case for the Ave, Leiria, and Oeste regions (Fig. 4). However, as internal travel within Portugal was not assessed in the present study, it cannot be ruled out that a seropositive result could be related to exposure in other parts of the country. In any case, these results raise the possibility of new foci of exposure to *Leishmania* in coastal and northern areas of Portugal (i.e., Ílhavo, Ovar, and Viana do Castelo) as a consequence of the expansion of the parasite due to global warming, as described in northwestern Spanish provinces previously considered non-endemic for leishmaniasis. However, autochthonous cases were first reported in the 1990s and since then several studies have detected seropositive dogs in the region [51–53]. In Portugal, two nationwide canine surveys performed 12 years apart, seroprevalence increased globally and in most regions; interestingly, some districts in the northern region showed marked (about three times) increases such as Porto (from 3.2% in 2009 to 9.2% in 2021) and Braga (from 2.1% in 2009 to 6.9% in 2021). In the present study, potential factors associated with seropositivity in multivariate analysis were male sex and older age. These findings are consistent with previous studies in the Mediterranean region [9, 13]. Older age could be related to cumulative exposure to *Leishmania*-infected sand flies [54]; no clear sociocultural explanation has been found in the present study for higher asymptomatic seroprevalence in the male sex. A higher incidence of disease in male sex has also been reported [55]; sex-related biological factors could play a role in the differential incidence of VL [56]. KPP results highlight that animal leishmaniasis is well-recognized among blood donors, especially in the central and southern parts of the country. Although dog owners had more frequently heard of the disease (78.2 vs. 68.0%), the percentage was still lower than described in previous studies in pet owners (83–91%) [20, 21], possibly because the proportion of participants from the Norte region was lower (26 vs. 34%) and also because these studies were performed in a veterinary clinic/hospital context. Human leishmaniasis, however, is less recognized (38.1%, very similar to a previous study [20]). Television was identified as the main source of information, possibly because leishmaniasis is mentioned in the insecticide and arthropod repellent advertisements broadcast. In many parts of the Norte region, where canine leishmaniasis is considered less endemic or non-endemic, it is expected, as seen in the present study, that television largely supersedes veterinary professionals as sources of information. Many respondents who had heard of leishmaniasis in animals were not able to describe any sign, and only approximately half of those who described them selected weight loss or skin lesions, which are commonly present in diseased dogs [57]. This could imply that tutors may theoretically recognize the risk of disease locally, but not be able to identify its signs early in their animals, leading to late seeking of veterinary attention. Leishmaniasis was mainly considered a dog's disease, but some donors also mentioned that cats could be affected. This could represent either a growing awareness in the veterinary and general community of the risk of feline leishmaniasis, following increasing scientific evidence [58], or a possible confusion with toxoplasmosis (supported in some questionnaires by the indication of hearing about it during pregnancy). It is interesting to note that regions where a higher percentage of donors reported knowing animals with leishmaniasis in the surroundings relate to the districts where canine seroprevalence was shown to be higher, namely Alto Alentejo, Médio Tejo, BSE, and Algarve [16]. The vaccination rate against canine leishmaniasis was higher than suggested in previous studies (21.7% vs. 4.5–14.9%) [16, 20], although awareness and use of vaccines were significantly different between regions. Compared to previous studies, similar rates of insecticide/repellent use in dogs were reported (82.2% vs. 69.6–92%) when considering all types of substances, regardless of their activity against sand flies [16, 20]. However, no information was collected regarding either the chemical substances or whether the products were being applied according to the manufacturer's recommendations. In the present study, a higher level of knowledge was associated with higher perception of leishmaniasis risk, but this did not translate into more protective practices. In fact, a higher Pra score was significantly associated with a lower Per score. Perhaps this could be partially explained by the fact that one of the practices considered protective was no ownership of dogs, but being a dog owner was significantly associated with a higher perception of disease. Additionally, vaccination was the only practice assessed specifically targeting leishmaniasis, and it is expensive; other practices considered protective against leishmaniasis are also protective for other arthropod-transmitted infections, so that they could be the result of the perception of risk of these other specific infections or of arthropod-borne infection in general. This could also explain why, in multivariate analysis, there was no significant association between the detection of anti-*Leishmania* antibodies and lower K, Per, or Pra scores related to leishmaniasis. These findings cannot be extrapolated to the general Portuguese mainland population, since only people aged 18–65 were included and the profile of people who donate blood could be different from the age-matched general population in each region and between regions. In addition, the representativeness even of the blood donor population itself could have been affected by the difficulty in obtaining a truly probabilistic sample, due to logistic constraints in some regions. Another limitation of the present study is the source of data, collected by questionnaires, which could present significant biases, including socially desirable responding.

5. Conclusions

A human *Leishmania* seroprevalence of 4.8% among blood donors in mainland Portugal was estimated in this study. Significant variations were found among regions. In some cases, the detection of positive or borderline results was very geographically restricted and could represent new foci of parasite circulation. These findings highlight the importance of following a One Health approach to tackling the challenges of climate change: investing in more detailed and localized studies of seroprevalence in human and canine populations, coupled with studies on the presence of *Leishmania* in phlebotomine sand flies. More consistent reporting of human and animal cases would also be vital to providing a complete picture of the national burden of disease. Seropositivity was associated with male sex and older age, but not with lower KPP, as defined by the score developed for this study. Future studies should follow probabilistic sampling approaches but should include a broader healthy population. Factors found to be significantly associated with higher levels of knowledge reinforce the need to educate older people and young adults, males, people from lower education levels, and those from less differentiated professional backgrounds. Knowledge regarding leishmaniasis is heterogeneous in the country, and education programs should target areas where emergence of this zoonosis is expected. Education programs could greatly impact canine and human health if they were directed towards people living in some of the coastal subregions of the NUTS2 Norte and Centro regions, where canine seroprevalence seems to be increasing and human seroprevalence was high. These programs should emphasize the practices associated with effectively lowering the risk of both animal and human infection; veterinarians and physicians could likely play a determinant role in potentiating perceptions and practices in pet owners and immunocompromised individuals, respectively.

Ethics statement

This study received approval from the ethics committees of the following institutions: Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (reference 1.22); Instituto Português do Sangue e da Transplantação; Centro Hospitalar Universitário do Algarve; Hospital do Espírito Santo de Évora; Unidade Local de Saúde do Norte Alentejano;

Unidade Local de Saúde do Baixo Alentejo; Unidade Local de Saúde do Litoral Alentejano. Additionally, the study was authorized by the administration councils of all the involved hospitals. All participants were informed about the study protocol and signed an informed consent form allowing for sample and data collection.

6. References

1. WHO, Health Organization Regional Office for Europe. Manual on case management and surveillance of the leishmaniases in the WHO European region. 2017. http://www.euro.who.int/pubrequest. Accessed 29 Apr 2020.

2. ECDC. Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. Stockholm; 2022. https://www.ecdc.europa.eu/sites/default/files/documents/leishmaniasis-surveillance-eu.pdf

3. SPMS. Doenças de Declaração Obrigatória . Portal da Transparência. 2018. https://transparencia.sns.gov.pt/explore/dataset/doencas-de-declaracao-obrigatoria/table/?disjunctive.regiao_de_saude_de_notificacao&disjunctive.residencia_nut_iii&disjunctive.doenca_de_declaracao_obrigatoria&disjunctive.sexo&disjunctive.grupo_etario&sort. Accessed 22 Feb 2023

4. Serrada E. A Leishmaniose visceral em Portugal Continental: 1999–2009. Universidade Nova de Lisboa. 2010. http://hdl.handle.net/10362/5546

5. Faucher B, Pomares C, Fourcade S, Benyamine A, Marty P, Pratlong L, et al. Mucosal *Leishmania infantum* leishmaniasis: specific pattern in a multicentre survey and historical cases. *J Infect.* 2011;**63**:76–82.

6. Marcoval J, Penín RM, Sabé N, Valentí-Medina F, Bonfill-Ortí M, Martínez-Molina L. Cutaneous leishmaniasis associated with anti-tumour necrosis factor-α drugs: an emerging disease. *Clin Exp Dermatol.* 2017;**42**:331–334.

7. Silva LP, Montenegro S, Werkauser R, da Sales KGS, Soares FCS, Costa VMA, et al. Asymptomatic *Leishmania* infection in blood donors from a major blood bank in northeastern Brazil: A cross-sectional study. *Rev Inst Med Trop Sao Paulo.* 2020;**15**:0238933.

8. de Oliveira FA, Soares LS, Pompilio MA, Tozetti IA, Bonin CM, Dorval MEMC. Cytokine profile in Leishmania-positive blood donors. *PLoS ONE.* 2020;**15**:e0238933.

9. Aliaga L, Ceballos J, Sampedro A, Cobo F, López-Nevot MÁ, Merino-Espinosa G, et al. Asymptomatic Leishmania infection in blood donors from the Southern of Spain. *Infection.* 2019;**47**:739–747.

10. Ibarra-Meneses AV, Corbeil A, Wagner V, Onwuchekwa C, Fernandez-Prada C. Identification of asymptomatic Leishmania infections: a scoping review. *Parasit Vect.* 2022;**15**:1–22.

11. Pederiva MMC, Santos Dos SM, Rivarola LGS, Guerreiro VJ, Lopes KS, Lima Junior MSC, et al. Asymptomatic Leishmania infection in humans: a systematic review. *J Infect Public Health.* 2023;**16**:286–294.

12. Ortuño M, Muñoz C, Spitzová T, Sumova P, Iborra MA, Pérez-Cutillas P, et al. Exposure to *Phlebotomus perniciosus* sandfly vectors is positively associated with Toscana virus and *Leishmania infantum* infection in human blood donors in Murcia Region, southeast Spain. *Transbound Emerg Dis.* 2022;**69**:1–11.

13. Pérez-Cutillas P, Goyena E, Chitimia L, De la Rúa P, Bernal LJ, Fisa R, et al. Spatial distribution of human asymptomatic *Leishmania infantum* infection in southeast Spain: a study of environmental, demographic and social risk factors. *Acta Trop.* 2015;**146**:127–134.

14. Moreira S. Avaliação do Grau de Exposição de Seres Humanos da Sub-Regiao da Cova da Beira ao Agente Zoonótico *Leishmania infantum*. Universidade da Beira Interior; 2016. http://hdl.handle.net/10400.6/5299

15. Fonseca M. Estudo da seroprevalência de Anticorpos Anti Leishmania spp. numa população que coabita com canídeos com leishmaniose. Universidade de Lisboa; 2009. https://repositorio.ul.pt/handle/10451/2383

16. Almeida M, Maia C, Cristóvão JM, Morgado C, Barbosa I, Ibars RF, et al. Seroprevalence and risk factors associated with *Leishmania* infection in dogs from Portugal. Microorganisms. 2022. https://www.mdpi.com/2076-2607/10/11/2262

17. Saleh F, Fazlarabbi Khan M, Rowshan KM. Awareness of residents about kala-azar and its related practices in two endemic areas of Bangladesh. *PLoS ONE.* 2019;**14**:e0219591.

18. Melkamu HT, Beyene AM, Zegeye DT. Knowledge, attitude and practices of the resident community about visceral leishmaniasis in West Armachiho district, Northwest Ethiopia. *Heliyon.* 2020;**6**:e03152.

19. Carrillo-Bonilla LM, Trujillo JJ, Álvarez-Salas L, Vélez-Bernal ID. Study of knowledge, attitudes, and practices related to leishmaniasis: Evidence of government neglect in the Colombian Darién. *Cad Saude Publica.* 2014;**30**:2134–2144.

20. Pereira A, Martins Â, Brancal H, Vilhena H, Silva P, Pimenta P, et al. Parasitic zoonoses associated with dogs and cats: a survey of Portuguese pet owners' awareness and deworming practices. *Parasit Vect.* 2016;**9**:245.

21. Matos M, Alho AM, Owen SP, Nunes T, de Madeira Carvalho L. Parasite control practices and public perception of parasitic diseases: A survey of dog and cat owners. *Prev Vet Med.* 2015;**122**:174–180.

22. INE. Resultados Provisórios–Censos 2021. Resultados Provisórios. 2021.

23. Sargent E. Epitools Epidemiological Calculators. Ausvet. 2018. https://epitools.ausvet.com.au/. Accessed 22 Feb 2023

24. Humphry RW, Cameron A, Gunn GJ. A practical approach to calculate sample size for herd prevalence surveys. *Prev Vet Med.* 2004;**65**:173–188.

25. Martín-Sánchez J, Rodríguez-Granger J, Morillas-Márquez F, Merino-Espinosa G, Sampedro A, Aliaga L, et al. Leishmaniasis due to *Leishmania infantum*: integration of human, animal and environmental data through a one health approach. *Transbound Emerg Dis.* 2020;**67**:2423–2434.

26. ESCO. Classification of Occupations. https://esco.ec.europa.eu/en/classification/occupation_main

27. Freguesias Rurais PDR2020 (nova Divisão Administrativa freguesias 2013). Programa de desenvolvimento rural 2014–

2020. https://www.gpp.pt/images/Estatisticas_e_analises/Estatisticas/associadasmedidasapoio/Territorios_Rurais.pdf

28. WHO. Number of cases of visceral leishmaniasis reported. The global health observatory. 2023 Accessed 24 Mar 2023. https://www.who.int/data/gho/data/indicators/indicator-details/GHO/number-of-cases-of-visceral-leishmaniasis-reported

29. Greiner M, Gardner IA. Application of diagnostic tests in veterinary epidemiologic studies. *Prev Vet Med.* 2000;**45**:43–59.

30. Fagerland MW, Hosmer DW. A generalized Hosmer-Lemeshow goodness-of-fit test for multinomial logistic regression models. *Stata J.* 2012;**12**:447–453.

31. Chitimia L, Muñoz-García CI, Sánchez-Velasco D, Lizana V, del Río L, Murcia L, et al. Cryptic Leishmaniosis by *Leishmania infantum*, a feature of canines only? A study of natural infection in wild rabbits, humans and dogs in southeastern Spain. *Vet Parasitol.* 2011;**181**:12–16.

32. Garrote JI, Gutiérrez MP, Izquierdo RL, Dueñas MAI, Zarzosa P, Canavate C, et al. Seroepidemiologic study of *Leishmania infantum* infection in Castilla-Leon. *Spain. Am J Trop Med Hyg.* 2004;**71**:403–406.

33. Riera C, Fisa R, López-Chejade P, Serra T, Girona E, Jimenez MT, et al. Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain) *Transfusion.* 2008;**48**:1383–1389.

34. Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Island, Spain) by different diagnostic methods. *Trans R Soc Trop Med Hyg.* 2004;**98**:102–110.

35. Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A, Ferroglio E. Asymptomatic *Leishmania infantum* infection in an area of Northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. *J Clin Microbiol.* 2010;**48**:131–136.

36. Scarlata F, Vitale F, Saporito L, Reale S, Vecchi VL, Giordano S, et al. Asymptomatic *Leishmania infantum*/*chagasi* infection in blood donors of western Sicily. *Trans R Soc Trop Med Hyg.* 2008;**102**:394–396.

37. Theocharidou D, Maltezos E, Constantinidis T, Papa A. Human visceral leishmaniasis in northern Greece: seroepidemiology and risk factors in endemic region. *J Vector Borne Dis.* 2019;**56**:244.

38. Ferroglio E, Battisti E, Zanet S, Bolla C, Concialdi E, Trisciuoglio A, et al. Epidemiological evaluation of *Leishmania infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy. *Zoonoses Public Health.* 2018;**65**:675–682.

39. Šiško-Kraljević K, Jerončić A, Mohar B, Punda-Polić V. Asymptomatic *Leishmania infantum* infections in humans living in endemic and non-endemic areas of Croatia, 2007 to 2009. *Eurosurveillance.* 2013;**18**:20533.

40. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, et al. Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. *PLoS Negl Trop Dis.* 2014;**8**:19.

41. de Oliveira França A, de Oliveira Ramos Pereira L, Ortiz Tanaka TS, de Oliveira M, Cavalheiros Dorval ME. Viability of Leishmania in blood donors: a tangible possibility of transfusion transmission. *J Microbiol Immunol Infect.* 2020;**12**:508.

42. Pereira LQ, Tanaka SCSV, Ferreira-Silva MM, Gomes FVBAF, Santana MP, Aguiar PR, et al. Leukoreduction as a control measure in transfusion transmission of visceral leishmaniasis. *Transfusion.* 2023;**63**:1044–1049.

43. Orsini M, Canela JR, Disch J, Maciel F, Greco D, Toledo A, et al. High frequency of asymptomatic *Leishmania* spp. infection among HIV-infected patients living in endemic areas for visceral leishmaniasis in Brazil. *Trans R Soc Trop Med Hyg.* 2012;**106**:283–288.

44. Moreno EC, Melo MN, Lambertucci JR, Serufo JC, Andrade ASR, Antunes CMF, et al. Diagnosing human asymptomatic visceral leishmaniasis in an urban area of the State of Minas Gerais, using serological and molecular biology techniques. *Rev Soc Bras Med Trop.* 2006;**39**:421–427.

45. Ortalli M, Lorrai D, Gaibani P, Rossini G, Vocale C, Re MC, et al. Serodiagnosis of visceral leishmaniasis in northeastern italy: Evaluation of seven serological tests. *Microorganisms.* 2020;**8**:1847.

46. Lévêque MF, Battery E, Delaunay P, Lmimouni BE, Aoun K, L'ollivier C, et al. Evaluation of six commercial kits for the serological diagnosis of Mediterranean visceral leishmaniasis. *PLoS Negl Trop Dis.* 2020;**14**:e0008139.

47. Gaspar C, Augusto G, Albuquerque M, Nascimento M, Vicêncio P, Nogueira P. Doenças de Declaração Obrigatória 2013–2016, Volume II-Portugal. 2017. http://hdl.handle.net/10400.26/22529

48. Campino L, Maia C. Epidemiology of leishmaniases in Portugal | Epidemiologia das leishmanioses em Portugal. *Acta Med Port.* 2010;**23**:859–864.

49. Infeção VIH e SIDA em Portugal-2019. Lisboa; 2019. http://repositorio.insa.pt/bitstream/10400.18/7093/1/DGS-INSA-RelatVIHSIDA2019.pdf

50. Cortes S, Vaz Y, Neves R, Maia C, Cardoso L, Campino L. Risk factors for canine leishmaniasis in an endemic Mediterranean region. *Vet Parasitol.* 2012;**189**:189–196.

51. Amusategui I, Sainz A, Aguirre E, Tesouro MA. Seroprevalence of *Leishmania infantum* in northwestern Spain, an area traditionally considered free of leishmaniasis. In: Annals of the New York Academy of Sciences. 2004.

52. Mirá G, Checa R, Montoya A, Hernández L, Dado D, Gálvez R. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. *Parasit Vect.* 2012;**5**:1–7.

53. Díaz-Regañón D, Roura X, Suárez ML, León M, Sainz Á. Serological evaluation of selected vector-borne pathogens in owned dogs from northern Spain based on a multicenter study using a commercial test. *Parasit Vect.* 2020;**13**:1.

54. Chapman LAC, Morgan ALK, Adams ER, Bern C, Medley GF, Hollingsworth TD. Age trends in asymptomatic and symptomatic Leishmania donovani infection in the Indian subcontinent: a review and analysis of data from diagnostic and epidemiological studies. *PLoS Negl Trop Dis.* 2018;**12**:e0006803.

55. Cloots K, Burza S, Malaviya P, Hasker E, Kansal S, Mollett G, et al. Male predominance in reported visceral leishmaniasis cases: Nature or nurture? A comparison of population-based with health facility-reported data. *PLoS Negl Trop Dis.* 2020;**14**:e0007995.

56. Lockard RD, Wilson ME, Rodríguez NE. Sex-related differences in immune response and symptomatic manifestations to infection with Leishmania species. *J Immunol Res.* 2019;**2019**:4103819.

57. Solano-Gallego L, Mirá G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, et al. leishvet guidelines for the practical management of canine leishmaniosis. *Parasit Vect.* 2011;**4**:1–6.

58. Pereira A, Maia C. Leishmania infection in cats and feline leishmaniosis: an updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Curr Res Parasitol Vector Borne Dis.* 2021;**1**:100035.

7. Supplementary data

Available at: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-023-05980-1

Additional file 1: Supplementary table 1. List of NUTS (Nomenclature of Territorial Units for Statistics) 2 and NUTS3 regions in Continental Portugal.

Additional file 2: Supplementary figure 1. Questionnaire about sociodemographic aspects and Knowledge, Perceptions and Practices regarding leishmaniasis.

Additional file 3: Supplementary table 2. Protocol implemented for scoring Knowledge, Perceptions and Practices of blood donors, according to the answers provided in the questionnaire

Additional file 4: Supplementary table 3. Sociodemographic characteristics of the participants, globally and by NUTS (Nomenclature of Territorial Units for Statistics) 3 region.

Additional file 5: Supplementary table 4. Answers to knowledge, perceptions, and practices questions, globally and by NUTS (Nomenclature of Territorial Units for Statistics) 3 region.

Additional file 6: Supplementary figure 2. Distribution of individual: a) Knowledge scores; b) Perceptions scores; c) Practices scores.

Additional file 7: Supplementary figure 3. Percentage of blood donors choosing different: a) sources of information about leishmaniasis; b) main routes of transmission of leishmaniasis; c) most frequent clinical signs in animals; d) most commonly affected organs in humans; e) types of repellent/insecticide product used in own dog(s).

Additional file 8: Supplementary table 5. Distribution of participants and K, Per and Pra scores by category, for sociodemographic variables.

CHAPTER 3

Knowledge, perceptions and practices of health students and professionals regarding leishmaniasis in Portugal.

This chapter is a transcription of the research article:

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. Knowledge, perceptions and practices of health students and professionals regarding leishmaniasis in Portugal: a cross-sectional study. Parasites & Vectors 16, 381 (2023). https://doi.org/10.1186/s13071-023-05982-z

Abstract

Background. Control of leishmaniasis in the Mediterranean Basin relies on the active contributions from researchers in the fields of animal, human and environmental health. The application of knowledge, perceptions and practices (KPP) questionnaires to health students and professionals in Europe could be fundamental to identify and explore gaps in KPP, highlighting the diversity of conceptions related to this disease between students and professionals active in (One) Health. The objective of this study was to characterize and compare the current knowledge, perceptions and practices regarding leishmaniasis among subgroups of students and health professionals in Portugal through the application of an online questionnaire.

Methods. A cross-sectional study targeted the population of health students and professionals in Portugal, including students in medicine, veterinary medicine and environmental health, physicians, veterinarians and environmental health technicians. Potential participants were approached by email via universities and professional societies and organizations and provided with the link to access the online questionnaire. Answers to the self-administered sociodemographic and KPP questionnaire were collected between July and December 2022. Individual KPP scores were calculated by summing grades defined for each question. Logistic regression models were used to search for potential associations, and the results were expressed at estimated crude and adjusted odds ratios with 95% confidence intervals.

Results. In total, 486 participants were included in this study: 254 students and 232 professionals. Overall, 75% of the participants reported having heard of both human and animal leishmaniasis, and > 80% reported hearing about the disease during their course work (although this was significantly lower among those in the field of environmental health). Around 90% of participants identified the pathogenic agent as a parasite, and an arthropod bite was identified as the main route of transmission by > 95%. Animal leishmaniasis was considered to be diagnosed in Portugal by 87% of participants and human leishmaniasis by only 69%. The main barriers pointed out by professionals to the control of leishmaniasis were: lack of knowledge in the general population, failures in the early diagnosis and treatment of diseased animals, absence/inefficacy of vector control programs and lack of knowledge in human health professionals. Median knowledge and perception scores were higher among professionals in the animal health field and higher in professionals than in students. Median practice scores were not significantly different between groups and subgroups. The multivariate analysis revealed that a longer period of study (for students) and having seen cases of leishmaniasis (for physicians) were associated with above-mentioned median knowledge score.

Conclusions. Most health students and professionals are knowledgeable about the cause and transmission route of leishmaniasis. However, recognition of the disease as autochthonous in humans is less common, highlighting the importance of promoting an approach to this infection through a One-Health lens. A national structured plan to control leishmaniasis could overcome some of the barriers pointed out by professionals, namely by implementing systematic phlebotomine surveillance and integrated reporting of animal and human cases of disease.

Keywords: Leishmaniasis, Awareness, Knowledge, Perceptions, Practices, Medicine, Veterinary, Environmental health, Portugal, One Health

1. Background

Leishmaniases are a group of diseases caused by protozoan parasites of the *Leishmania* genus that are transmitted through the bite of phlebotomine sand flies. In southern Europe, including Portugal, *Leishmania infantum* is the only endemic human pathogenic species and is maintained in zoonotic cycles where dogs are the most important domestic reservoirs [1]. Human infection by parasites of the *Leishmania* genus is asymptomatic in most cases [2]. However, some individuals progress to clinically recognizable disease, which can be grouped in the following syndromic forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML/MCL). In Portugal, symptomatic infection by the endemic species *L. infantum* most often results in VL [3], and the reporting of VL cases to central public health authorities is mandatory. Between 2014 and 2018, 6–14 cases were reported each year nationwide [4], although these numbers likely reflect a significant underreporting at the hospital level, as the numbers were higher in previous periods [5]. CL is considered to be rare in Portugal and is not listed as a mandatory declaration disease, so available data of cases are dispersed and mostly derived from the few case reports published in national and international literature [6]. Regarding canine leishmaniasis (CanL), there is also no national, integrated and standardized reporting and surveillance system in place, and only cases suspected by municipal veterinarians during rabies control campaigns are reported to the General Directorate for Food and Veterinary; consequently, the number of cases diagnosed annually at a global and regional level is unknown [3]. However, seroprevalence studies performed in dogs, at both national and regional levels, have provided some insight into the distribution of infection, helping to define regions where exposure to *Leishmania* parasites is expected to be more frequent/ intense, namely in the districts of Beja, Portalegre and Castelo Branco [7, 8]. Despite the wide distribution of leishmaniasis and the health impacts on the population in endemic areas, knowledge, perceptions and practices (KPP) regarding this disease are not homogeneous between countries and between regions of the same country, or even different sectors of the population, including health professionals, health sciences students and animal owners. Studies to assess the knowledge, perceptions, attitudes and practices regarding VL have been conducted mostly in South Asia [9–11], South America [12, 13] and East Africa [14], with the target study populations consisting predominantly of resident communities in highly endemic areas. In the Mediterranean region, where VL is also endemic, the few studies dedicated to analyzing knowledge, attitudes and practices (KAP) of the resident general population were mostly directed to animal owners, including three studies performed in Portugal [15–17]. With respect to studies directed to the population of health professionals, there is an abundant body of literature on the knowledge and practices of veterinary doctors in Mediterranean countries, including Portugal, generally with a focus on the epidemiology and clinical approach to CanL [18–21]. However, no studies carried out in Mediterranean countries have included medical doctors and environmental health technicians (EHTs) in the target population. On a worldwide perspective, few studies have addressed these groups, although it is generally recognized that they play an important role in controlling leishmaniasis, under a One Health lens. In a number of studies, physicians were enrolled in completing structured questionnaires, either self-administered (online or paper) or by interview, in endemic areas such as South Asia [22, 23], North Africa [24, 25], Middle East [26, 27] and South America [28,29]. Both primary healthcare physicians and specialists (such as dermatologists) were included in these studies. Some published studies included educational interventions, showing a significant increase in knowledge following the intervention [26, 28]. These studies showed that knowledge on the cause, transmission route, clinical presentation, diagnosis, treatment and perception of risk were very diverse among regions. Even though the designation EHT is not employed homogeneously across countries, professionals involved in environmental health were involved in at least one study in Brazil [30] in which correct answers were compared between different professional groups; The highest average score was achieved by veterinarians, followed by physicians and EHTs, although few participants were recruited in each group. Some observational and interventional studies targeted high-school students in countries such as Iran and Ethiopia [26, 31, 32]. University students in health sciences, including medicine, veterinary medicine and environmental health, have only rarely been included in KAP/KPP studies and never in the context of a Mediterranean country, as shown in a recent review article on CL [33]; however, an understanding of the current knowledge and perceptions of future professionals could be an essential step towards raising awareness and improving practices in the health community. One study involving medical students in Latin America participating in an online questionnaire [34] showed that most students were aware that leishmaniasis was caused by a parasite transmitted by sand flies and recognized ways of preventing the disease, but they were less knowledgeable regarding the clinical presentation of disease and treatment. In Portugal, incomplete reporting and insufficient characterization of symptomatic leishmaniasis cases (human and canine) may result in gaps in KPP regarding leishmaniasis in health professionals. These gaps may be perpetuated due to neglect of leishmaniasis in the training of students in these areas. The application of KPP questionnaires to students and professionals could be fundamental to identifying and exploring these gaps, highlighting the diversity of conceptions related to this disease between students and professionals in (One) Health. Therefore, the aim of this project was to characterize and compare current KPP regarding leishmaniasis among subgroups of students and health professionals, in Portugal, through the application of an online questionnaire, in a sample of each of these groups.

2. Methods

2.1. Study population and sample size calculation

This cross-sectional, observational study was carried out from July to December 2022, in Portugal, which is located in southwest Europe, bordering Spain and the Atlantic Ocean. The study consisted of the self-administration of a structured, anonymous, online questionnaire aimed at collecting sociodemographic data and information on KPP related to leishmaniasis. The populations targeted were students of medicine, veterinary medicine and environmental health in Portuguese public and private higher education institutions as well as health professionals (medical doctors, veterinary doctors and EHTs) working in public and private institutions in Portugal. The most recent statistics of the Portuguese Order of Physicians show that 59,545 professionals were registered in 2021 [35]. Most of these were women (56.7%), and the districts where most professionals worked were Lisbon (29.0%), Porto (22.1%) and Coimbra (9.7%) [35]. The age groups with the highest proportion of registered doctors < 31 years (17.4%) and > 65 years (24.0%). The medical specialties that could potentially be involved in a more detailed approach to leishmaniasis represented a significant fraction of the registered specialists: Internal Medicine (8.7%, *n* = 3165), Pediatrics (6.3%, *n* = 2297), Public Health (1.6%, *n* = 582), Dermatology (1.2%, *n* = 427) and Infectious Diseases (0.6%, *n* = 227) [36]. It should be noted that at least 1.1% of registered doctors completed their medical training in countries or regions where cases of leishmaniasis are rare or absent, such as Portuguese-speaking African countries, non-Mediterranean European countries, North America and Oceania [37]. Based on data from the National Institute of Statistics, 12,449 medical students were registered in 2021, with a predominance of women (69.7%), of whom 18.4% were registered for the first time in that year [38]. These students were registered in programs offered by eight faculties (7 public and 1 private), located in five Portuguese cities (Braga, Coimbra, Covilhã, Lisboa and Porto), according to data available in the Directorate General for Higher Education (DGES) [39].

Statistics from the Portuguese Order of Veterinary Doctors show that 6788 active members were registered in 2022, most of whom were women (65.2%). In terms of distribution by regions, most actively working members were in the districts of Lisbon (26.3%, n = 1783), Porto (15.6%, n = 1057) and Setúbal (8.2%, n = 556) [40]. Based on data from the DGES, 2959 veterinary medicine students were registered in the 2019–2020 academic year, most of whom were (77.0%). Veterinary medicine courses are currently offered in eight faculties (4 public and 4 private) located in six Portuguese cities (Almada, Coimbra, Évora, Lisboa, Porto and Vila Real) [39]. Lastly, the global number of EHT professionals in Portugal could not be ascertained via official available sources. However, based on data from the DGES, there were 387 environmental health students (EHSs) registered in the 2019–2020 academic year, with a predominance of women (70.3%) [39]. This bachelor's course is offered in three public institutes, located in the cities of Coimbra, Lisbon and Porto. The sample size and geographic distribution of each student/professional group were not determined a priori and depended on the rates of participation from each institution. However, a standardized protocol was used to approach potential participants in each group in terms of number, frequency, media and content of contacts, as explained in the following text. To ensure a nationwide coverage of sampling, collaboration in this study was proposed to all of the faculties and institutes where the target courses were available, as detailed above, and to the professional orders, societies and associations of the three targeted professional categories.

2.2. Eligibility criteria

Individuals included in this study cumulatively fulfilled the following criteria:

- Being registered (in Portugal) as a student in the academic year of 2022–2023 in one of three degree programs, namely Integrated Master's in Medicine, Integrated Master's in Veterinary Medicine or Bachelor's in Environmental Health, or having completed training in medicine, veterinary medicine or environmental health and actively practicing (in Portugal) in these professional fields in 2022.
- Age between 18 and 70 years, inclusive.
- Access to an electronic device for filling in the online questionnaire.
- Consenting to the informed consent form to participate in the study.

2.3. Data and sample collection

All of the higher education institutions in Portugal offering the Integrated Master's in Medicine, Integrated Master's in Veterinary Medicine and Bachelor's in Environmental Health were contacted with requests for collaboration in disseminating the questionnaire to the students enrolled in these courses. Collaboration was achieved in four of the eight medical faculties, five of the eight veterinary faculties and two of the three environmental health institutes. The link to access the questionnaire was sent to all students enrolled in these courses via their institutional emails; this first email was followed by two subsequent emails at 3 and 6 weeks after the first. Additionally, the Portuguese Order of Physicians (OM), the Portuguese Order of Veterinary Doctors (OMV) and the Portuguese Association of Environmental Health (APSAi) were contacted and requested to disseminate the questionnaire among their registered professionals or members. For the APSAi, we used the same approach as that for the faculties. For the OMV, however, the link to the questionnaire was posted one the APSAi website and, due to low visibility and adherence, the questionnaire was also disseminated through posting in specific Facebook® groups, following a similar timeline (3 posts separated by 3 weeks). Collaboration of the OM was not possible, so professional medical societies were contacted to request their collaboration (specialties of Infectious Diseases, Pediatrics, Anatomopathology, Clinical Pathology, Dermatology, Internal Medicine and Public Health). Only the first three specialties collaborated, and the questionnaire was sent via email to the associates following a similar timeline; this was complemented by posting in specific Facebook® medical groups. The questionnaire was constructed specifically for this study, although some questions were adapted from previous KPP research on leishmaniasis. The questions were designed to address all of the relevant topics regarding knowledge on leshmaniasis (epidemiology, presentation, diagnosis and management aspects) and professional and personal practices, while avoiding redundancy. Questions considered to be appropriate for the purpose of the project based on consensus of all authors were included in the final version of the questionnaire. To improve ease/speed of filling in the questionnaire, most questions were designed as multiple choice; possible answers were selected by the authors to include all "correct" knowledge based on current scientific evidence and all the expected most common practices; additional plausible options were added to allow greater discrimination. Likert scales were used as answers to perception questions. The questionnaire was pre-tested on a convenience sample of health students and professionals and readapted to achieve conformity. The first page of the form consisted of an information text and request for consent. Progression required consent, selection of the professional/student category and confirmation of current activity. Participants went on to fill in a self-administered online questionnaire about sociodemographic and professional/academic aspects and KPP regarding leishmaniasis. This questionnaire was built upon RedCap® (Research Electronic Data Capture—a secure web application for building and managing online surveys and databases), and different versions were available for different groups, although many questions overlapped, and some were directed specifically to one or a few groups (Additional file 1: Figure S1). The latter was the case for professional activity-related questions and for questions targeting biological details of vectors. Additionally, some questions were only visible to participants who selected specific answers to previous questions, in a drop-off fashion. For most questions, an answer was not mandatory for progressing to the next question in the questionnaire. The total number of accesses to the questionnaire was registered, but only submitted forms were saved and used for analysis. Categorical variables extracted from the questionnaire were analyzed mostly using the original categories provided as answer options, but regrouping was performed in some cases. Districts of work/study in mainland Portugal were grouped into five regions (Norte, Centro, Lisboa e Vale do Tejo [LVT], Alentejo and Algarve) according to the areas of activity of the five Regional Coordination and Development Commissions (Comissões de Coordenação e Desenvolvimento Regional [CCDR]). For questions answered in an ordinal scale with k options, answers were rated from 0 to k (with the k value attributed to the highest frequency, importance or agreement).

2.4. Statistical analysis

Absolute and relative frequencies, hypothesis testing and logistic regressions were performed using IBM® SPSS® Statistics Version 29.0 (SPSS IBM Corp, Armonk, NY, USA). Bar and pie charts were built using Microsoft® Excel® (Microsoft Corp., Redmond, WA, USA). Answers to each KPP question were scored according to the criteria presented in Additional file 2: Table S1. A total score for each individual was calculated for knowledge (K score), for perceptions (Per score) and for practices (Pra score), by totaling the scores for all the questions in each category. The range of possible values for each score was 0–8 for K, 0–11 for Per and 0–3.5 for Pra. Descriptive statistics were expressed as absolute frequencies and percentages for categorical variables and as means with standard deviations or medians with interquartile ranges for continuous variables (e.g. age, and K, Per and Pra scores). Comparisons between groups were performed using the Pearson Chi-square test ($\chi 2$) for categorical variables (or Fisher's exact test in case of failure of the assumptions of the $\chi 2$test). For continuous variables, after checking the assumptions of normality and homogeneity of the variances, instead of the t-test and analysis of variance (ANOVA), we used the Mann–Whitney U-test or the Kruskal–Wallis test for comparing ≥ 2 independent groups, respectively. For variables rated in an ordinal scale, the median value was calculated and presented in the tables, and statistical significance between groups was assessed comparing distributions using the Mann–Whitney U-test or the Kruskal–Wallis test (≥ 2 independent groups, respectively). A value of $P < 0.05$ was considered statistically significant. Recoding into binary variables was performed. Participants were divided in two groups for K, Per and Pra scores: those with scores above the global median score value and those with scores equal or below this value (K = 6.5, Per = 9, Pra = 1.5). Multivariate analyses were conducted to identify sociodemographic and occupational factors associated with higher K, Per or Pra scores. These analyses were performed through multiple binary logistic regression models, analyzing variables with statistical meaning in the univariate analysis ($P < 0.05$) and some biologically relevant or potentially confounding variables. The reference categories used for each independent variable are specified in each multivariate analysis results table. For those variables that remained significant, crude odds ratios (ORs) were updated to adjusted odds ratios (aORs) with 95% confidence intervals (CIs). The Hosmer–Lemeshow test was used to assess goodness of fit in each multiple logistic regression model [41].

3. Results

3.1. Sociodemographic and occupational characteristics

In total, 486 consented to participate in the study, of whom 254 were students and 232 were professionals. The sociodemographic characteristics of these participants are summarized in Table 1. Median age was 21 years old for students and 38 years for professionals; differences in age were not statistically significant among subgroups of students nor among subgroups of health professionals. Female gender was predominant in all subgroups (> 70%). The distribution of participants by region was significantly different between students and professionals and among subgroups, but Lisboa e Vale do Tejo (LVT) and Norte were the most represented (except for EHSs).

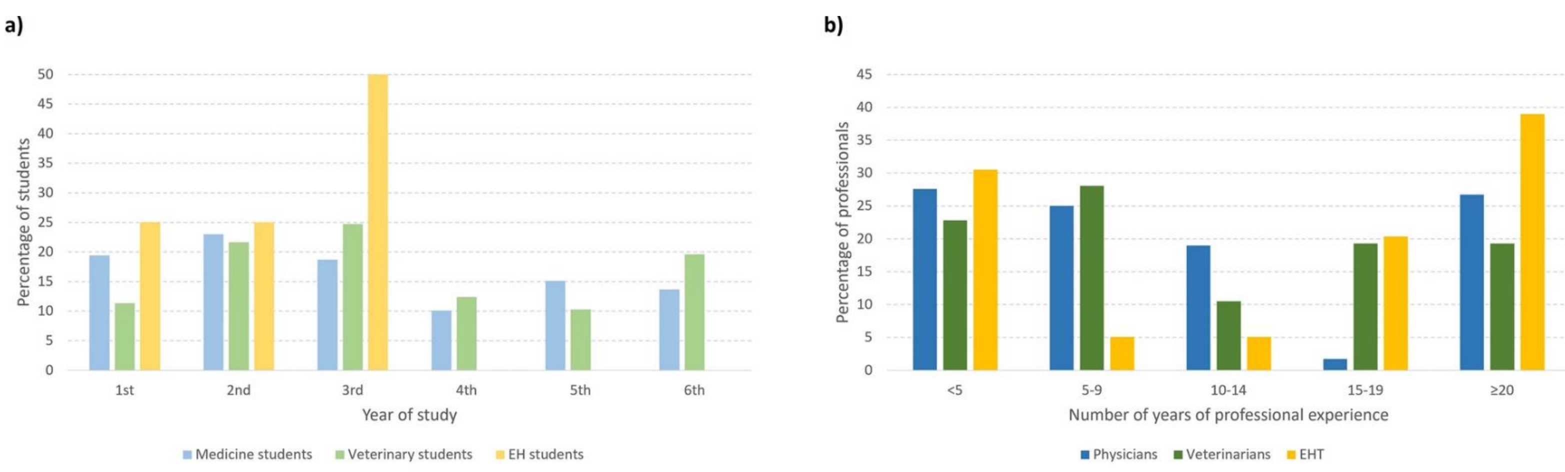
Table 1. Sociodemographic characteristics of the participants, for students and professionals.

	Global students	Medicine students	Veterinary students	EH students	*p*-value students	Global profs	Physicians	Vets	EH technicians	*p*-value profs
n	254	140	98	16		232	116	57	59	
Median age (IQR)	21 (19-24)	21 (19-23)	21 (20-24)	20.5 (20-21)	0.146 (H = 3.8, df = 2)	38 (30-46)	36 (30-46)	36 (30-46)	42.5 (32,25-45,75)	0.350 (H = 2.1, df = 2)
Gender										
Male	16.7 (42/252)	20.1 (28/139)	12.4 (12/97)	12.5 (2/16)	0.259 ($\chi2$ = 2.7, df = 2)	23.3 (54/232)	28.4 (33/116)	17.5 (10/57)	18.6 (11/59)	0.174 ($\chi2$ = 3.5, df = 2)
Female	83.3 (210/252)	79.9 (111/139)	87.6 (85/97)	87.5 (14/16)		76.7 (178/232)	71.6 (83/116)	82.5 (47/57)	81.4 (48/59)	
Region of work/study										
Norte	26.2 (66/252)	20.1 (28/139)	37.1 (36/97)	12.5 (2/16)	<0.001 (FET = 70.5)	32.5 (75/231)	36.5 (42/115)	21.1 (12/57)	35.6 (21/59)	0.011 (FET = 21.0)
Centro	10.7 (27/252)	11.5 (16/139)	0 (0/97)	68.8 (11/16)		18.2 (42/231)	23.5 (27/115)	8.8 (5/57)	16.9 (10/59)	
LVT	55.6 (140/252)	68.3 (95/139)	43.3 (42/97)	18.7 (3/16)		38.5 (89/231)	33.0 (38/115)	54.4 (31/57)	33.9 (20/59)	
Alentejo	7.5 (19/252)	NA	19.6 (19/97)	NA		3.0 (7/231)	1.7 (2/115)	7.0 (4/57)	1.7 (1/59)	
Algarve	NA	NA	NA	NA		5.6 (13/231)	3.5 (4/115)	8.8 (5/57)	6.8 (4/59)	
Açores/Madeira	NA	NA	NA	NA		2.2 (5/231)	1.7 (2/115)	0 (0/57)	5.1 (3/59)	

Abbreviations: *n* - number; IQR - interquartile range; LVT - Lisboa e Vale do Tejo; profs – professionals; EH – Environmental Health; FET – Fisher's exact test
*Statistically significant

Distribution of students by year of study is shown in Fig. 1a (for medicine and veterinary students: Chi-square test, $\chi 2 = 5.9$, df = 5, $P = 0.324$) and distribution of professionals by number of years of experience is shown in Fig. 1b (Kruskal–Wallis test, H = 3.5, df = 2, $P = 0.170$). Distribution of students by university and faculty is shown in Additional file 3: Figure S2. The type of work most performed by physicians and veterinarians was consultations (63.8% and 84.2%, respectively), followed by infirmary visits (62.1% and 42.1%, respectively). Most physicians were specialists (64.7%, *n* = 75) or specialty residents (31.9%, *n* = 37) and were practicing in the following specialties: Pediatrics (38.8%, *n* = 45), Infectious Diseases (20.7%, *n* = 24), Anatomopathology (8.6%, *n* = 10), Public Health (6.9%, *n* = 8), Internal Medicine and Family Medicine (5.2% each, *n* = 6), Clinical Pathology (3.4%, *n* = 4) and Dermatology (0.9%, *n* = 1). Types of institutions where most of the professionals worked were: Public Health Units (88.1%, *n* = 52) for EHTs; public hospitals (79.3%, *n* = 92), for physicians; veterinary clinics (78.9%, *n* = 45) for veterinarians. Most veterinarians reported working with companion animals (94.7%, *n* = 54), but also with livestock (12.3%, *n* = 7), exotic animals (10.5%, *n* = 6) and horses (7.0%, *n* = 4).

Figure 1. Distribution of (a) medical and veterinary students by year of study (a) and of professionals by number of years of professional experience (b).



Abbreviations: EHT – Environmental Health Technicians

3.2. Knowledge results

Answers to individual knowledge questions are summarized in Table 2. Overall, 75.3% of participants reported having heard of both human and animal leishmaniasis, although this percentage was significantly higher in professionals (Fisher's exact test, 49.8, $P < 0.001$). Over 80% of participants reported hearing about the disease during their courses; the number was similar between students and professionals globally, but was significantly lower among people in the field of EH (Chi-square test, $\chi2 = 14.2$, df = 2, $P = 0.001$). The course year most reported by students for hearing of leishmaniasis was the second, both for veterinary students (71.1%) and for medical students (60.8%). Hearing about the disease during professional activity was more commonly reported by veterinarians (94.7%), followed by physicians (82.8%) and EHTs (77.2%). Hearing about animal leishmaniasis in professionals during their career in human was less frequently reported than hearing about human leishmaniasis careers in animal health (42.9% vs 77.7%, $P < 0.001$). Professionals reported hearing about leishmaniasis relatively less often outside their course/work (Chi-square test, $\chi2 = 63.2$, df = 1, $P < 0.001$). Figure 2 presents the percentage of participants reporting hearing of leishmaniasis in different contexts at work, during their course work and outside of their work/course work. The most reported contexts for hearing of leishmaniasis at work were the observation of animals/patients, conversation with work colleagues, performance of diagnostic tests and courses/workshops/congresses; the first three categories were significantly more selected by veterinarians than by physicians. In course work, theoretical classes were the context most often selected, followed by practical classes; compared to medical and EH students, veterinary students reported hearing of leishmaniasis significantly more often in practical classes, workshops/congresses and informal talks with peers or professors. Outside of the work/course context, the two most reported contexts for hearing of leishmaniasis were advertisements on TV and conversation with a veterinary, for animal leishmaniasis; internet searches and conversation with friends/family, for human leishmaniasis.

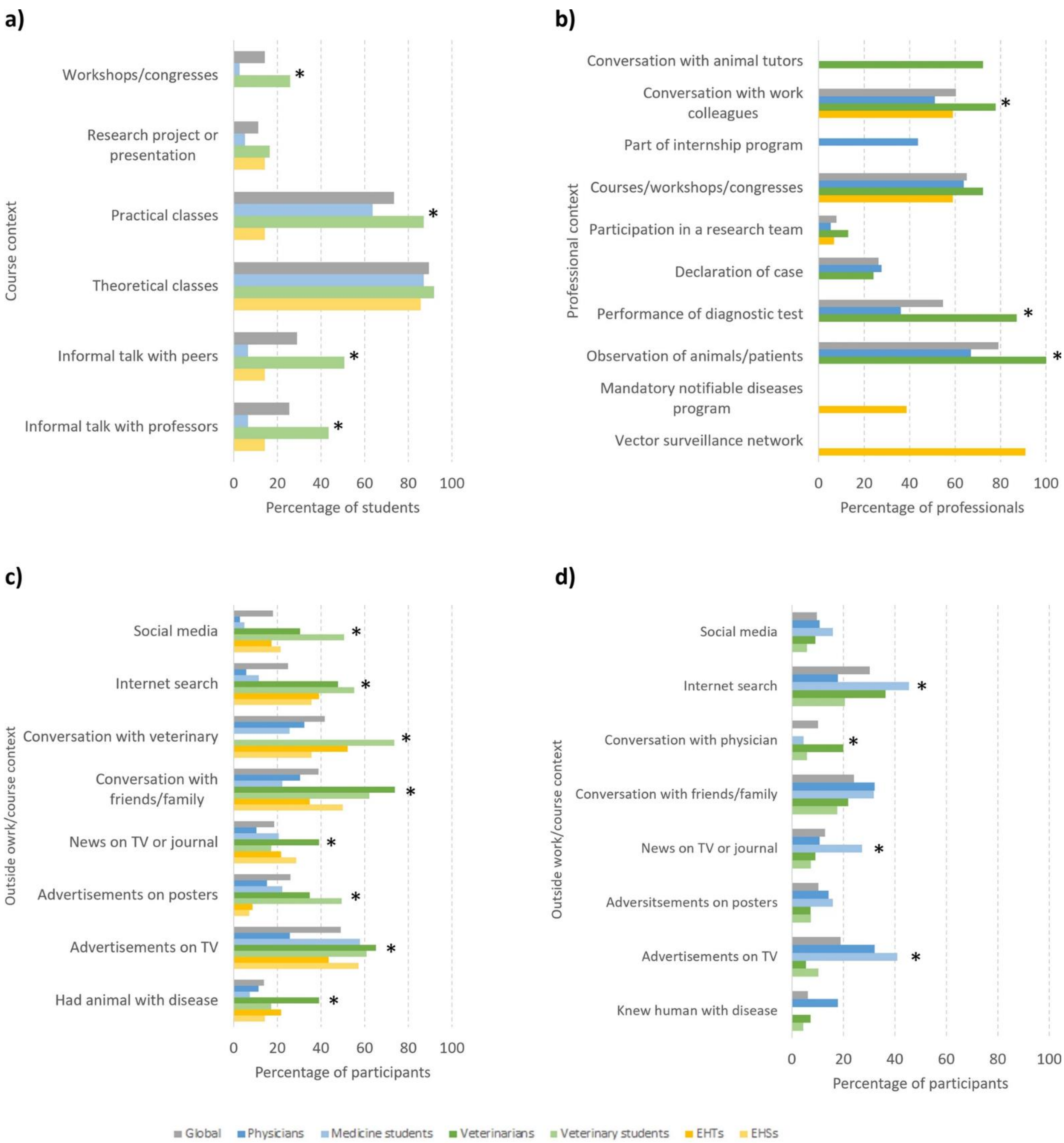
Table 2. Answers to knowledge questions, globally and by student/professional group.

	All	Global students	Med students	Vet students	EH students	*p*-value students	Global profs	Med profs	Vet profs	EH profs	*p*-value profs	*p*-value global
Heard of leishmaniasis?												
Animals only	17.7 (86/486)	28.3 (72/254)	26.4 (37/140)	29.6 (29/98)	37.5 (6/16)	0.094 (FET = 10.8)	6.0 (14/232)	2.6 (3/116)	3.5 (2/57)	11.9 (7/59)	0.004* (FET = 19.4)	<0.001* (FET = 49.8)
Humans only	4.3 (21/486)	3.9 (10/254)	6.4 (9/140)	0 (0/98)	6.2 (1/16)		4.7 (11/232)	9.5 (11/116)	0 (0/57)	0 (0/59)		
Humans and animals	75.3 (366/486)	63.4 (161/254)	60.0 (84/140)	70.4 (69/98)	50.0 (8/16)		88.4 (205/232)	87.9 (102/116)	96.5 (55/57)	81.4 (48/59)		
No/DK/CR	2.7 (13/486)	4.3 (11/254)	7.1 (10/140)	0 (0/98)	6.2 (1/16)		0.9 (2/232)	0 (0/116)	0 (0/57)	6.8 (4/59)		
Heard during course?												
Yes	82.4 (361/438)	82.0 (169/206)	82.8 (77/93)	86.7 (85/98)	46.7 (7/15)	0.001* (χ^2 = 14.2, df = 2)	82.8 (192/232)	85.3 (99/116)	100 (57/57)	63.2 (36/57)	<0.001* (FET = 26.1)	0.843 (χ^2 = 0.04, df = 1)
Heard outside course/work?												
Yes	50.2 (220/438)	70.4 (145/206)	47.3 (44/93)	88.8 (87/98)	93.3 (14/15)	<0.001* (FET = 43.4)	32.3 (75/232)	25.0 (29/116)	40.4 (23/57)	40.4 (23/57)	0.046* (χ^2 = 6.2, df = 2)	<0.001* (χ^2 = 63.2, df = 1)
Pathogenic agent												
Bacteria	1.9 (9/469)	2.5 (6/241)	3.9 (5/128)	1.0 (1/98)	0 (0/15)	0.231 (FET = 8.1)	1.3 (3/228)	0 (0/114)	0 (0/57)	5.3 (3/57)	0.001* (FET = 22.0)	0.145 (FET = 5.4)
Virus	5.5 (26/469)	7.1 (17/241)	5.5 (7/128)	9.2 (9/98)	6.7 (1/15)		3.9 (9/228)	0 (0/114)	1.8 (1/57)	14.0 (8/57)		
Parasite	89.1 (418/469)	85.9 (207/241)	83.6 (107/128)	88.8 (87/98)	86.7 (13/15)		92.5 (211/228)	96.5 (110/114)	98.2 (56/57)	78.9 (45/57)		
Other/DK	3.4 (16/469)	4.6 (11/241)	7.0 (9/128)	1.0 (1/98)	6.7 (1/15)		2.2 (5/228)	3.5 (4/114)	0 (0/57)	1.8 (1/57)		
Same species in animals/humans?												
Yes	74.4 (265/356)	64.7 (99/153)	52.4 (44/84)	79.7 (55/69)	NA NA	<0.001* (χ^2 = 12.4, df = 1)	81.8 (166/203)	74.0 (74/100)	89.1 (49/55)	89.6 (43/48)	0.018* (χ^2 = 8.0, df = 2)	<0.001* (χ^2 = 13.4, df = 1)
Main route of transmission												
Arthropod bite	97.1 (442/455)	97.8 (225/230)	96.6 (115/119)	99.0 (96/97)	100 (14/14)		96.4 (217/225)	92.8 (103/111)	100 (57/57)	98.2 (56/57)		
Mosquito bite	35.3 (156/442)	43.6 (98/225)	53.9 (62/115)	29.2 (28/96)	57.1 (8/14)		26.7 (58/217)	41.7 (43/103)	8.8 (5/57)	17.9 (10/56)		
San dfly bite	72.4 (320/442)	66.2 (149/225)	56.5 (65/115)	84.4 (81/96)	21.4 (3/14)		78.8 (171/217)	65.0 (67/103)	100 (57/57)	83.9 (47/56)		
Flea bite	7.9 (35/442)	13.3 (30/225)	17.4 (20/115)	7.3 (7/96)	21.4 (3/14)		2.3 (5/217)	4.9 (5/103)	0 (0/57)	0 (0/56)		
Tick bite	9.0 (40/442)	14.2 (32/225)	19.1 (22/115)	6.2 (6/96)	28.6 (4/14)		3.7 (8/217)	5.8 (6/103)	1.8 (1/57)	1.8 (1/56)		
Direct contact with animals	12.7 (58/455)	13.5 (31/230)	19.3 (23/119)	8.2 (8/97)	0 (0/14)		12.0 (27/225)	15.3 (17/111)	3.5 (2/57)	14.0 (8/57)		
Animal bite/scratch	7.5 (34/455)	10.4 (24/230)	13.4 (16/119)	8.2 (8/97)	0 (0/14)		4.4 (10/225)	4.5 (5/111)	1.8 (1/57)	7.0 (4/57)		
Blood transfusion	21.1 (96/455)	21.3 (49/230)	13.4 (16/119)	32.0 (31/97)	14.3 (2/14)		20.9 (47/225)	18.0 (20/111)	31.6 (18/57)	15.8 (9/57)		
Vertical	14.5 (66/455)	13.0 (30/230)	10.9 (13/119)	16.5 (16/97)	7.1 (1/14)		16.0 (36/225)	13.5 (15/111)	26.3 (15/57)	10.5 (6/57)		
Organ transplant	8.8 (40/455)	7.0 (16/230)	4.2 (5/119)	11.3 (11/97)	0 (0/14)		10.7 (24/225)	13.5 (15/111)	12.3 (7/57)	3.5 (2/57)		
DK/CR	3.8 (18/473)	5.3 (13/243)	8.5 (11/130)	1.0 (1/98)	6.7 (1/15)	0.046* (FET = 6.2)	2.2 (5/230)	4.3 (5/116)	0 (0/57)	0 (0/57)	0.515 (FET = 1.3)	0.071 (χ^2 = 3.3, df = 1)
Is animal leishmaniasis diagnosed in Portugal?												
Yes	87.4 (333/381)	85.8 (188/219)	79.3 (96/121)	93.9 (92/98)	80.0 (12/15)	0.008* (FET = 9.6)	89.5 (145/162)	83.8 (88/105)	100 (57/57)	96.5 (55/57)	0.002* (FET = 12.4)	0.287 (χ^2 = 1.1, df = 1)

Is human leishmaniasis diagnosed in Portugal?												
Yes	69.4 (218/314)	49.3 (73/148)	51.2 (41/80)	47.1 (32/68)	NA NA	0.611 (χ2 = 0.26, df = 1)	87.3 (145/166)	91.9 (102/111)	78.2 (43/55)	NA NA	0.012* (χ2 = 6.3, df = 1)	<0.001* (χ2 = 53.3, df = 1)
Is leishmaniasis endemic in Portugal?												
Yes	98.6 (354/359)	99.3 (144/145)	97.6 (40/41)	100 (92/92)	100 (12/12)	0.280 (FET = 2.5)	98.1 (210/214)	97.1 (99/102)	100 (57/57)	98.2 (54/55)	0.850 (FET = 0.32)	0.349 (FET = 0.65)
Animal species most affected												
Dogs	98.0 (345/352)	97.0 (194/200)	97.9 (95/97)	96.7 (88/91)	91.7 (11/12)	0.372 (FET = 1.5)	99.3 (151/152)	100 (86/86)	100 (11/11)	98.2 (54/55)	0.434 (FET = 2.9)	0.146 (FET = 2.4)
Cats	19.9 (70/352)	26.5 (53/200)	37.1 (36/97)	14.3 (13/91)	33.3 (4/12)		11.3 (17/151)	15.1 (13/86)	0 (0/11)	7.3 (4/55)		
Other(s)[a]	19.6 (69/352)	20.0 (40/200)	21.6 (21/97)	20.9 (19/91)	0 (0/12)		19.2 (29/151)	20.9 (18/86)	9.1 (1/11)	18.2 (10/55)		
DK/CR	2.5 (9/361)	2.9 (6/206)	2.0 (2/99)	4.2 (4/95)	0 (0/12)	0.609 (FET = 1.5)	1.9 (3/155)	2.3 (2/88)	9.1 (1/12)	0 (0/55)	0.164 (FET = 1.8)	0.734 (FET = 0.35)
Presentation of *Leishmania* infection												
Always symptomatic	7.0 (21/301)	3.5 (5/142)	1.8 (1/55)	3.8 (3/79)	12.5 (1/8)	0.304 (FET = 4.8)	10.1 (16/159)	15.1 (8/53)	1.8 (1/57)	14.3 (7/49)	0.006* (FET = 14.4)	0.015* (χ2 = 8.3, df = 2)
Mostly symptomatic	68.4 (206/301)	66.2 (94/142)	60.0 (33/55)	68.4 (54/79)	87.5 (7/8)		70.4 (112/159)	58.5 (31/53)	73.7 (42/57)	79.6 (39/49)		
Mostly asymptomatic	24.6 (74/301)	30.3 (43/142)	38.2 (21/55)	27.8 (22/79)	0 (0/8)		19.5 (31/159)	26.4 (14/53)	24.6 (14/57)	6.1 (3/49)		
DK/CR	19.1 (71/372)	30.7 (63/205)	40.9 (38/93)	18.6 (18/97)	46.7 (7/15)	0.001* (χ2 = 13.0, df = 2)	4.8 (8/167)	0 (0/53)	0 (0/57)	14.0 (8/57)	0.006* (FET = 10.2)	<0.001* (χ2 = 40.1, df = 1)
Is there any treatment in humans?												
Yes	68.8 (159/231)	57.4 (81/141)	70.3 (45/64)	44.1 (30/68)	66.7 (6/9)	0.008* (FET = 9.6)	86.7 (78/90)	83.3 (35/42)	NA NA	89.6 (43/48)	0.384 (χ2 = 0.76, df = 1)	<0.001* (χ2 = 21.9, df = 1)
Is there any treatment in animals?												
Yes	67.1 (114/170)	57.3 (63/110)	NA NA	56.2 (54/96)	64.3 (9/14)	0.570 (χ2 = 0.32, df = 1)	85.0 (51/60)	NA NA	100 (6/6)	83.3 (45/54)	0.415 (FET = 0.58)	<0.001* (χ2 = 13.5, df = 1)
Vaccine available for dogs?												
Yes	76.3 (190/249)	82.8 (82/99)	NA NA	84.1 (74/88)	72.7 (8/11)	0.346 (FET = 0.40)	72.0 (108/150)	64.7 (55/85)	100 (11/11)	77.8 (42/54)	0.068 (FET = 5.4)	0.049* (χ2 = 3.9, df = 1)

[a]Including horses, sheep, goats, cattle, rabbits, other domestic animals, wild carnivores, other wild animals
Abbreviations: profs - professionals; DK/CR - Don't know/Can't remember; EH – Environmental Health; vet – veterinary; med – medicine; FET – Fisher's exact test
*Statistically significant

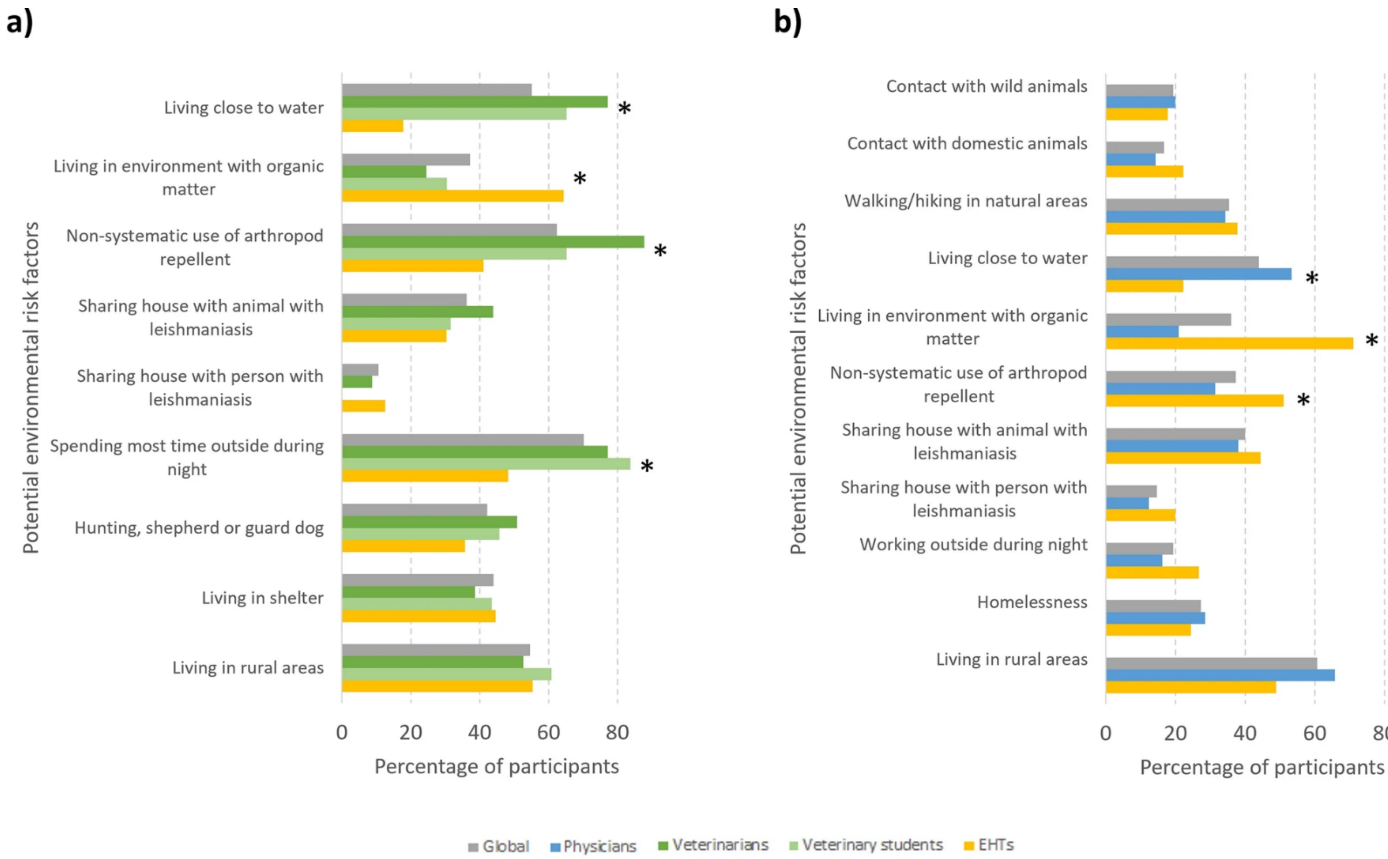
Figure 2. Percentage of participants, globally and by student/professional group, reporting having heard of leishmaniasis in different contexts: a) during the course, b) during professional activities, c) outside the course/work (animal leishmaniasis), d) outside the course/work (human leishmaniasis). Asterisk indicates a significant difference at $^*P < 0.05$.



$^*p<0.05$
Abbreviations: EHTs – Environmental Health Technicians; EHSs – Environmental Health Students; TV – television

Around 90% of participants identified the pathogenic agent as a parasite. Of those who recognized animal and human disease, approximately 75% answered that the same species of *Leishmania* infects both animals and humans. Arthropod bite was identified as the main route of transmission by 97.1% of participants; among these, sand fly bite was the predominant answer, followed by mosquito bite, although the difference between the two was less pronounced for students. More than 10% of participants answered that leishmaniasis could be transmitted by direct contact with animals. When questioned about the periods of highest phlebotomine activity, >50% of the EHTs selected each month between May and September (inclusively); 82.2% selected dusk and 51.1% selected night. The preferred sand fly breeding grounds pointed by the EHTs were domestic animal shelters (67.4%), decomposing vegetal matter (50.0%), wild animal burrows (32.6%) and small water bodies (28.3%). Individual risk factors for leishmaniasis most often selected by medical students, physicians and EHTs were HIV infection/AIDS and use of immunosuppressive drugs; physicians who had previously diagnosed VL also recognized active malignancy and solid organ transplant as important risk factors. Both veterinarians and veterinary students selected the use of immunosuppressive drugs as the highest risk factor; however, the second most often selected answer was animal breed for veterinarians and juvenile age for students. For all groups, male sex was the least frequently selected risk factor. Figure 3 shows the percentage of participants who selected each potential environmental risk factor for animal or human leishmaniasis. The three most selected environmental risk factors for animal leishmaniasis were: (i) spending most time outside during night; (ii) non-systematic use of arthropod repellent; and (iii) living close to water. Veterinary students and veterinarians selected these three risk factors significantly more often than EHTs, who selected living in an environment with organic matter significantly more often than veterinary students and veterinarians. For human leishmaniasis, living in rural areas was clearly the most selected risk factor; living close to water was significantly more selected by physicians and living in an environment with organic matter was significantly more selected by EHTs.

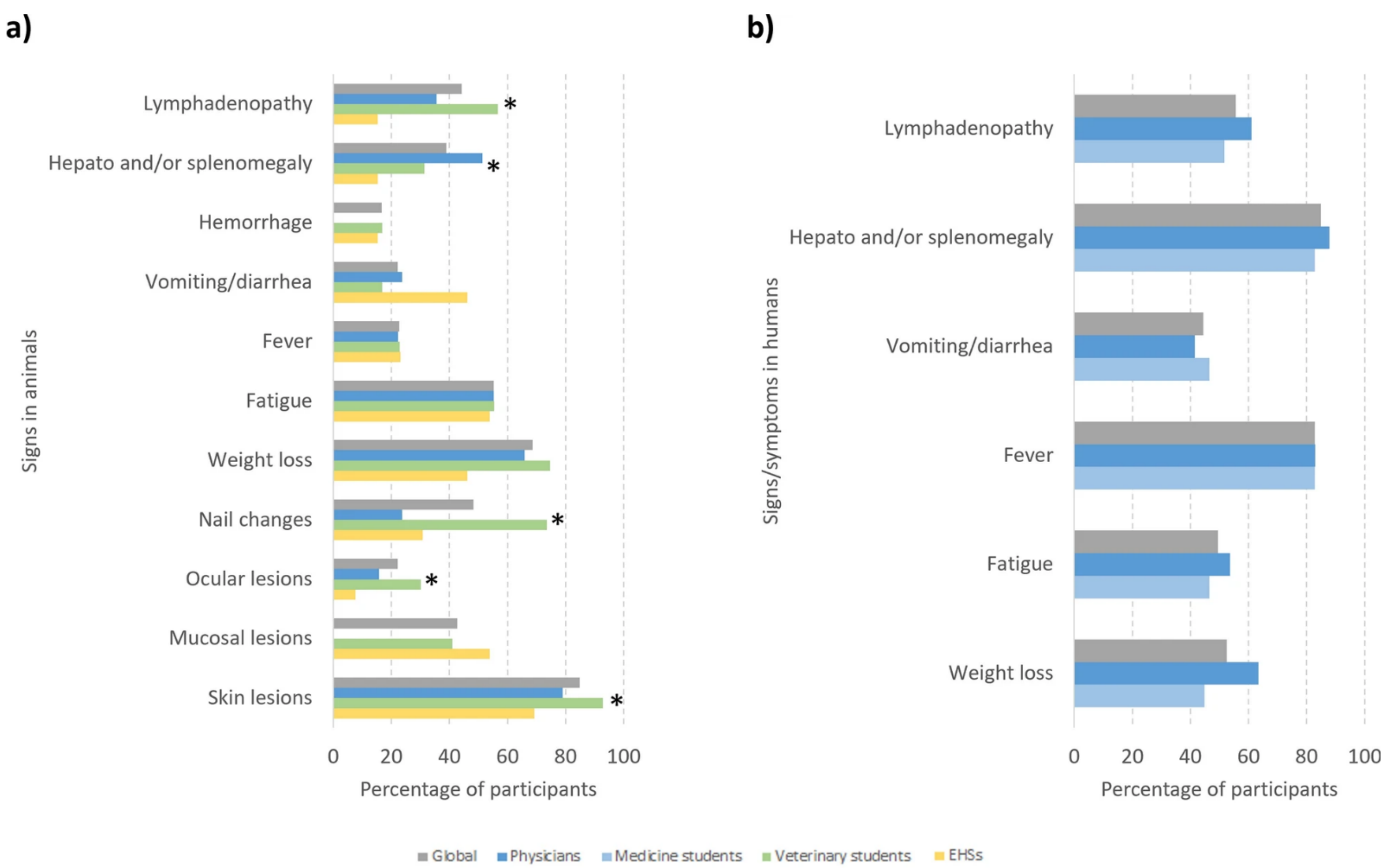
Figure 3. Percentage of participants, globally and by student/professional group, who selected each potential environmental risk factor for animal leishmaniasis (a) and human leishmaniasis (b). Asterisk indicates a significant difference at *$P < 0.05$.



*p<0.05
Abbreviations: EHTs – Environmental Health Technicians

Animal leishmaniasis was considered to be diagnosed in Portugal by 87.4% of participants and human leishmaniasis by 69.4%. Among human health students/professionals, diagnosis of VL in Portugal was recognized more commonly than diagnosis of CL (73.9% vs 60.6%, P = 0.009). Almost every participant who considered leishmaniasis was diagnosed in Portugal answered that the disease was endemic. The percentage of physicians who considered the disease was imported in all or most cases was 24.1% for VL and 54.0% for CL; for medical students, however, it was 82.4% for VL and 56.2% for CL. On the other hand, 92.2% of veterinarians considered cases of CanL were all or mostly autochthonous. Regarding animal hosts, dogs were almost universally selected as the species most affected by leishmaniasis; 19.9% of participants selected cats. All groups considered that *Leishmania* infection was mostly symptomatic. A significant proportion of students assumed not knowing or not remembering the signs of animal leishmaniasis (27.6% of medical students, 13.5% of veterinary students and 7.1% of EHSs). Among those participants who reported knowing the signs/symptoms of *Leishmania* infection, the percentage who selected each sign/symptom associated with animal or human leishmaniasis is shown in Fig. 4. In animal leishmaniasis, skin lesions, weight loss and fatigue were the most selected signs (no significant difference in percentage between groups, except for skin lesions, being less selected by EHSs); nail changes, ocular lesions and lymphadenopathy were significantly more recognized by veterinary students. In human leishmaniasis, hepato- and/or splenomegaly and fever were the most selected signs of disease; no significant difference in percentage was seen for any of the signs/symptoms analyzed between medical students and physicians.

Figure 4. Percentage of participants, globally and by student/professional group, who selected each sign/symptom associated with animal leishmaniasis (a) and human leishmaniasis (b).



*p<0.05
Abbreviations: EHSs – Environmental Health Technicians

Regarding the diagnosis of leishmaniasis, both medical students and physicians who had never seen cases of leishmaniasis considered blood to be the type of sample most used (selected by 65.6% of students and 81.6% of physicians); as the second most sample type used, physicians considered bone marrow (47.4%) and medical students considered lymph node (26.2%). Blood was also the type of sample most selected by veterinary students (89.8%), followed by lymph node (30.7%). Around 15% of veterinary and medical students assumed that they did not know the diagnostic techniques most often used. Among those who did know, the techniques most selected were serology (50.6%) and microscopy (33.7%) for veterinary students, and microscopy (61.1%) for medicine students; in contrast, 78.4% of physicians who had never seen cases of leishmaniasis selected PCR and 64.9% microscopy. Leishmaniasis was considered to be treatable in humans by 68.8% of participants and treatable in animals by 67.1%. In addition, 76.3% of participants recognized that a vaccine against leishmaniasis was available for dogs. The declaration of cases to Public or Animal Health authorities was considered to be mandatory by 43.9% of veterinarians for CanL and by 96.9% and 66.2% of physicians for VL and CL, respectively.

3.3. Results on perceptions

Answers to individual questions on perceptions are summarized in Table 3. Most veterinarians (77.4%) considered that there were > 50 cases of leishmaniasis diagnosed in their region of work. Among physicians who had previously diagnosed leishmaniasis, 86.8% considered that there was a "low" risk of a person developing the disease in their area of work. Additionally, the number of VL cases diagnosed in their region in the last 10 years was considered to be decreasing (48.7%) or stable (41.0%). For CL, 90.0% considered the number of cases to be stable. In contrast, 56.5% of veterinarians who had previously diagnosed leishmaniasis considered the number of cases to have increased in the last 10 years.

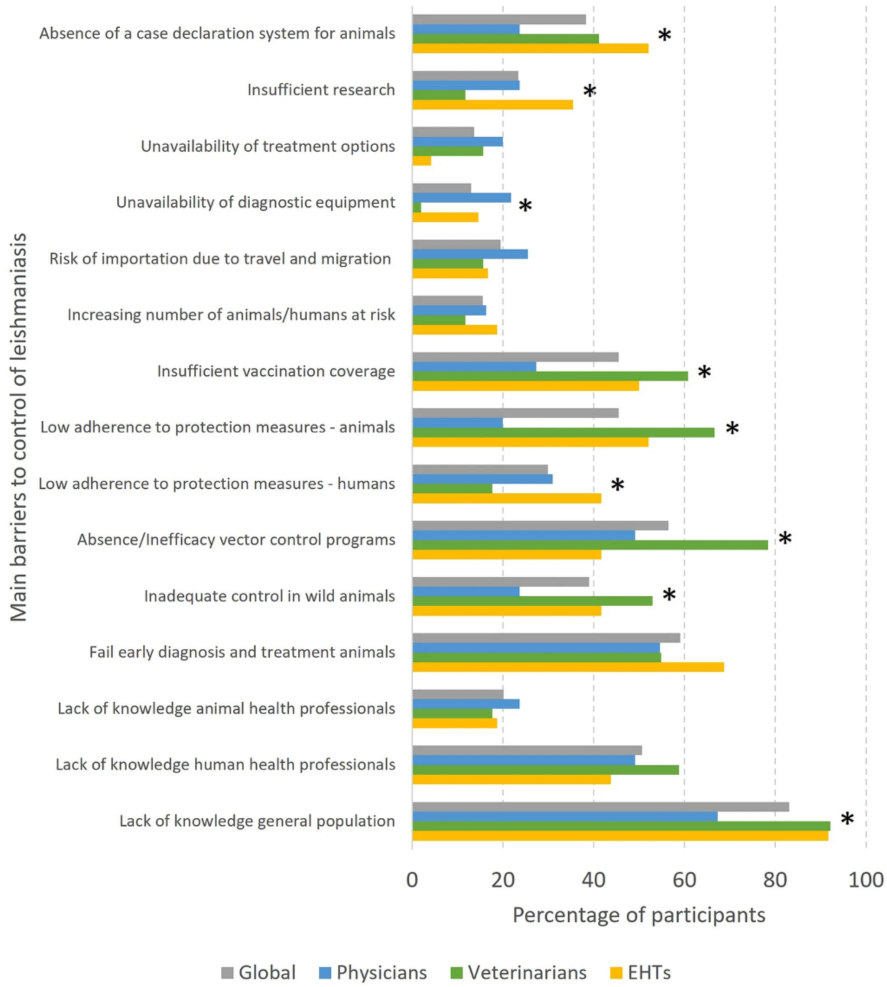
Table 3. Answers to the questions on perceptions, globally and by student/professional group.

	All	**Global students**	**Med students**	**Vet students**	**EH students**	***p*-value students**	**Global profs**	**Med profs**	**Vet profs**	**EH profs**	***p*-value profs**	**p-value global**
How important is it to include leishmaniasis in the training of (0-4):												
Veterinary students	3.71	3.70	3.64	3.76	3.92	0.063 (H = 5.5, df = 2)	3.71	3.53	3.88	3.86	<0.001* (H = 25.6, df = 2)	0.711 (H = 0.1, df = 1)
Medicine students	3.16	3.01	2.80	3.25	3.44	<0.001* (H = 14.1, df = 2)	3.27	2.93	3.55	3.75	<0.001* (H = 48.7, df = 2)	0.002* (H = 9.7, df = 1)
EH students	3.61	.	.	.	2.87		.	.	.	3.80		<0.001* (H = 15.7, df = 1)
Profs of respective group	.	.	.	.	.		.	3.25	3.73	3.77	<0.001* (H = 35.1, df = 2)	
How important do you think is the collaboration between physicians, veterinarians and environmental health technicians to eliminate leishmaniasis (0-3):												
	2.89	2.92	2.88	2.96	3.00	0.150 (H = 3.8, df = 2)	2.87	2.82	2.87	2.98	0.026* (H = 7.3, df = 2)	0.154 (H = 2.0, df = 1)

Abbreviations: profs – professionals; EH – Environmental Health; vet – veterinary; med - medicine
*Statistically significant

Inclusion of leishmaniasis in the veterinary curriculum was considered similarly important by students and professionals and among different subgroups of students; among professionals, however, physicians considered the inclusion of leishmaniasis as less important. All groups considered it to be more important to include leishmaniasis in the veterinary curriculum than in the human medicine curriculum; it was considered to be less important by more students than health professionals, and to be less important by students/professionals in the human health field. Inclusion of leishmaniasis in the academic program was considered to be more important by EHSs than by professionals. Regarding the inclusion of leishmaniasis in the training of professionals, physicians assessed as a group considered it to be less relevant for themselves. In terms of medical specialties for which training on leishmaniasis was considered to be more important, 95.5% selected Infectious Diseases, 87.5% Internal Medicine, 83.0% Pediatrics, 74.1% Dermatology, 56.2% Anatomopathology and 19.6% Others. The importance of collaboration between sectors was rated equally by students and professionals (Kruskal–Wallis test, H = 2.0, df = 1, $P = 0.154$); among professionals, importance was rated in descending order as: EHTs, veterinarians, physicians (Kruskal–Wallis test, H = 7.3, df = 2, *P* = 0.026). The creation of national guidelines for the diagnosis and management of leishmaniasis was considered to be "very important" by 84.0% of physicians for VL and 71.4% for CL. The implementation of a national structured plan to control leishmaniasis was considered to be "very important" by 89.6% of veterinarians and 87.5% of EHTs. Additionally, only 38.6% of veterinarians and 12.5% of physicians were satisfied with the information on animal or human leishmaniasis that appeared on official platforms, respectively (the remaining participants were not or had no opinion). Similarly, only 42.2% of veterinary students and 15.8% of medicine students were "very satisfied" with the quantity and quality of information presented in their courses. The main barriers pointed by professionals to the control of leishmaniasis were (note: options selected by > 50% of respondents): lack of knowledge in the general population, failure in early diagnosis and treatment of diseased animals, absence/inefficacy of vector control programs and lack of knowledge by human health professionals. Significant differences were reported for some of these barriers, as shown in Fig. 5. Low adherence to protection measures in animals, insufficient vaccination coverage and inadequate control in wild animals were the most common barriers considered by veterinarians, while absence of a case declaration system for animals, low adherence to protection measures in humans and insufficient research were pointed out mostly by EHTs. Unavailability of diagnostic equipment was the most common barrier considered by physicians.

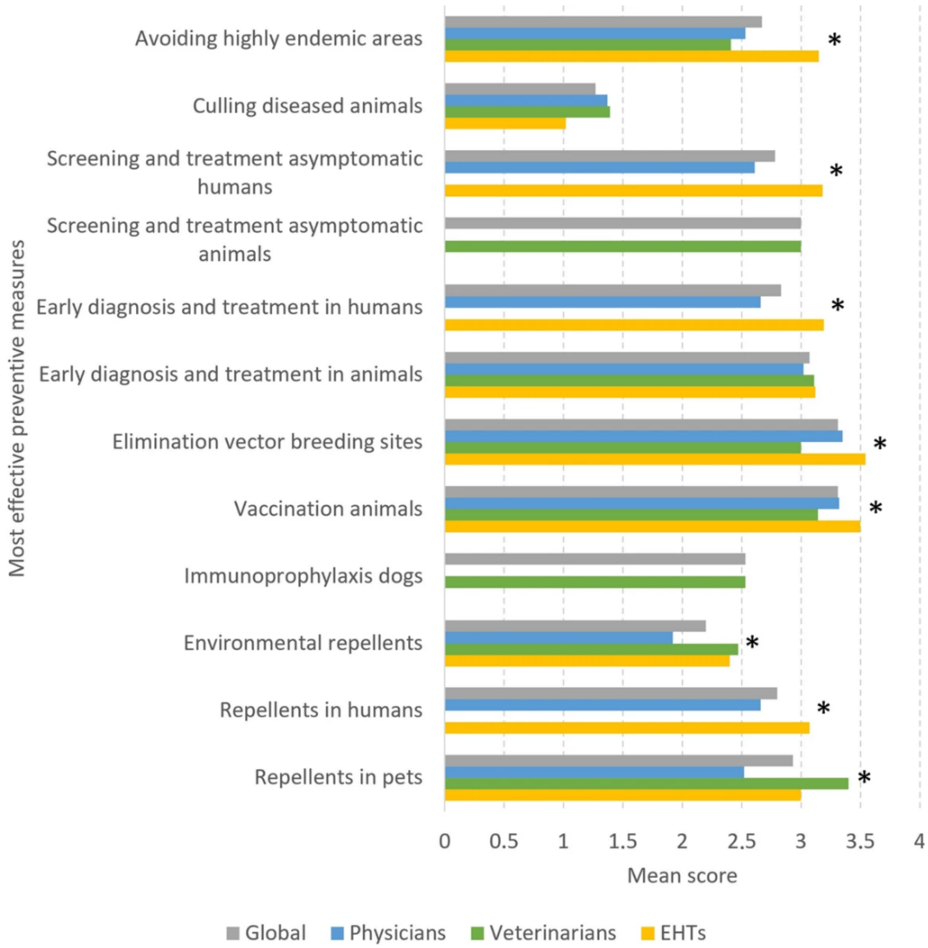
Figure 5. Percentage of participants, globally and by student/professional group, who selected each potential main barrier to the control of leishmaniasis. Asterisk indicates a significant difference at $*P < 0.05$.



*p<0.05
Abbreviations: EHTs – Environmental Health Technicians

The mean scores for effective measures in leishmaniasis control is shown in Fig. 6. Overall, vaccination of animals, elimination of vector breeding sites, early diagnosis and treatment of diseased animals and use of repellents in pets were considered to be the most effective measures. Most measures evaluated were scored significantly differently between groups: use of repellent in pets and use of environmental repellents were scored higher by veterinarians; vaccination of animals, elimination of vector breeding sites and avoiding highly endemic areas were scored higher by EHTs. Early diagnosis and treatment in animals and culling of diseased animals were not scored significantly differently.

Figure 6. Mean score for each possibly most effective measure in leishmaniasis control, globally and by student/professional group. Asterisk indicates a significant difference at *$P < 0.05$.



*$p<0.05$
Abbreviations: EHSs – Environmental Health Technicians

3.4. Results on general practices

Answers to individual questions on general practices are summarized in Table 4. Seeing cases of leishmaniasis in course/work was more common for professionals than for students (Chi-square test, $\chi2 = 76.5$, df = 1, $p < 0.001$) and in the animal health field than in the human (Chi-square test, $\chi2 = 27.5$, df = 1, $p < 0.001$).

Table 4. Table 4 Answers to general practices questions, globally and by student/professional group.

	All	Global students	Med students	Vet students	EH students	*p*-value students	Global profs	Med profs	Vet profs	EH profs	*p*-value profs	p-value global
Seen cases in course/work?												
Yes	39.1 (127/325)	13.8 (21/152)	0 (0/71)	25.9 (21/81)	NA	<0.001* (FET = 18.6)	61.3 (106/173)	48.3 (56/116)	89.5 (51/57)	NA	<0.001* (χ^2 = 27.5, df = 1)	<0.001* (χ^2 = 76.5, df = 1)
Repellent use in outdoor activities during dusk/night												
Always	4.7 (19/403)	5.1 (11/215)	6.6 (8/122)	2.6 (2/78)	6.7 (1/15)	0.772 (FET = 1.8)	4.3 (8/188)	3.2 (3/95)	2.1 (1/47)	8.7 (4/46)	0.471 (FET = 3.5)	0.020* (χ^2 = 7.9, df = 2)
Sometimes	32.8 (132/403)	38.6 (83/215)	36.9 (45/122)	41.0 (32/78)	40.0 (6/15)		26.1 (49/188)	24.2 (23/95)	29.8 (14/47)	26.1 (12/46)		
Never	62.5 (252/403)	56.3 (121/215)	56.6 (69/122)	56.4 (44/78)	53.3 (8/15)		69.7 (131/188)	72.6 (69/95)	68.1 (32/47)	65.2 (30/46)		
Not applicable	16.7 (81/484)	15.0 (38/253)	12.9 (18/140)	19.6 (19/97)	6.2 (1/16)		18.6 (43/231)	17.4 (20/115)	17.5 (10/57)	22.0 (13/59)		
Nets in doors/windows												
All	4.1 (20/484)	5.9 (15/253)	6.4 (9/140)	5.2 (5/97)	6.2 (1/16)	0.974 (FET = 0.49)	2.2 (5/231)	3.5 (4/115)	0 (0/57)	1.7 (1/59)	0.405 (FET = 4.0)	0.051 (χ^2 = 6.0, df = 2)
Some	15.3 (74/484)	17.0 (43/253)	15.7 (22/140)	18.6 (18/97)	18.8 (3/16)		13.4 (31/231)	10.4 (12/115)	12.3 (7/57)	20.3 (12/59)		
No	80.6 (390/484)	77.1 (195/253)	77.9 (109/140)	76.3 (74/97)	75.0 (12/16)		84.4 (195/231)	86.1 (99/115)	87.7 (50/57)	78.0 (46/59)		
Pet ownership												
Dog(s)	47.3 (229/484)	57.7 (146/253)	48.9 (68/139)	72.4 (71/98)	43.8 (7/16)	<0.001* (FET = 36.8)	35.9 (83/231)	28.7 (33/115)	52.6 (30/57)	33.9 (20/59)	<0.001* (χ^2 = 36.8, df = 4)	<0.001* (χ^2 = 23.0, df = 2)
Other(s)	25.8 (125/484)	20.9 (53/253)	16.5 (23/139)	24.5 (24/98)	37.5 (6/16)		31.2 (72/231)	23.5 (27/115)	45.6 (26/57)	32.2 (19/59)		
No	26.9 (130/484)	21.3 (54/253)	34.5 (48/139)	3.1 (3/98)	18.7 (3/16)		32.9 (76/231)	47.8 (55/115)	1.8 (1/57)	33.9 (20/59)		
Dog(s) spend(s) time outdoors during dusk/night?												
Yes	72.5 (166/229)	77.4 (113/146)	83.8 (57/68)	73.2 (52/71)	57.1 (4/7)	0.139 (FET = 3.9)	63.9 (53/83)	75.8 (25/33)	50.0 (15/30)	65.0 (13/20)	0.104 (χ^2 = 4.5, df = 2)	0.027* (χ^2 = 4.9, df = 1)
Repellent use in dog(s)												
Yes	78.5 (179/228)	77.2 (112/145)	80.6 (54/67)	74.6 (53/71)	71.4 (5/7)	0.659 (FET = 0.84)	80.7 (67/83)	69.7 (23/33)	90.0 (27/30)	85.0 (17/20)	0.107 (FET = 4.5)	0.538 (χ^2 = 0.38, df = 1)
All year round	78.8 (141/179)	82.1 (92/112)	81.5 (44/54)	81.1 (43/53)	100 (5/5)	0.730 (FET = 0.63)	73.1 (49/67)	65.2 (15/23)	81.5 (22/27)	70.6 (12/17)	0.417 (χ^2 = 1.7, df = 2)	0.154 (χ^2 = 2.0, df = 1)
Some months	21.2 (38/179)	17.9 (20/112)	18.5 (10/54)	18.9 (10/53)	0 (0/5)		26.9 (18/67)	34.8 (8/23)	18.5 (5/27)	29.4 (5/17)		
Spot-on	57.4 (103/179)	59.8 (67/112)	61.1 (33/54)	54.7 (29/53)	100 (5/5)		53.7 (36/67)	65.2 (15/23)	40.7 (11/27)	58.8 (10/17)		
Collar	54.7 (98/179)	57.1 (64/112)	51.9 (28/54)	64.2 (34/53)	40.0 (2/5)		50.7 (34/67)	47.8 (11/23)	63.0 (17/27)	35.3 (6/17)		
Pills	38.0 (68/179)	39.3 (44/112)	38.9 (21/54)	43.4 (23/53)	0 (0/5)		35.8 (24/67)	47.8 (11/23)	33.3 (9/27)	23.5 (4/17)		
Shampoo	8.4 (15/179)	8.0 (9/112)	7.4 (4/54)	7.5 (4/53)	20.0 (1/5)		9.0 (6/67)	21.7 (5/23)	0 (0/27)	5.9 (1/17)		
Spray	5.0 (9/179)	5.4 (6/112)	3.7 (2/54)	7.5 (4/53)	0 (0/5)		4.5 (3/67)	8.7 (2/23)	0 (0/27)	5.9 (1/17)		
Vaccination of dog(s)												
Every year	42.4 (73/172)	42.4 (56/132)	41.2 (28/68)	45.6 (26/57)	28.6 (2/7)	0.660 (FET = 0.83)	42.5 (17/40)	47.6 (10/21)	25.0 (1/4)	40.0 (6/15)	0.682 (FET = 0.76)	0.993 (χ^2 < 0.01, df = 1)
Some years/No/DK/CR	57.6 (99/172)	57.6 (76/132)	58.8 (40/68)	54.4 (31/57)	71.4 (5/7)		57.5 (23/40)	52.4 (11/21)	75.0 (3/4)	60.0 (9/15)		
Veterinary observation of dog(s)												
At least once a year	91.7 (209/228)	89.0 (129/145)	88.1 (59/67)	90.1 (64/71)	85.7 (6/7)	0.891 (FET = 0.23)	96.4 (80/83)	97.0 (32/33)	100 (30/30)	90.0 (18/20)	0.452 (FET = 1.6)	0.051 (χ^2 = 3.8, df = 1)
Less than once a year	8.3 (19/228)	11.0 (16/145)	11.9 (8/67)	9.9 (7/71)	14.3 (1/7)		3.6 (3/83)	3.0 (1/33)	0 (0/30)	10.0 (2/20)		

Abbreviations: profs - professionals; DK/CR - Don't Know/Can't remember; EH – Environmental Health; vet – veterinary; med – medicine
*Statistically significant

Most participants reported "Never" to using arthropod repellent during outdoor activities at dusk/night, with the proportion of "Never' responses being significantly higher in professionals (Chi-square test, $\chi 2 = 7.9$, df = 2, $P = 0.020$). Most participants mentioned having "No" nets in doors/windows, and this proportion was similar between groups (Chi-square test, $\chi 2 = 6.0$, df = 2, $P = 0.051$). Approximately half of the participants (47.3%) were dog owners, although dog ownership was significantly more common among students (57.7 vs 35.9%) and professionals in the animal health field (Fisher's exact test, 36.8, $P < 0.001$). Most participants reported that their dog(s) spent time outside between dusk and dawn, although this response was higher for students (Chi-square test, $\chi 2 = 4.9$, df = 1, $p = 0.027$). Of the dog owners who mentioned that their dogs spent time outdoors during the night, 77.1% selected garden/yard, 30.1% street/road, 9.6% forest/bush and 5.4% other. The use of arthropod repellent on dog(s) was reported by > 75% of participants and was year-round for most dogs (78.8%), with no significant differences between groups. In general, spot-on treatments were the mostly commonly used arthropod repellent method used by both students and professionals, followed by collars and pills. Among veterinarians and veterinary students, however, collars were preferred over spot-on treatments. Approximately 40% of the participants reported vaccinating their dog(s) "every year," with similar proportions among groups (Chi-square test, $\chi 2 < 0.01$, df = 1, $p = 0.993$). Over 90% of participants mentioned taking their dog(s) for veterinary observation "at least once a year" and this was similar between groups (Chi-square test, $\chi 2 = 3.8$, df = 1, $p = 0.051$).

3.5. Results on professional practices

Answers to individual questions on professional practices are summarized in Table 5. Among professionals who had seen leishmaniasis, 77.1% of veterinarians and 11.3% of physicians had seen > 10 cases. Of physicians who reported having been involved in diagnosing/treating patients with leishmaniasis, 75.0% mentioned only VL, 3.6% only CL and 21.4% both.

Table 5. Answers to questions on professional practices, globally and by professional group.

	Veterinarians CanL	**Physicians** VL	***p*-value**
Number of cases seen (%)			
1-5	14.6 (7/48)	75.5 (40/53)	<0.001* ($\chi2$ = 46.2, df = 2)
6-10	8.3 (4/48)	13.2 (7/53)	
11-50	39.6 (19/48)	11.3 (6/53)	
>50	37.5 (18/48)		
Type of sample used for diagnosis (0-3)			
Bone marrow biopsy/aspirate	0.49	2.22	<0.001* (U = 298.0)
Liver biopsy/aspirate	.	0.85	
Lymph node biopsy/aspirate	1.00	1.12	0.485 (U = 1129.5)
Spleen biopsy/aspirate	0.20	0.43	0.074 (U = 1005.5)
Skin	0.78	.	
Blood	2.84	2.18	<0.001* (U = 808.5)
Type of laboratory exam used for diagnosis (0-3)			
Culture	0.24	0.96	<0.001* (U = 722.0)
Microscopy of smear/histology	0.78	2.08	<0.001* (U = 501.5)
PCR	1.32	2.19	<0.001* (U = 647.5)
Quantitative serology	2.53	1.77	<0.001* (U = 623.5)
Qualitative serology	2.23		
Drug used for treatment (0-4)			
Miltefosine	1.75	1.11	<0.001* (U = 824.5)
MA/Pentavalent antimonials	2.02	0.80	<0.001* (U = 536.0)
Allopurinol	3.30	NA	
Allopurinol+MA	2.26	NA	
Allopurinol+miltefosine	1.90	NA	
Liposomal amphotericin B	NA	3.32	
Paromomycin	NA	0.26	
Amphotericin B deoxycholate	NA	0.24	
Pentamidine	NA	0.12	
Recommended individual protective measures to patients (%)			
Often/always	93.0 (53/57)	8.1 (8/99)	<0.001* ($\chi2$ = 109.5, df = 1)

Abbreviations: CanL - canine leishmaniasis; VL - visceral leishmaniasis; PCR - polymerase chain reaction; MA - meglumine antimoniate;
*Statistically significant

The most commonly used samples for diagnosing VL were bone marrow biopsy/aspirate and blood. PCR was the most common technique used for diagnosis, followed by microscopy. The factors reported to most influence the choice of diagnostic method for VL were availability (72.9%), sensitivity (72.9%), specificity (64.6%) and speed (54.2%) of test; need to send a sample to an external laboratory (16.7%); and cost (2.1%). Liposomal amphotericin B (LAmB) was the drug reported as most often used to treat VL, followed by miltefosine. LAmB was considered to be more effective than miltefosine, and both drugs were suggested as being more effective in immunocompetent than immunosuppressed patients. Only 6/44 physicians reported ever using amphotericin B deoxycolate, 7/45 paromomycin and 4/42 pentamidine. The factors reported out to most influence the choice of treatment for VL were availability (83.0%), side effects (55.3%), degree of immunosuppression (40.4%), formulation (27.7%) and cost (10.6%). In the case of failure/relapse, 58.6% of physicians reported initiating treatment with combination of drugs and 24.1% reported continuing with the same drug for a longer duration. Only 14/116 physicians reported having seen cases of CL (all but one mentioned 1–5 cases). Of these, 61.5% reported having seen CL cases resulting from infection in Latin America, 38.5% from infection in southern Europe, 30.8% from infection in the Middle East and 15.4% from infection in North Africa. The most frequently observed type of lesion was an ulcer, and 86.7% of physicians reported obtaining samples by excisional biopsy. The techniques most often used for CL diagnosis were (in descending order): microscopy, PCR, serology and culture. Only 20% of respondents answered that they “Always” or “Often” identified the infecting species of *Leishmania* in their cases. Among the species identified, *L. infantum* was mentioned most (n = 6). The most commonly used treatment strategies for CL were watch and wait, LAmB, miltefosine and intralesional antimonial; none of these strategies was frequently used by > 30% of clinicians. Factors most selected as influencing the choice of treatment were availability (70%), number (60%) and location of lesions (50%) and side effects (50%). Among clinicians, 58.3% considered CL treatment to be “moderately effective.” For CanL, serology was the technique reported to be most used for diagnosis. Most veterinarians (66.7%) said they “always” recommended treatment of *Leishmania* infection in dogs while 25.5% initiated treatment only if dogs were symptomatic, regardless of severity. Allopurinol alone was the treatment regimen most often reported, followed by a combination of allopurinol + meglumine antimoniate (MA) and MA alone. Allopurinol + MA was considered to be more effective than single drug treatments, both in moderate/mild and in severe disease. Seeing cases of leishmaniasis in other animals was reported by 14.0% (8/57) of veterinarians, all of whom mentioned cats (1–2 cases each) and one mentioned a horse. Most veterinarians (93.0%) but only 8.1% of physicians recommended individual protection measures to patients.

3.6. Scoring KPP and associations with sociodemographic factors

The distribution of individual K, Per and Pra scores is shown in Additional file 4: Figure S3. Median K and Per scores were higher in professionals than in students. Among students, median K and Per scores were significantly higher in veterinary students, while among professionals, they were significantly higher in veterinarians and EHTs. Median Pra scores were not significantly different between groups and subgroups. Factors associated with higher K score in the univariate analysis were age > 20 years (for students) or > 30 years (for professionals), higher academic year of study, more years of professional experience, Infectious Diseases specialty, having seen cases at work and specialist level. In the multivariate analysis, however, for students, only higher academic year of study was associated with a K score higher than the median K score (OR 3.49, 95% CI 1.48–8.21, $P = 0.004$) and for professionals, only for physicians was having seen cases of leishmaniasis associated with a K score higher than the median K score (OR 14.23, 95% CI 3.79–53.45, $P < 0.001$). Factors associated with higher Per score in the univariate analysis were professional status, age > 25 years, residing outside the Centro or LVT regions, no dog ownership and K score above the median K score (> 6.5). However, in the multivariate analysis, no dog ownership (OR 2.19, 95% CI 1.46–3.27, $P < 0.001$) and K score above the median K score (> 6.5) (OR 4.69, 95% CI 3.03–7.28, $p < 0.001$) were the only factors associated with a higher than median Per score globally. Factors associated with higher Pra score in the univariate analysis were age > 30 years old and living outside the Norte region; in the multivariate analysis, both factors remained significant (OR 1.88, 95% CI 1.08–3.26, $P = 0.025$; and OR 1.56, 95% CI 1.03–2.34, $P = 0.035$, respectively) (Table 6).

Table 6. Potential factors for knowledge (students and professionals), perception and practices scores above the respective median score, according to logistic regression models to estimate crude and adjusted odds ratio values.

a)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
K >6.5 (students)	Female gender	83.3	1.37	0.62-3.03	1.65	0.71-3.80	0.242
	Age >20 yo	58.3	2.67	1.43-4.97	1.37	0.62-3.00	0.434
	Year of study >2nd	60.9	4.23	2.08-8.61	3.49	1.48-8.21	**0.004**
	No ownership of dog(s)	50.4	1.54	0.87-2.70	1.49	0.82-2.73	0.193
	Constant				0.036		<0.001
	Hosmer and Lemeshow Test				Sig.=0.975		

b)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
K >6.5 (professionals)	Male gender	28.4	3.01	1.25-7.25	1.48	0.43-5.12	0.534
	Age >30 yo	72.4	3.76	1.58-8.97	1.12	0.14-8.70	0.913
	Nyp experience ≥10	47.4	2.77	1.30-5.91	1.93	0.47-7.87	0.360
	ID specialty	23.1	14.88	3.28-67.63	2.05	0.34-12.26	0.432
	Seen cases of leishmaniasis	48.3	14.36	5.76-35.83	14.23	3.79-53.45	**<0.001**
	Specialist level	64.7	5.40	2.26-12.89	2.47	0.33-18.22	0.376
	No ownership of dog(s)	51.7	2.54	1.20-5.41	2.57	0.87-7.56	0.087
	Constant				0.043		0.092
	Hosmer and Lemeshow Test				Sig.=0.419		

c)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
Per >9 (global)	Professional	47.7	2.20	1.53-3.17	1.61	0.74-3.49	0.226
	Female gender	80.2	1.27	0.80-2.00	1.57	0.94-2.61	0.085
	Age >25 yo	54.2	1.98	1.37-2.86	1.42	0.65-3.13	0.381
	Residing outside Centro/LVT	38.3	1.62	1.12-2.35	1.51	0.99-2.28	0.051
	No ownership of dogs	47.4	2.23	1.54-3.23	2.19	1.46-3.27	**<0.001**
	K score >6.5	45.5	5.11	3.47-7.53	4.69	3.03-7.28	**<0.001**
	Constant				0.452		0.116
	Hosmer and Lemeshow Test				0.470		

d)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude OR	95% CI	Adjusted OR	95% CI	*p*-value
Pra >1.5 (global)	Professional	47.7	1.10	0.76-1.57	1.45	0.84-2.51	0.180
	Age >30 yo	37.4	1.45	1.01-2.10	1.88	1.08-3.26	**0.025**
	Residing outside Norte	70.4	1.54	1.03-2.32	1.56	1.03-2.34	**0.035**
	K score >6.5	45.5	1.24	0.87-1.78	1.17	0.78-1.76	0.439
	Constant				0.235		0.003
	Hosmer and Lemeshow Test				0.934		

Abbreviations: yo - years old; Nyp - number of years of professional; K - Knowledge; Per - Perceptions; Pra - Practices; OR - odds ratio; CI - confidence interval; ID - Infectious Diseases; LVT - Lisboa e Vale do Tejo. Reference categories:

a) Male gender; Age ≤20 yo; year of study 1st or 2nd; ownership of dogs
b) Female gender; age ≤30 yo; Nyp experience <10; non-ID specialty; not previously seen cases of leishmaniasis; trainee level; ownership of dogs
c) Student; male gender; age ≤25 yo; residing in Centro or LVT region; ownership of dogs; K score ≤6.5
d) Student; age ≤30 yo; residing in Norte region; K score ≤6.5

4. Discussion

This study represents the first national study of knowledge, perceptions and practices (KPP) on leishmaniasis in health students and professionals, including veterinarians. At a global level, few studies have addressed the human medicine and environmental health fields, although the zoonotic and vector-borne nature of *Leishmania* infection implies that all these fields are actively involved in its management and treatment. In the present study, not only had > 95% of students and professionals heard previously of leishmaniasis, but also the majority acknowledged it as a zoonosis, although the majority was lower in the human medicine side. Comparisons of different student categories should be seen in the light of different distribution of students by year of study; similarly, professionals of different fields were not distributed equally by years of professional experience. Leishmaniasis seems to be consistently included in the courses in the three groups, especially in theoretical classes; it is possible that the disease is a focus of study earlier in the veterinary than in the medical curriculum. In the professional context, in addition to the direct observation of cases of disease, workshops, congresses, courses and conversations with colleagues were most often mentioned as the sources of information on leishmaniasis, highlighting the importance of continuous education and informal and peer education. Approximately half of the participants reported having heard about leishmaniasis outside of their work, with particular sources being television advertisements and conversation with veterinarians on animal leishmaniasis and via an internet search for human leishmaniasis. Although no studies in Europe have previously addressed the non-occupational sources of information on leishmaniasis, it is likely that television plays an important role via pesticide repellent advertisements for pets; however, human disease is not addressed in this platform and much less disseminated in all communication media (all less selected for human vs animal leishmaniasis). Even though arthropod bite was correctly identified as a main route of transmission by almost every participant, mosquitoes were commonly pointed as the arthropod vector. This could explain why many participants considered living close to water to be a relevant environmental risk factor for both animal and human leishmaniasis and why only EHTs more often pointed to organic matter as a relevant risk factor. This lack of knowledge on the vector could lead to inefficient/incorrect counseling of animal owners and at-risk human groups on specific prevention strategies against phlebotomine sand flies, such as no accumulation of decaying organic matter [42]; in other endemic settings, such as in areas of Brazil, veterinarians systematically recommend keeping the domestic environment free from organic matter [43]. It should be notes that direct contact with infected animals was also considered to be an important route of transmission by > 10% of participants, similarly by students and professionals, although this route has rarely been documented, and then only in dogs [44], possibly leading to inaccurate information being provided to dog/animal owners regarding their personal risk. EHTs seem to be adequately informed about periods of activity and breeding grounds of sand flies according to current knowledge on their biology [45]. Physicians and medical students referred to HIV infection/AIDS and use of immunosuppressive drugs as the most significant individual host risk factors, which is in accordance with the literature, which suggests increased risk of progression to disease in persons with HIV infection/AIDS [46], and with national data revealing that 51.8% of persons with VL diagnosed between 1999 and 2009 were coinfected with HIV and 6.5% had other immunosuppressive condition [5]. Although male sex was the least frequently selected risk factor among our respondents, studies in South Asia suggest that it as a biological risk factor, regardless of sociocultural differences based in gender [47]. The participants in our questionnaire survey considered that animal leishmaniasis was more often diagnosed in Portugal than human leishmaniasis, likely reflecting the higher incidence of the former disease and the high seroprevalence in the canine population (7). CanL and VL cases were considered to be mostly autochthonous by professionals; in available data, 76.4% of cases of VL were assumed to be autochthonous, since they were living in known endemic foci in Portugal [5]. Students were unaware of this. Information on the situation of CL is scarce in Portugal since notification of the disease is not mandatory, while in other southern European countries it has been reported that approximately one half of CL cases seem to be imported (in Spain [48] and France [49]). In the present study, clinicians were polarized between either mostly imported or mostly autochthonous. Asymptomatic infection has been extensively described as the most common result of exposure to *Leishmania* parasites, but only approximately 25% of students and professionals were aware of this fact, although it could be an important consideration for interpretation of positive serological results in sick individuals in endemic areas, where it could represent an incidental finding [2]. On the other hand, asymptomatic, latent infections represent both an individual and public health problem since they can reactivate and progress to overt disease in specific settings, such as in the context of iatrogenic/pharmacologic immunosuppression (in transplant, autoimmune diseases, etc.) and older age, with an aged population representing an increasing share of the population [50]. Additionally undetected and/or neglected asymptomatic infections could compromise disease control in Portugal, as it is being increasingly recognized that asymptomatic individuals can transmit the parasite to phlebotomine sand flies, especially when immunosuppressed [51]. Common signs of animal leishmaniasis (skin lesions, weight loss, fatigue) were correctly identified by most students and professionals not active in diagnosing CanL [52], although other common signs were specifically mentioned more frequently by veterinary students, such as nail and ocular changes. For human leishmaniasis, hepatosplenomegaly and fever were more often chosen by the participants, consistent with case series of VL [53]. Regarding diagnosis, students and physicians who had never seen cases of VL more often chose blood as the sample type for testing; however, bone marrow seems to be preferentially used in Portugal (5). Due to the low incidence of the disease, most physicians have never seen any case of VL, but this could change in the future, raising the possibility of a gap in knowledge of locally available diagnostic protocols and techniques. Mandatory declaration of VL cases was correctly indicated by most physicians, possibly suggesting that underreporting (as shown in a previous study [5]) may be related to other issues in addition to lack of knowledge, such as forgetting to report, lack of time, complexity or low user friendliness, lack of feedback on notified cases; some of these have already been reported in other countries for mandatory declaration diseases in general [54]. Even though CL is a non-notifiable disease in Portugal, it was considered to be otherwise by most physicians. Perceptions of health professionals in terms of trend in number of cases in their region of work are compatible with the decrease in the number of VL cases reported annually in the period of 2014–2018 [4] compared to pre-2010 [5] and with increasing national canine seroprevalence [7, 8]. Inclusion of leishmaniasis in the curriculum and training of physicians and veterinarians is perceived as important, but for participants who recognize the disease as zoonotic, the lower rating for human health students and professionals could be related to the low incidence of the human disease [3] and to the fact that these cases are usually seen only by certain medical specialists. Although regional European guidelines for the management of leishmaniasis have been developed [1], most clinicians considered the creation of national guidelines to be very important and were not satisfied with the information available in official platforms. Although systematic surveillance of phlebotomine sand flies is included in the Vector Surveillance Network (REVIVE [Rede de Vigilância de Vetores]), no national program is currently implemented to control leishmaniasis, which could explain why absence/inefficacy of vector control programs was often perceived as a barrier to controlling leishmaniasis. Low adherence to protection measures in animals and insufficient vaccination coverage were also barriers often pointed by the veterinarians. In Portugal, although > 90% of dog owners seem to use ectoparasiticides for their dogs, the type of drug used and the frequency of application are often inappropriate to adequately prevent sand fly bites (15, 16). The use of vaccination as a preventative strategy is estimated at around 15% [7]. All of the interventions considered to be most effective (vaccination of animals, early diagnosis and treatment of diseased animals and use of repellents in animal pets) have been related in previous studies to control of the disease in diverse settings [55], except for the elimination of vector breeding sites. Practices among physicians highlight that CL was less commonly seen, and that when seen it was often by professionals who also reported seeing cases of VL; it is possible that most diagnosed CL cases present simultaneous visceral involvement, as shown in data from inpatients in Portugal, where only 3/21 had isolated CL (5). It would appear likely that many cases of isolated, uncomplicated CL do not come to medical attention and are not diagnosed; it should be noted, however, that only one dermatologist was enrolled in the present study. Diagnosis of VL has often relied on testing samples of bone marrow, although European guidelines suggest serology as the first-line approach [1], possibly because bone marrow aspirate or biopsy could be more informative in terms of differential diagnoses and also because serology may not be widely available. It should be noted that PCR was reported as the most used technique, which contrasts with data from 1999 to 2009 (use of PCR in only approx. 25% of cases [5]) and probably relates to a wider availability of this technique in more recent years. These assumptions are supported by the finding that availability was the factor most often selected as influencing the choice of diagnostic method. Preferential use of LAmB is consistent with previously reported data [5], and the seldom use of other regimens could also be related to weaker evidence for the use of miltefosine in the European region [1] and limited availability. Answers provided by physicians regarding CL suggest that each professional has seen few cases, reflecting low experience in diagnosing and treating this disease. Samples were most often reported to be obtained by excisional biopsy and tested by histopathology. A significant proportion of cases observed were suspected to originate from Latin America, consistent with an increasing migrant population from Brazil and more intense travel to the region [56]. Systematic identification of *Leishmania* species was reported by a minority of clinicians, suggesting treatment selection could be performed by inferring species identification based on geographical location. Additionally, monitoring of potential import and establishment of new species in the country is limited. Concerning CanL, the use of serology for diagnosis was commonly reported, following the LeishVet diagnostic approach [52]. Treatment strategies favored allopurinol alone or allopurinol + MA, reflecting that veterinarians are likely following recommendations for mild or moderate/severe disease, respectively. It is interesting to note that 8/57 veterinarians reported having seen cases of feline leishmaniasis, reflecting a raising awareness of this endemic infection in cats and suggesting clinical cases are likely to be more common than reported [57]. The results of the present study overlap with findings from a previous Portuguese study that involved 141 veterinarians, with the results showing that > 50% of the veterinarians saw > 10 cases of leishmaniasis per year; serology was reported to be the preferred method for diagnosis (especially the immunofluorescence antibody test [IFAT]), followed by PCR in lymph nodes and/or bone marrow; allopurinol + MA was the most commonly used treatment regime. Two other recent national studies [21, 58] showed similar findings regarding diagnosis and treatment; additionally, in these latter studies, 31.3% of veterinarians reported not following any guidelines, even though 93.0% of responders were aware of their existence. In would appear that owner financial restraints negatively influenced veterinary follow-up and relapse recognition. Accordingly, two international studies including veterinarians in Portugal [59, 60] showed that veterinarians in Portugal took a relatively higher consideration of the impact of the socioeconomic situation on the veterinary care of dogs affected by leishmaniasis, in comparison to other countries. Additionally, these studies showed that rapid diagnostic testing was more commonly used for diagnosis in Portugal as compared to other European countries. Globally, the approach to diagnosis and treatment of VL and CanL in Portugal generally follows regional (European) guidelines, but limitations in terms of access and lack of national specific recommendations could lead to some heterogeneity. Median K and Per scores were significantly higher in professionals, and especially in animal and environmental health professionals, but this did not translate into higher Pra scores. A possible explanation is that the practices evaluated can be performed generally to prevent arthropod-borne infections, and not specifically for leishmaniasis (except for vaccination). It has been suggested in Brazil that veterinarians need to have increased knowledge on leishmaniases [30]. Results from the multivariate analysis reinforce the importance of practical experience as the means to increase knowledge in professionals and to promote that knowledge being effectively increased according to progression in disease course. Higher knowledge was associated with higher perception of the importance of education/training and of collaboration. Lower median Pra score in the Norte region could be related to a lower perception of risk for vector-borne infections, also likely associated with lower canine *Leishmania* seroprevalence in many subregions, especially in coastal areas [7]. This study has a number of important limitations. First, not every faculty in Portugal participated in the study. The courses that were contacted but did not collaborate, such as medial courses from Algarve, Madeira and Açores, were not actively contacted for collaboration in the study since they were not listed in the DGES website, which was used as a reference for this study [39]. In addition, adherence was generally very low. The participants of this questionnaire were probably students/professionals who were most likely to know/be aware of leishmaniasis (as the participation was voluntary, those who did not know/care about leishmaniasis might have been less likely to respond). Physicians and veterinarians were approached via social network publications due to visibility and adherence limitations with dissemination by email, and this could have resulted in a selection bias; professionals who take part in these Facebook® groups could be non-representative of the class in terms of age and other sociodemographic factors. Very few Internal Medicine physicians participated, although they likely represent the most involved specialty in diagnosis of adult VL in smaller hospitals. Similarly, the first approach to many patients, in primary health care, was not assessed since few family medicine physicians participated. Also, other professionals involved in healthcare were not enrolled in this study, such as nurses (human and veterinary), who are increasingly responsible for health education, but not involved in diagnosis/management or entomologic surveillance programs in Portugal. The results of this study could also have been affected by social desirability or conformity bias, since some questions addressed points that could represent professional competence or student performance. Participation in the study was unsupervised, so individuals could have shared opinions or experiences or used external sources of information while filling in the questionnaire. Additionally, other biases associated with online participation could be presumed, including nonresponse bias and question order bias.

5. Conclusions

Inclusion of leishmaniasis in the curriculum of health students is perceived as important and seems to be associated with an increase in knowledge of the disease. A national structured program to control leishmaniasis could overcome some of the barriers pointed out by professionals, namely by implementing systematic phlebotomine surveillance and integrated reporting of animal and human cases of disease. Joint efforts and collaboration are recognized as crucial to fight this zoonosis, following a One Health approach.

Ethics statement

This study was approved by the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (reference 12.22). All participants were informed about the study protocol and signed an informed consent form allowing for data collection.

6. References

1. Gradoni L, López-Vélez RMM. *Manual on case management and surveillance of the leishmaniasis in the WHO European Region.* Geneva: World Health Organization; 2017.

2. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic leishmania infection: a new challenge for leishmania control. *Clin Infect Dis.* 2014;**58**:1424. doi: 10.1093/cid/ciu102.

3. European Centre for Disease Prevention and Control (ECDC). Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. 2022. https://www.ecdc.europa.eu/sites/default/files/documents/leishmaniasis-surveillance-eu.pdf. Accessed 22 Feb 2023.

4. Servicos Partilhados do Ministerio de Saude (SPMS). Doenças de Declaração Obrigatória. Portal da Transparência. 2018. https://transparencia.sns.gov.pt/explore/dataset/doencas-de-declaracao-obrigatoria/table/?disjunctive.regiao_de_saude_de_notificacao&disjunctive.residencia_nut_iii&disjunctive.doenca_de_declaracao_obrigatoria&disjunctive.sexo&disjunctive.g.rupo_etario&sort. Accessed 22 Feb 2023.

5. Serrada E. A Leishmaniose visceral em Portugal Continental: 1999–2009. Universidade Nova de Lisboa. 2010. http://hdl.handle.net/10362/5546. Accessed 22 Feb 2023.

6. Campino L, Abranches P. Cutaneous leishmaniasis. Unusual disease in Portugal? *Acta Med Port.* 2002;**15**:387–90.

7. Almeida M, Maia C, Cristóvão JM, Morgado C, Barbosa I, Ibars RF, et al. Seroprevalence and risk factors associated with Leishmania infection in dogs from Portugal. *Microorganisms.* 2022;**10**:2262. doi: 10.3390/microorganisms10112262.

8. Cortes S, Vaz Y, Neves R, Maia C, Cardoso L, Campino L. Risk factors for canine leishmaniasis in an endemic Mediterranean region. *Vet Parasitol.* 2012;**189**:189. doi: 10.1016/j.vetpar.2012.04.028.

9. Singh SP, Reddy DCS, Mishra RN, Sundar S. Knowledge, attitude, and practices related to Kala-azar in a rural area of Bihar State. *India Am J Trop Med Hyg.* 2006;**75**:505. doi: 10.4269/ajtmh.2006.75.505.

10. Govil D, Sahoo H, Pedgaonkar SP, Das KC, Lhungdim H. Assessing knowledge, attitudes, and preventive practices related to kala-a: a study of Rural Madhepura, Bihar India. *Am J Trop Med Hyg.* 2018;**98**:857. doi: 10.4269/ajtmh.16-0978.

11. Saleh F, Fazlarabbi Khan M, Rowshan Kabir M. Awareness of residents about kala-azar and its related practices in two endemic areas of Bangladesh. *PLoS ONE.* 2019;**17**:e0270656. doi: 10.1371/journal.pone.0219591.

12. Carrillo-Bonilla LM, Trujillo JJ, Álvarez-Salas L, Vélez-Bernal ID. Study of knowledge, attitudes, and practices related to leishmaniasis: Evidence of government neglect in the Colombian Darién. *Cad Saude Publica.* 2014;**30**:2134. doi: 10.1590/0102-311X00139713.

13. Borges BKA, Da Silva JA, Haddad JPA, Moreira ÉC, De Magalhães DF, Ribeiro LML, et al. *Assessment of knowledge and preventive attitudes concerning visceral leishmaniasis in Belo Horizonte, Minas Gerais State.* Rio de Janeiro: Cad Saude Publica; 2008. p. 777.

14. López-Perea N, Sordo L, Gadisa E, Cruz I, Hailu T, Moreno J, et al. Knowledge, attitudes and practices related to visceral Leishmaniasis in Rural communities of amhara state: a longitudinal

study in Northwest Ethiopia. *PLoS Negl Trop Dis.* 2014;**8**:e2799. doi: 10.1371/journal.pntd.0002799.

15. Pereira A, Martins Â, Brancal H, Vilhena H, Silva P, Pimenta P, et al. Parasitic zoonoses associated with dogs and cats: a survey of Portuguese pet owners' awareness and deworming practices. *Parasit Vectors.* 2016;**9**:245. doi: 10.1186/s13071-016-1533-2.

16. Matos M, Alho AM, Owen SP, Nunes T, Madeira de Carvalho L. Parasite control practices and public perception of parasitic diseases: a survey of dog and cat owners. *Prev Vet Med.* 2015;**122**:174. doi: 10.1016/j.prevetmed.2015.09.006.

17. Mateus TL, Moreira S, Maia RL. Unawareness about vector-borne diseases among citizens as a health risk consequence of climate change—a case study on leishmaniosis in Northwest Portugal. In: Filho WL, Guedes DG, Dinis MAP, editors. Climate change and health hazards addressing hazards to human and environmental health from a changing climate. Cham: Springer Nature Switzerland; 2023. pp. 197–208.

18. Montoya A, Gálvez R, Checa R, Sarquis J, Plaza A, Barrera JP, et al. Latest trends in L. infantum infection in dogs in Spain, Part II: current clinical management and control according to a national survey of veterinary practitioners. *Parasit Vectors.* 2020;**13**:1. doi: 10.1186/s13071-020-04080-8.

19. Le Rutte EA, van Straten R, Overgaauw PAM. Awareness and control of canine leishmaniosis: a survey among Spanish and French veterinarians. *Vet Parasitol.* 2018;**253**:87. doi: 10.1016/j.vetpar.2018.01.013.

20. Ruiz de Ybáñez R, del Río L, Martínez-Carrasco C, Segovia M, Cox J, Davies C, et al. Questionnaire survey on Canine Leishmaniosis in southeastern Spain. *Vet Parasitol.* 2009;**164**:123. doi: 10.1016/j.vetpar.2009.06.013.

21. Pereira MA, Santos R, Nóbrega C, Mega C, Cruz R, Esteves F, et al. A questionnaire-based survey on the long-term management of canine leishmaniosis by veterinary practitioners. *Animals.* 2022;**12**:731. doi: 10.3390/ani12060731.

22. Hewawasam C, Weerakoon HS, Thilakan V, Lelwala T, Prasanka K, Rathnayaka AS, et al. Is leishmaniasis adequately notified in Sri Lanka? A survey among doctors from an endemic district. *Sri Lanka. BMC Public Health.* 2020;**20**:913. doi: 10.1186/s12889-020-09066-w.

23. Kumar N, Singh SP, Mondal D, Joshi A, Das P, Sundar S, et al. How do health care providers deal with kala-azar in the Indian subcontinent? *Indian J Med Res.* 2011;**134**:349.

24. El-Mouhdi K, Fekhaoui M, Elhamdaoui F, Guessioui H, Chahlaoui A. Knowledge and experiences of health professionals in the peripheral management of Leishmaniasis in morocco (ELHajeb) *J Parasitol Res.* 2020;**2020**:8819704. doi: 10.1155/2020/8819704.

25. Ali RM, Loutfy NF, Awad OM. Attitude and knowledge of primary health care physicians and local inhabitants about leishmaniasis in west Alexandria. *Trop Med Int Heal.* 2015;**9**:2167.

26. Kashfi SM, Jeihooni AK, Rezaianzadeh A. Effect of health workers' training programs on preventive behavior of leishmaniosis based on BASNEF model. *J Res Health Sci.* 2012;**12**:114.

27. Qurtas DS, Shabila NP. Using Q-methodology to understand the perspectives and practical experiences of dermatologists about treatment difficulties of cutaneous leishmaniasis. *BMC Infect Dis.* 2020;**20**:645. doi: 10.1186/s12879-020-05365-0.

28. de Carvalho AG, Alves I, Borges LM, Spessatto LB, Castro LS, Luz JGG. Basic knowledge about visceral leishmaniasis before and after educational intervention among primary health care professionals in midwestern Brazil. *Rev Inst Med Trop Sao Paulo.* 2021;**63**:e56. doi: 10.1590/s1678-9946202163056.

29. Ruoti M, Oddone R, Lampert N, Orué E, Miles MA, Alexander N, et al. Mucocutaneous leishmaniasis: Knowledge, attitudes, and practices among Paraguayan communities, patients, and health professionals. *J Trop Med.* 2013;**2013**:538629. doi: 10.1155/2013/538629.

30. Menezes JA, Maia KN, Verne RN, Madureira AP, Schall VT, de Souza CM. Leishmanioses: o conhecimento dos profissionais de saúde em área endêmica. *Rev Bras Promoção da Saúde.* 2014;**27**:207. doi: 10.5020/18061230.2014.p207.

31. Ghodsi M, Maheri M, Joveini H, Rakhshani MH, Mehri A. Designing and evaluating educational intervention to improve preventive behavior against cutaneous leishmaniasis in endemic areas in Iran. *Osong Public Heal Res Perspect.* 2019;**10**:253. doi: 10.24171/j.phrp.2019.10.4.09.

32. Dires A, Kumar P, Gedamu S, Yimam W, Ademe S. Knowledge, attitude and prevention measures of students towards cutaneous leishmaniasis in Delanta district Northeast Ethiopia. *Parasite Epidemiol Control.* 2022;**17**:e00241. doi: 10.1016/j.parepi.2022.e00241.

33. Alidosti M, Heidari Z, Shahnazi H, Zamani-Alavijeh F. Behaviors and perceptions related to cutaneous leishmaniasis in endemic areas of the world: a review. *Acta Trop.* 2021;**223**:106090. doi: 10.1016/j.actatropica.2021.106090.

34. Rios-González C, Flores J, Calvopiña M. Knowledge of Leishmania spp. and leishmaniasis in medical students in Latin America, 201. *Rev Chil Infectol.* 2016;**33**:481–2. doi: 10.4067/S0716-10182016000400020.

35. Ordem dos Médicos. Estatísticas nacionais 2021, por Distrito. 2022. https://ordemdosmedicos.pt/wp-content/uploads/2022/01/ESTATISTICAS_DISTRITO_2021.pdf. Accessed 22 Feb 2023.

36. Ordem dos Médicos. Estatísticas Nacionais 2021, por Especialidades. 2022. https://ordemdosmedicos.pt/wp-content/uploads/2022/01/ESTATISTICAS_ESPECIALIDADES_2021.pdf. Accessed 22 Feb 2023.

37. Ordem dos Médicos. Estatísticas Nacionais 2021, por país de formação. 2022. https://ordemdosmedicos.pt/wp-content/uploads/2022/01/ESTATISTICAS_PAIS_FORMACAO_2021.pdf. Accessed 22 Feb 2023.

38. Fundação Francisco Manuel dos Santos. PORDATA Base de Dados de Portugal Contemporâneo. Alunos matriculados no ensino superior na área da Saúde: total, por área de educação e formação e sexo. https://www.pordata.pt/portugal/diplomados+no+ano++no+ensino+superior+na+area+da+saude+total++por+area+de+educacao+e+formacao+e+sexo-804. Accessed 22 Feb 2023.

39. Ministério da Educação e Ciência. Direção-Geral do Ensino Superior. Guia das provas de ingresso—Índice por Curso e Instituição. https://www.dges.gov.pt/guias/indcurso.asp?letra=M. Accessed 22 Feb 2023.

40. Ordem dos Médicos Veterinários. Estatísticas. Atualizado a 19 de janeiro de 2022. https://www.omv.pt/omv/estatisticas. Accessed 22 Feb 2023.

41. Fagerland MW, Hosmer DW. A generalized Hosmer-Lemeshow goodness-of-fit test for multinomial logistic regression models. *Stata J.* 2012;**12**:447. doi: 10.1177/1536867X1201200307.

42. Feliciangeli MD. Natural breeding places of Phlebotomine sandflies. *Med Vet Entomol.* 2004;**18**:71. doi: 10.1111/j.0269-283X.2004.0487.x.

43. Camargo T, Bondan E. Awareness of visceral leishmaniasis in the population of Cotia (SP), Brazil, and participation of local veterinarians in the spread of preventive measures. *R Bras Ci Vet.* 2015;**22**:28–33. doi: 10.4322/rbcv.2015.315.

44. Naucke TJ, Amelung S, Lorentz S. First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. *Parasit Vectors.* 2016;**9**:256. doi: 10.1186/s13071-016-1551-0.

45. Talbi FZ, El Ouali Lalami A, Fadil M, Najy M, Ech-Chafay H, Lachhab M, et al. Entomological investigations, seasonal fluctuations and impact of bioclimate factors of Phlebotomines sand flies (Diptera: Psychodidae) of an emerging focus of cutaneous leishmaniasis in Aichoun central Morocco. *J Parasitol Res.* 2020;**2020**:6495108. doi: 10.1155/2020/6495108.

46. Monge-Maillo B, Norman FF, Cruz I, Alvar J, López-Vélez R. Visceral Leishmaniasis and HIV coinfection in the mediterranean region. *PLoS Negl Trop Dis.* 2014;**8**:e3021. doi: 10.1371/journal.pntd.0003021.

47. Cloots K, Burza S, Malaviya P, Hasker E, Kansal S, Mollett G, et al. Male predominance in reported visceral leishmaniasis cases: nature or nurture? A comparison of population-based with health facility-reported data. *PLoS Negl Trop Dis.* 2020;**14**:e0007995. doi: 10.1371/journal.pntd.0007995.

48. Silgado A, Armas M, Sánchez-Montalvá A, Goterris L, Ubals M, Temprana-Salvador J, et al. Changes in the microbiological diagnosis and epidemiology of cutaneous leishmaniasis in real-time PCR era: a six-year experience in a referral center in Barcelona. *PLoS Negl Trop Dis.* 2021;**15**:e0009884. doi: 10.1371/journal.pntd.0009884.

49. Pasquier G, Demar M, Lami P, Zribi A, Marty P, Buffet P, et al. Leishmaniasis epidemiology in endemic areas of metropolitan France and its overseas territories from 1998 to 2020. *PLoS Negl Trop Dis.* 2020;**16**:e0010745. doi: 10.1371/journal.pntd.0010745.

50. Collective Eurosurveillance editorial team Note from the editors: Eurosurveillance special issue on leishmaniasis painting a picture of the situation in Europe. *Eurosurveillance.* 2013;**18**:20529. doi: 10.2807/ese.18.29.20529-en.

51. Molina R, Jiménez M, García-Martínez J, San Martín JV, Carrillo E, Sánchez C, et al. Role of asymptomatic and symptomatic humans as reservoirs of visceral leishmaniasis in a mediterranean context. *PLoS Negl Trop Dis.* 2020;**14**:4. doi: 10.1371/journal.pntd.0008253.

52. Solano-Gallego L, Mirá G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, et al. LeishVet guidelines for the practical management of canine leishmaniosis. *Parasit Vectors.* 2011;**4**:86. doi: 10.1186/1756-3305-4-86.

53. Horrillo L, Castro A, Matía B, Molina L, García-Martínez J, Jaqueti J, et al. Clinical aspects of visceral leishmaniasis caused by L. infantum in adults. Ten years of experience of the largest outbreak in Europe: what have we learned? *Parasit Vectors.* 2019;**12**:359. doi: 10.1186/s13071-019-3628-z.

54. Al-Hashimi DA, Al-Roomi K, Al-Sayyad AS. Reasons for under-reporting of notifiable communicable diseases in the Kingdom of Bahrain: a health-centers based survey. *J Bahrain Med Soc.* 2014;**25**:75. doi: 10.26715/jbms.25_2_3.

55. Vulpiani MP, Iannetti L, Paganico D, Iannino F, Ferri N. Methods of control of the Leishmania infantum dog reservoir: state of the art. *Vet Med Int.* 2011;**2011**:215964. doi: 10.4061/2011/215964.

56. Instituto Nacional de Estatística (INE). Resultados Provisórios—Censos 2021. Resultados Provisórios. 2021. https://censos.ine.pt/xportal/xmain?xpgid=censos21_main&xpid=CENSOS21&xlang=pt. Accessed 22 Feb 2023.

57. Pereira A, Maia C. Leishmania infection in cats and feline leishmaniosis: an updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Curr Res Parasitol Vector-Borne Dis.* 2021;**1**:100035. doi: 10.1016/j.crpvbd.2021.100035.

58. Monteiro M, Prata S, Cardoso L, Pereira da Fonseca I, Leal RO. Diagnosis and clinical management of canine leishmaniosis by general veterinary practitioners: a questionnaire-based survey in Portugal. *Parasit Vectors.* 2021;**14**:306. doi: 10.1186/s13071-021-04799-y.

59. Bourdeau P, Saridomichelakis MN, Oliveira A, Oliva G, Kotnik T, Gálvez R, et al. Management of canine leishmaniosis in endemic SW European regions: a questionnaire-based multinational survey. *Parasit Vectors.* 2014;**7**:110. doi: 10.1186/1756-3305-7-110.

60. Mattin MJ, Solano-Gallego L, Dhollander S, Afonso A, Brodbelt DC. The frequency and distribution of canine leishmaniosis diagnosed by veterinary practitioners in Europe. *Vet J.* 2014;**200**:410–419. doi: 10.1016/j.tvjl.2014.03.033.

7. Supplementary data

Available at: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-023-05982-z

Additional file 1: Supplementary Figure 1. Online questionnaire about sociodemographic and professional/academic aspects and about knowledge, perceptions and practices regarding leishmaniasis

Additional file 1: Supplementary Table 1. Protocol implemented for scoring Knowledge, Perceptions and Practices of students and professionals, according to the answers provided in the questionnaire

Additional file 2: Supplementary figure 2. Distribution of students by university and faculty of study for: a) Integrated Master's in Medicine; b) Integrated Master's in Veterinary Medicine; c) Bachelor's in Environmental Health.

Additional file 1: Supplementary Figure 3. Distribution of individual: a) Knowledge scores; b) Perceptions scores; c) Practices scores.

CHAPTER 4

Knowledge and practices regarding leishmaniasis in Portugal – comparison between blood donors and health students/professionals.

This chapter is a transcription of the research article:

Rocha, R., Conceição, C., Gonçalves, L., Maia, C. Knowledge and practices regarding leishmaniasis in Portugal - Comparison between blood donors and health students/professionals. One Health. 2024 Mar 2;18:100697.
https://doi.org/10.1016/j.onehlt.2024.100697.

Knowledge and practices regarding leishmaniasis in Portugal – comparison between blood donors and health students/professionals.

Abstract

Objective. To compare the current knowledge and practices regarding leishmaniasis among blood donors and health students and professionals, in Portugal.

Material and methods. Data were collected through the application of two questionnaires (one online and one in paper) with similar questions in two distinct cross-sectional independent studies, each targeting one of the groups. Descriptive statistics and hypothesis testing were performed using IBM® SPSS® Statistics.

Results. In total, 3763 blood donors, 254 students and 232 professionals were included in the comparative analysis. Over 95% of students and professionals, but only around 70% of blood donors had previously heard of leishmaniasis. Over 90% of participants in each group admitted leishmaniasis affected animals, but only in the professional group over 90% were aware of human leishmaniasis.

Conclusions. Even though canine leishmaniasis is recognized by many blood donors and by most students and professionals, awareness of the disease in humans is less common, highlighting the importance of promoting an approach to this infection through a One Health lens.

Keywords: Practices, Leishmaniasis, Portugal, Blood donors, Health professionals, Students, One Health

1. Introduction

Control of leishmaniasis in the Mediterranean basin relies on individual contributions by the general population and on active interventions from the fields of animal, human and environmental health. Application of knowledge and practices (KP) questionnaires in Europe could be fundamental from a Public (One) Health perspective, to highlight the diversity of conceptions related to this disease among the students/professionals and the populations at risk in endemic areas. Non-standardized KP questionnaires applied in *Leishmania* endemic areas, such as South America [1,2], South Asia [3-5] and East Africa [6], showed heterogenous results among countries, regions and different sectors of the general population.

Although the Mediterranean region is also endemic, few studies have addressed the KP of the resident population and these were mostly directed to animal owners, including three studies performed in Portugal [7-9]. In these studies, 83–91% of the owners heard of animal leishmaniasis, but only 38.6% of human leishmaniasis. Hearing of leishmaniasis was significantly associated with non-rural areas and academic degree. Concerning health professionals, studies directed at KP of veterinary doctors have been performed in the Mediterranean region, generally focusing on the epidemiology and clinical approach to canine leishmaniasis [10-13]. However, in this region, medical doctors and environmental health technicians (EHTs), as well as students, have not been systematically included. Lastly, no studies have attempted to compare the KP of the general population with that of the professionals/students, even though this approach could help understand if differential knowledge between groups could explain distinct practices related to leishmaniasis and/or to arthropod-borne infections in general. The aim of this work is to compare the current knowledge and practices regarding leishmaniasis among blood donors and health students and professionals, in Portugal, through the application of an online or paper questionnaire.

2. Methods

Data for this work were collected via two distinct cross-sectional independent studies. One targeting the population of health students and professionals in Portugal: students currently enrolled in the course of Medicine, Veterinary Medicine, or Environmental Health in public or private higher education institutions; actively practicing physicians, veterinarians, and Environmental Health technicians [14]. Potential participants were approached by email via universities and professional societies and organizations, providing the link to access the online, anonymous, questionnaire (built on Redcap®). Answers to the self-administered sociodemographic and KP questionnaire were collected between July and December 2022. The second study targeted the population of people who donate blood in mainland Portugal through the Portuguese Institute of Blood and Transplantation (IPST) or the Immunohemotherapy departments (IHDs) of public hospitals in the Alentejo and Algarve regions [15]. Although not representative of the general Portuguese population, this target population was chosen due to ease of nation-wide sampling. Participants, distributed proportionally by municipality, aged between 18 and 65 years old, were selected randomly in 347 blood collection points, between February and June 2022, and filled in a self-administered paper sociodemographic and KP questionnaire. For the comparative analysis, absolute and relative frequencies and hypothesis testing were performed using IBM® SPSS® Statistics Version 29.0. Descriptive statistics were expressed as absolute frequencies and percentages for categorical variables or as a median with interquartile ranges (IQRs) for the continuous variable (age). Comparisons between groups were performed using Pearson Chi-Square test for categorical variables (or Fisher's exact test in case of failure of the assumptions of the Chi-square test). For the continuous variable, after checking the assumptions of normality and homogeneity of the variances, the Kruskal-Wallis test was used, for comparing more than two independent groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

In total, 3763 blood donors, 254 students and 232 professionals were included in the comparative analysis. Female sex was predominant among students and professionals, but not among blood donors. Findings from the knowledge and practices questions are summarized in Table 1 and Table 2, respectively. Over 95% of students and professionals had previously heard of leishmaniasis, compared to only around 70% of blood donors ($p < 0.001$). Television advertisements and conversation with a veterinarian were common non-academic sources of information for all groups. Unawareness regarding mode of transmission of leishmaniasis was more common in blood donors ($p < 0.001$); arthropod bite was the mode of transmission most often pointed by participants, especially health students/professionals; sand flies were identified as the vectors much more commonly by students/professionals than by blood donors ($p < 0.001$), who preferentially selected mosquito bites. Over 10% of participants in every group considered direct contact with animals could be a major route of transmission. Over 90% of participants in each group admitted leishmaniasis affected animals; however, only in the professional group over 90% of participants were aware of human leishmaniasis. All groups equally acknowledged dogs as the most affected animal species ($p = 0.319$) and recognized the endemic status of the disease ($p = 0.085$). In humans, however, endemicity was recognized more often by professionals and less by students ($p < 0.001$). Leishmaniasis was more often considered treatable (both in animals and in humans) by professionals than by students or blood donors ($p = 0.001$ and $p < 0.001$, respectively). Students and professionals were more frequently aware of availability of vaccination against canine leishmaniasis ($p < 0.001$) and were almost twice likely to provide it yearly for their dogs. Use of insecticide/repellent in dogs was reported in similar proportions in all groups ($p = 0.317$), but use of collars and administration all year round were more frequently stated by students and professionals ($p = 0.001$ and $p < 0.001$, respectively). Regular veterinarian follow-up (at least once a year) was reported in similar proportions (89.0–96.4%, $p = 0.155$).

Table 1. Answers to knowledge questions concerning source of information, route of transmission, affliction of animals or humans, by group (blood donors, health students, health professionals).

	Global blood donors	Global health students	Global health professionals	*p*-value
Total (*n*)	3763	254	232	
Median age (y) (IQR)	41 (31-48)	21 (19-24)	38 (30-46)	**<0.001**
Male sex (%)	49.8 (1867/3749)	16.7 (42/252)	23.3 (54/232)	**<0.001**
Heard of leishmaniasis (%)	72.3 (2704/3740)	95.7 (243/254)	99.1 (230/232)	**<0.001**
Source of information (%)				
Television	53.2 (1406/2643)	58.8 (143/243)	40.0 (92/230)	**<0.001**
Veterinarian	48.1 (1273/2643)	40.3 (98/243)	39.1 (68/174)	**0.006**
Route of transmission (%)				
DK/CR	19.5 (526/2704)	5.3 (13/243)	2.2 (5/230)	**<0.001**
Arthropod bite	88.2 (1922/2178)	97.8 (225/230)	96.4 (217/225)	**<0.001**
Sand fly bite	13.5 (260/1922)	66.2 (149/225)	78.8 (171/217)	**<0.001**
Contact with animals	19.6 (426/2178)	13.5 (31/230)	12.0 (27/225)	**0.003**
Affects animals (%)	91.0 (2451/2693)	95.9 (233/243)	95.2 (219/230)	**0.004**
Species most affected (%)				
Dogs	97.4 (1893/1937)	97.0 (194/200)	99.3 (151/152)	0.319
Cats	32.6 (632/1937)	26.5 (53/200)	11.3 (17/151)	**<0.001**
Present in Portugal (%)	86.1 (2081/2418)	85.5 (200/234)	91.3 (200/219)	0.085
Treatable in animals (%)	62.8 (1397/2226)	57.3 (63/110)	85.0 (51/60)	**0.001**
Vaccine available (%)	52.2 (1092/2090)	82.8 (82/99)	72.0 (108/150)	**<0.001**
Affects humans (%)	53.8 (1433/2666)	70.4 (171/243)	93.9 (216/230)	**<0.001**
Present in Portugal (%)	78.7 (1135/1442)	49.3 (73/148)	87.3 (145/166)	**<0.001**
Treatable in humans (%)	55.6 (772/1388)	57.4 (81/141)	86.7 (78/90)	**<0.001**

Abbreviations: n - number; y - years; IQR - interquartile range; DK/CR - don't know/can't remember

Table 2. Answers to practices questions concerning ownership of dogs, by group (blood donors, health students, health professionals).

	Global blood donors	Global health students	Global health professionals	*p*-value
Nets in some/all windows/doors (%)	23.7 (848/3572)	22.9 (58/253)	15.6 (36/231)	**0.017**
Dog ownership (%)	48.1 (1775/3688)	57.7 (146/253)	35.9 (83/231)	**<0.001**
Dog outdoors during nighttime	63.8 (1055/1653)	77.4 (113/146)	63.9 (53/83)	**0.004**
Use of repellents/insecticides (%)	82.2 (1320/1605)	77.2 (112/145)	80.7 (67/83)	0.317
Spot-on	55.0 (726/1320)	59.8 (67/112)	53.7 (36/67)	0.594
Collar	40.9 (540/1320)	57.1 (64/112)	50.7 (34/67)	**0.001**
All year round	62.8 (829/1320)	82.1 (92/112)	73.1 (49/67)	**<0.001**
Regular veterinarian follow-up (at least once a year) (%)	90.6 (1472/1625)	89.0 (129/145)	96.4 (80/83)	0.155
Use of vaccine against canine leishmaniasis every year (%)	21.7 (385/1775)	42.4 (56/132)	42.5 (17/40)	**<0.001**

4. Discussion

Awareness of leishmaniasis was high both in blood donors and in health students/professionals. The role of veterinarians as providers of information regarding leishmaniasis was highlighted by every group; in this sense, academic training and continuous education of veterinarians in this disease could be a decisive strategy for control of leishmaniasis in Portugal, as well as in other zoonotic settings. In blood donors, identification of phlebotomine sand flies as vectors is insufficient; potential sandfly breeding sites, such as animal burrows and shelters and leaf litter, differ from mosquito breeding sites (small or large bodies of water); as such, confusion between these two arthropods could lead to improper individual management of potential *Leishmania* vector breeding sites. Additionally, belief of transmission via direct contact (excluding bites or scratches), even in professionals, could lead to inadequate isolation measures or rejection of diseased animals. In blood donors, decreased recognition of potential human infection with *Leishmania*, including in endemic areas in Portugal, could implicate a lower perception of individual or community risk, even in areas where canine cases are seen, and a low stimulus to implement animal protective measures. Lower awareness of vaccine availability for dogs and lower effective implementation by blood donors could also represent barriers to disease control. Although systematic use of repellents in dogs was consistently reported by students/professionals and blood donors, the latter were less likely to apply them spanning all the phlebotomine season (in Portugal, mostly from May to October [16]); other practices that could be different between groups and impact prevention, such as type of repellent substance used, frequency and mode of application, were not assessed in these studies.

A national structured plan to control leishmaniasis could overcome some of these challenges, namely by implementing systematic surveillance and integrated reporting of animal and human cases of disease and by investing in health education and promotion concerning vector-borne infections. A limitation of this comparative analysis is that participants in each group were not equally distributed regarding sex and region of residence, which could have impacted knowledge, since endemicity of leishmaniasis and access to information is not homogeneous across the country. Future studies should target a broader sector of the Portuguese population and could prospectively evaluate the effect of education in changing practices.

5. Conclusions

Even though canine leishmaniasis is recognized by many blood donors and most students and professionals, awareness of the disease in humans is less common, highlighting the importance of promoting an approach to this infection through a One Health lens. Gaps in knowledge in the general population could explain insufficient protective practices, such as lower adherence to canine vaccination against leishmaniasis. Health professionals from different fields could play an important role in promoting health related to vector-borne infections.

Ethics statement

For this study, only data produced in two previous studies were used. The study in health students and professionals received a favorable opinion of the Ethics Committee of the Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL) (reference 12.22). The study in blood donors received a favorable opinion of the Ethics Committees of the following institutions: IHMT, UNL (reference 1.22); Instituto Português do Sangue e da Transplantação; Centro Hospitalar Universitário do Algarve; Hospital do Espírito Santo de Évora; Unidade Local de Saúde do Norte Alentejano; Unidade Local de Saúde do Baixo Alentejo; Unidade Local de Saúde do Litoral Alentejano. Additionally, the study was authorized by the Administration Council of all the involved Hospitals. All participants in the two studies were informed about the study protocol and signed an informed consent form allowing for data collection.

6. References

1. L.M. Carrillo-Bonilla, J.J. Trujillo, L. Álvarez-Salas, I.D. Vélez-Bernal. Study of knowledge, attitudes, and practices related to leishmaniasis: evidence of government neglect in the Colombian Darién. Cad. Saude Publ., 30 (10) (2014), pp. 2134-2144

2. B.K.A. Borges, J.A. da Silva, J.P.A. Haddad, E.C. Moreira, D.F. de Magalhães, L.M.L. Ribeiro, et al. Assessment of knowledge and preventive attitudes concerning visceral leishmaniasis in Belo Horizonte, Minas Gerais state, Brazil. Cad Saude Publ. [Internet]., 24 (4) (2008 Apr), pp. 777-784.

3. S.P. Singh, D.C.S. Reddy, R.N. Mishra, S. Sundar. Knowledge, attitude, and practices related to kala-azar in a rural area of Bihar state, India. Am. J. Trop. Med. Hyg., 75 (8) (2006), pp. 505-508

4. D. Govil, H. Sahoo, S.P. Pedgaonkar, K.C. Das, H. Lhungdim. Assessing knowledge, attitudes, and preventive practices Related to kala-a: a study of rural Madhepura, Bihar, India. Am. J. Trop. Med. Hyg., 98 (3) (2018), pp. 857-863

5. F. Saleh, M. Fazlarabbi Khan, Kabir M. Rowshan. Awareness of residents about kala-azar and its related practices in two endemic areas of Bangladesh. PLoS One, 14 (7) (2019), Article e0219591

6. N. López-Perea, L. Sordo, E. Gadisa, I. Cruz, T. Hailu, J. Moreno, et al. Knowledge, attitudes and practices related to visceral Leishmaniasis in rural communities of Amhara state: a longitudinal study in Northwest Ethiopia. PLoS Negl. Trop. Dis., 8 (4) (2014), p. e2799

7. A. Pereira, Â. Martins, H. Brancal, H. Vilhena, P. Silva, P. Pimenta, et al. Parasitic zoonoses associated with dogs and cats: a survey of Portuguese pet owners' awareness and deworming practices. Parasit. Vectors, 9 (1) (2016), p. 245

8. M. Matos, A.M. Alho, S.P. Owen, T. Nunes, L. Madeira de Carvalho. Parasite control practices and public perception of parasitic diseases: A survey of dog and cat owners. Prev Vet. Med., 122 (1–2) (2015), pp. 174-180

9. T.L. Mateus, S. Moreira, R.L. Maia. Unawareness about Vector-Borne Diseases among Citizens as a Health Risk Consequence of Climate Change—A Case Study on Leishmaniosis in Northwest Portugal. Climate Change Management (2023)

10. A. Montoya, R. Gálvez, R. Checa, J. Sarquis, A. Plaza, J.P. Barrera, et al. Latest trends in L. infantum infection in dogs in Spain, part II: current clinical management and control according to a national survey of veterinary practitioners. Parasit. Vect. [Internet]., 13 (1) (2020 Dec 21), p. 205.

11. E.A. Le Rutte, R. Vvan Straten, P.A.M. Overgaauw. Awareness and control of canine leishmaniosis: A survey among Spanish and French veterinarians. Vet. Parasitol., 253 (2018), pp. 87-93

12. R. Ruiz de Ybáñez, L. del Río, C. Martínez-Carrasco, M. Segovia, J. Cox, C. Davies, et al. Questionnaire survey on canine leishmaniosis in southeastern Spain. Vet. Parasitol., 164 (2–4) (2009), pp. 124-133

13. M.A. Pereira, R. Santos, C. Nóbrega, C. Mega, R. Cruz, F. Esteves, et al. A questionnaire-based survey on the long-term Management of Canine Leishmaniosis by veterinary practitioners. Animals, 12 (6) (2022), p. 731

14. R. Rocha, C. Conceição, L. Gonçalves, C. Maia. Knowledge, perceptions and practices of health students and professionals regarding leishmaniasis in Portugal: a cross-sectional study. Parasit. Vect. [Internet], 16 (1) (2023 Oct 25), p. 381

15. R. Rocha, L. Gonçalves, C. Conceição, P. Andrade, J.M. Cristóvão, J. Condeço, et al. Prevalence of asymptomatic Leishmania infection and knowledge, perceptions, and practices in blood donors in mainland Portugal. Parasit. Vect. [Internet], 16 (1) (2023 Oct 10), p. 357

16. L. Zé-Zé, F. Amaro, H.C. Osório, M. Giovanetti, J. Lourenço, M.J. Alves. Molecular identification and ecology of Portuguese wild-caught Phlebotomine Sandfly specimens Zoonot. Dis., 2 (1) (2022), pp. 19-31

CHAPTER 5

Epidemiological and Clinical Aspects of Cutaneous and Mucosal Leishmaniases in Portugal.

This chapter is a transcription of the research article:

Rocha, R., Conceição, C., Gonçalves, L., Carvalho, AC., Maia, A., Martins, A., Carujo, A., Maio, A., Forra, C., Melita, C., Couto, D., Fernandes, D., Pereira, D., Leal, E., Sarmento, H., Sousa, I., Gonçalves, JP., Marinho, J., Vasconcelos, J., Cunha, J., Rodrigues, J., Silva, JM., Caley, L., Malheiro, L., Santos, L., Garcia, M., Cunha, M., Lima, M., Andrade, MM., Marques, M., Alpalhão, M., Silva, M., Ferraz, R., Soares, R., Fernandes, S., Llobet, S., Cruz, S., Guimarães, T., Branco, T., Robalo-Nunes, T., Almeida, V., Maia, C. Epidemiological and Clinical Aspects of Cutaneous and Mucosal Leishmaniases in Portugal: Retrospective Analysis of Cases Diagnosed in Public Hospitals and Reported in the Literature between 2010 and 2020. Microorganisms. 2024 Apr 18;12(4):819. https://doi: 10.3390/microorganisms12040819

Abstract

Leishmania infantum, a zoonotic vector-borne parasite, is endemic in the Mediterranean region, presenting mostly as visceral (VL), but also as cutaneous (CL) and mucosal leishmaniasis (ML). This study aimed to describe the epidemiological and clinical aspects of the CL and ML cases diagnosed in mainland Portugal between 2010 and 2020. Collaboration was requested from every hospital of the Portuguese National Health System. Cases were screened through a search of diagnostic discharge codes or positive laboratory results for *Leishmania* infection. Simultaneously, a comprehensive literature search was performed. Descriptive statistics and hypothesis testing were performed using IBM® SPSS® Statistics. A total of 43 CL and 7 ML cases were identified, with a predominance of autochthonous cases (86%). In CL, immunosuppressed individuals constituted a significant proportion of patients (48%), and in this group, disseminated CL (22%) and simultaneous VL (54%) were common. In autochthonous cases, lesions, mostly papules/nodules (62%), were frequently observed on the head (48%). The approach to treatment was very heterogeneous. ML cases were all autochthonous, were diagnosed primarily in older immunosuppressed individuals, and were generally treated with liposomal amphotericin B. The findings suggest a need for enhanced surveillance and reporting, clinical awareness, and diagnostic capacity of these forms of leishmaniasis to mitigate underdiagnosis and improve patient outcomes. A holistic One Health approach is advocated to address the multifaceted challenges posed by leishmaniases in Portugal and beyond.

Keywords: *Leishmania*, leishmaniasis, cutaneous, mucosal, Portugal, 2010–2020

1. Introduction

Leishmaniases are a group of diseases caused by protozoan parasites of the genus *Leishmania*. These parasites are transmitted by infected female phlebotomine sand flies, and the disease is zoonotic in most settings [1]. The clinical spectrum of symptomatic disease is usually grouped into two main syndromes, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) [1], both of which are endemic and geographically widespread in the Mediterranean region. In this region, *L. infantum*, which belongs to the *L. donovani* complex, is the etiologic species of most autochthonous human leishmaniasis cases [2]. Infection with *L. infantum*, when symptomatic, usually presents as VL, although cases of simultaneous or independent CL and mucosal leishmaniasis (ML) caused by this species are increasingly recognized [3]. In the western Mediterranean regions where *L. infantum* is endemic, including in Portugal, *Phlebotomus perniciosus* is the main vector [4], and dogs are the main reservoir for human infection [5].

Cutaneous leishmaniasis is traditionally considered a rare disease in Portugal, with very few cases documented prior to 2002 [6]. However, since reporting of CL is not mandatory, information regarding cases of this clinical form is dispersed between databases of different hospitals and a few publications in national and international journals and master/doctoral theses. Most cases described after 2000 are locally acquired and likely caused by *L. infantum* (even though species identification was seldom reported [7,8]); few cases suggested imported disease by dermotropic species from the New World (including those from the *L. Viannia* subgenus [9]). ML cases caused by endemic *L. infantum* have also been described [10,11,12]. In contrast to other European endemic countries, such as France, where CL cases are mostly imported [13], in Portugal, most published cases seem to be autochthonous. In most of these countries, however, CL is not regularly monitored at a national level [2]; in Portugal, no reports or reviews addressed CL nationally, and only one did so at a regional level (in Cova da Beira and Beira Interior Norte, describing 13 patients [14]). Consequently, the epidemiology of CL in Portugal is largely unknown, and attention should be focused on understanding the contexts of endemic disease, as well as the trends in imported cases following recent waves of migration from and increased travel to dermotropic species endemic countries, especially those in South America [15].

Therefore, this study aimed to describe the cases of cutaneous or mucosal leishmaniases diagnosed in hospitals of the Portuguese National Health Service between 2010 and 2020, or reported in the scientific literature in an equivalent period.

2. Materials and Methods

2.1. Study Population

Mainland Portugal is located in southwest Europe, bordering Spain and the Atlantic Ocean, and is divided into seven NUTS2 regions and 24 NUTS3 regions [16]. According to the 2021 national census, the population of mainland Portugal was 9,857,593 inhabitants [17]. Between 2010 and 2020, hospital-based healthcare services were provided by the Portuguese National Health Service (NHS) in approximately 100 general and specialized hospitals in mainland Portugal, according to data from the Directorate-General for Health (DGS) of Portugal [18]. Some of these hospitals are grouped into hospital centers. By protocol, every episode of emergency or hospitalization in these hospitals is given a code on discharge for primary and secondary diagnoses, following the International Classification of Diseases (ICD).

In this multicenter retrospective study, individuals diagnosed with cutaneous and/or mucosal leishmaniases in one of the hospitals of the Portuguese NHS, located in mainland Portugal, between 2010 and 2020, inclusively, were included in this study. Only laboratory confirmed cases were included, which consisted of the presence of a compatible clinical picture and meeting at least one of the following criteria: (i) detection of *Leishmania* DNA in cutaneous and/or mucosal samples; (ii) visualization of intracellular organisms in macrophages, compatible with *Leishmania* amastigotes in biopsy material or cytological examination; (iii) growth of *Leishmania* from a clinical sample inoculated in a specific culture medium.

2.2. Data Collection

Every hospital or hospital center of the NHS in mainland Portugal was contacted, and collaboration in this study was requested. Cases in each included hospital were screened through a search of the following diagnostic discharge codes: 085, 085.1, 085.2, 085.3, 085.4, 085.5, 085.9 (ICD-9); B55, B55.1, B55.2, B55.9 (ICD-10). In hospitals where codification of diagnosis was incomplete or unavailable for the whole or parts of the period of analysis, the listing of cases was complemented by searching skin/mucosa samples in which

Leishmania DNA was detected by PCR and cytology, as well as histopathology reports in which observation of *Leishmania* amastigotes was mentioned. Sociodemographic and clinical data for the cases identified was extracted from the medical records of each episode, codified, and inserted into a digital database. Data extraction was carried out by different professionals; a common database was used, and a protocol for filling in the required information was provided to every collaborator.

Additionally, a comprehensive literature search was performed on 3 March 2024 by sourcing National Library of Medicine (NLM) resources through PubMed (https://pubmed.ncbi.nlm.nih.gov/, accessed on 3 March 2024) using the following Boolean string: ("cutaneous" OR "mucosal" OR "mucocutaneous") AND "leishmaniasis" AND "Portugal". Search results were saved as a comma-separated value (CSV) file, and subsequently imported into Microsoft Excel® (Version Office 365, Microsoft Corp, Redmond, USA) Study eligibility was manually assessed. All records were screened according to the title and abstract, if available. Only studies published between 2011 and 2022 and in which at least one of the affiliations of the authors was a Portuguese hospital were included. This time frame was selected to match the cases diagnosed in the hospitals between 2010 and 2020, considering a 1–2 year delay between the diagnosis and publishing of the cases. Only case reports or series of confirmed cutaneous and/or mucosal leishmaniases were retained, including those published in English or Portuguese languages.

Cases of CL/ML obtained from the two sources (hospitals and publications) were matched, considering the following individual details, whenever available: age and sex of patient, comorbidities, region of residence at the time of diagnosis, year of admission to the hospital, diagnostic techniques, and treatment strategy. For duplicated cases, data from both sources were merged into a single entry in the final database. Categorical variables extracted from the clinical records or scientific articles were analyzed, mostly using the categories provided as options in the standardized database, but regrouping was performed in some cases. NUTS regions and municipalities were defined according to the latest organizational definition, implemented in 2024. The term "migrant" was used for people born abroad. Cases were defined as autochthonous if no species other than *L. infantum* was identified and if (a) there was no lifetime history of travel or residence abroad in CL endemic regions (any of the countries listed as endemic by the WHO for 2022 [19]); (b) there was a history of travel or residence in CL endemic region(s), but it occurred more than 12 months before the beginning of symptoms, and there was no change in immune status since the stay abroad; (c) or there was no information regarding travel history. Cases not meeting any of these criteria were considered as imported. Time to presentation represented the amount of time elapsed since the beginning of signs/symptoms related to leishmaniasis and the first visit to healthcare providers/institutions. Time to diagnosis represented the amount of time elapsed since presentation to healthcare providers and the confirmation of the diagnosis of leishmaniasis (according to the criteria above). Time to treatment was measured as the amount of time elapsed since the confirmation of diagnosis and the start of *Leishmania* directed therapy. For the purposes of this study, a patient was considered immunosuppressed if one or more of the following conditions were present: HIV infection with a CD4 cell count <500/µL; any primary immunodeficiency; active solid or hematologic malignancy; prior solid organ or bone marrow transplantation; current treatment with immunosuppressive/ immunomodulatory drugs (as listed in [20]). The types of lesions were defined according to clinical records. Disseminated CL was defined by the presence of over 10 lesions in multiple non-contiguous sites; MCL (mostly associated with *L. braziliensis* complex) was defined as a condition in which, following (or simultaneously with) a non-adjacent primary cutaneous lesion, parasites disseminate towards the mucosa; ML was defined as a condition in which localized *Leishmania* lesions in the mucosa occur without primary skin involvement, or in which skin involvement presents concurrently only in contiguous areas [21]. Non-improvement was defined as persistence or worsening of signs/symptoms, despite appropriate therapy, and was assessed at seven and thirty days after starting treatment. These two timeframes were defined by the authors to allow homogeneous data collection regarding outcome in the different hospitals involved. European guidelines propose a definition of non-response for CL as no clinical improvement at four weeks after start of therapy [21]. Relapses were defined as recurrence of signs/symptoms and positive culture/PCR/microscopy in a skin/mucosa sample after completing primary treatment with clinical improvement at 30 days.

2.3. Statistical Analysis

Mean annual incidence of CL was estimated based on the following formula: Incidence = (New Cases)/(Population × Timeframe), considering a timeframe of 11 years and an at-risk population, for each region, consisting of the average value between the number of inhabitants estimated in the census of 2011 and the census of 2021, according to the National Institute of Statistics [17]. The corresponding 95% confidence intervals (CIs) for the incidence rate were obtained using a substitution method [22].

Descriptive statistics and hypothesis testing were performed using IBM® SPSS® Statistics Version 29.0. Bar charts were built using Microsoft® Excel®. Geographical representation and analysis of results were achieved using QGIS® Version 3.22.

For categorical variables, absolute frequencies and percentages were calculated. Symmetric continuous variables were summarized by means with standard deviations, and asymmetric continuous variables (e.g., age, lesion size) by medians with interquartile intervals (IQIs). Missing or unknown data were excluded from denominators, unless stated otherwise.

Comparisons between CL and ML were performed using the Pearson Chi-square test (CST) for categorical variables, or Fisher's exact test (FET), in case of failure of the assumptions of the CST. For continuous variables, after checking the assumptions of normality and homogeneity of the variances, the Mann–Whitney U test (MWT) was used for comparing two independent groups.

3. Results

Data from 42 of the 45 hospitals or hospital centers in mainland Portugal were available for analysis. A total of 42 cases of CL and 7 cases of ML were diagnosed between 2010 and 2020 in the hospitals included. A total of 79 articles were obtained from the PubMed database search. Of these, six articles were selected, according to selection criteria, representing a total of three cases of CL [8,9,23] and three cases of ML [10,11,12]. Of these cases, five were matched with cases retrieved through the hospital searches. Consequently, combining the two sources of data, 43 cases of CL and 7 cases of ML were available for analysis.

3.1. Sociodemographic Characteristics and Comorbidities

Sociodemographic characteristics of CL/ML cases are represented in Table 1. The median age was 48 years old (IQI 33–61.2) and was significantly higher in ML patients compared to CL patients (66 vs. 47, $p = 0.026$, U = 71.0). Male sex was predominant globally and in both forms of leishmaniasis. Seven cases of CL (16.3%) were imported (from Brazil $n = 3$, Morocco $n = 2$, Mexico $n = 1$, Tunisia $n = 1$); all cases of ML were autochthonous. Migrants represented approximately 25% of the patients diagnosed.

Table 1. Sociodemographic characteristics and comorbidities of leishmaniasis cases, globally and by form of disease.

	Global	CL	ML	*p*-value
Number	50	43	7	
Median age, years (IQI)	48 [33 - 61.25]	47 [33 - 59]	66 [50 - 75]	**0.026** (U = 71.0)
Male sex (%)	68.0 (34/50)	69.8 (30/43)	57.1 (4/7)	0.666 (FET)
Country of birth (%)				
Native	75.6 (34/45)	72.5 (29/40)	100 (5/5)	0.313 (FET)
Migrant	24.4 (11/45)	27.5 (11/40) [1]	0 (0/5)	
Origin of infection (%)				
Autochthonous	86.0 (43/50)	83.7 (36/43)	100 (7/7)	0.573 (FET)
Imported	14.0 (7/50)	16.3 (7/43) [2]	0 (0/7)	
Immunosuppression (%)				
Yes	48.0 (24/50)	46.5 (20/43)	57.1 (4/7)	0.697 (FET)
Unknown/Not reported	2.0 (1/50)	0 (0/43)	14.3 (1/7)	
HIV infection/AIDS				
Yes (%)	32.7 (16/49)	34.9 (15/43)	16.7 (1/6)	0.649 (FET)
CD4 cell count <200/μL (%)	78.6 (11/14)	84.6 (11/13)	0 (0/1)	0.214 (FET)
Chronic pharmacologic immunosuppression (%)				
Inflammatory/autoimmune diseases [3]	12.2 (6/49)	9.3 (4/43) [4]	33.3 (2/6) [5]	0.151 (FET)
Other	4.1 (2/49)	2.3 (1/43)	16.7 (1/6) [7]	
Chronic dysfunction/condition (%)				
Yes	28.6 (12/42)	19.4 (7/36) [8]	83.3 (5/6) [9]	**0.005** (FET)

[1] Brazil n = 4; Cape Verde n = 2; Guinea-Bissau n = 1; Morocco n = 1; São Tomé e Príncipe n = 1; Senegal n = 1; unknown n = 1
[2] Brazil n = 3; Morocco n = 2; Mexico n = 1; Tunisia n = 1
[3] Systemic lupus erythematosus n = 2; Ankylosing spondylitis n = 1; Crohn's disease n = 1; Psoriasis n = 1; Reumatoid arthritis n = 1
[4] Adalimumab n = 1, Adalimumab+methotrexate n = 1, Methotrexate n = 1, Methotrexate+prednisolone n = 1
[5] Methotrexate n = 1; Mycophenolate mofetil+prednisolone n = 1
[6] Lymphoma n = 1
[7] Kidney transplant n = 1
[8] Chronic kidney disease n = 6; Chronic heart failure n = 1; Chronic obstructive respiratory disease n = 1; Chronic hepatic disease n = 1; Diabetes mellitus n =1
[9] Chronic kidney disease n = 2; Chronic heart failure n = 1; Chronic obstructive respiratory disease n = 1; Diabetes mellitus n = 3
Abbreviations: CL - Cutaneous leishmaniasis; ML - Mucosal leishmaniasis; HIV - Human immunodeficiency virus; AIDS - Acquired Immunodeficiency syndrome; IQI - Interquartile interval; FET - Fisher's exact test

Immunosuppressing conditions were present in 48.0% of patients, including HIV infection/AIDS, reported in nearly one-third of patients (78.6% had CD4 cell counts <200/μL). Additionally, chronic pharmacologic immunosuppression for inflammatory diseases was reported in 12.2% of patients (*n* = 6), most commonly consisting of regimens containing anti-TNFα (*n* = 2) and methotrexate (*n* = 3). Chronic organ dysfunction was present in 28.6% of patients, especially those with ML (*p* = 0.005, FET).

Of the 50 cases of CL/ML identified, 46 were considered primary (or incident) cases, and 4 were relapsing cases (first episode diagnosed before 2010). The estimated annual incidence of CL/ML in mainland Portugal between 2010 and 2020 was 0.036 cases/100,000 population/year. Table 2 and Figure 1 show the number of cases of CL/ML diagnosed between 2010 and 2020 and the estimated annual incidence in this period by NUTS2 and NUTS3 region.

Figure 1. Estimated mean annual incidence, per 100,000 population, between 2010 and 2020 of cutaneous leishmaniasis by NUTS3 region.

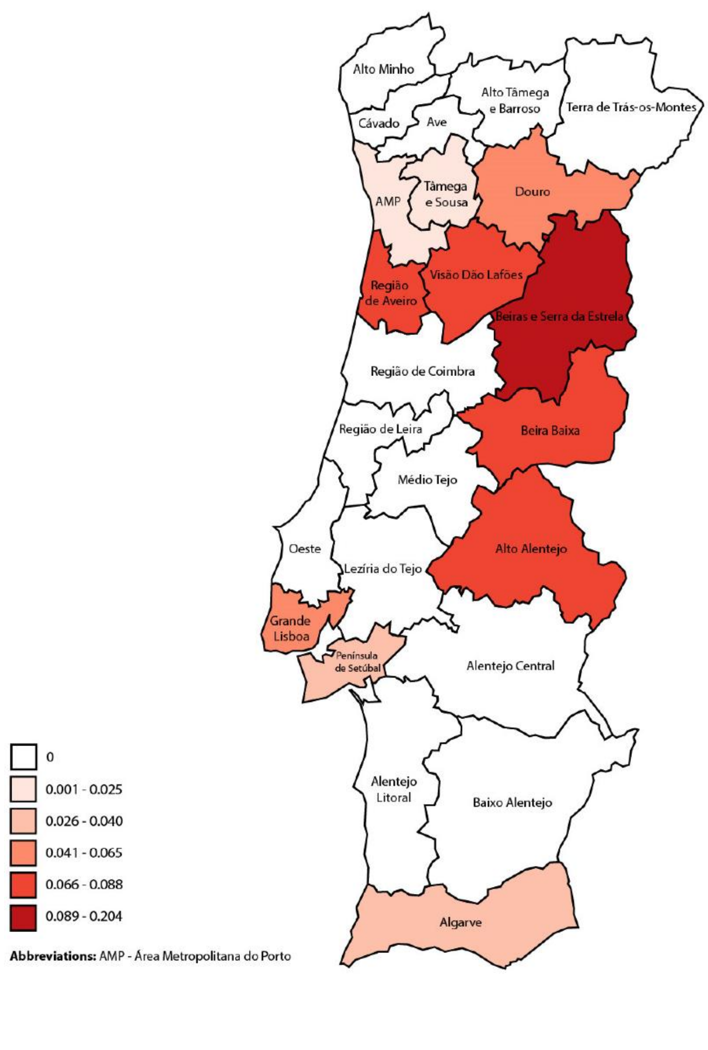


Table 2. Number of cases of cutaneous and mucosal leishmaniasis diagnosed between 2010 and 2020, inclusively, and mean annual incidence in this period, per 100,000 population, by NUTS2 and NUTS3 region.

Region	Average population in 2011-2021*	Number of CL/ML cases	Mean annual CL/ML incidence**	95% CI
Mainland Portugal	9,951,765	39	0.036	0.024-0.047
Norte	3,638,134	7	0.017	0.006-0.033
Alto Minho	238,051	0	0	NA
Cávado	413,387	0	0	NA
Ave	421,933	0	0	NA
Área Metropolitana do Porto	1,747,876	4	0.021	0.003-0.046
Alto Tâmega	89,195	0	0	NA
Tâmega e Sousa	420,776	1	0.022	0.001-0.120
Douro	194,516	1	0.047	0.001-0.260
Terras de Trás-os-Montes	112,399	0	0	NA
Centro	1,695,204	13	0.070	0.037-0.119
Região de Aveiro	368,898	3	0.074	0.015-0.216
Região de Coimbra	448,500	0	0	NA
Região de Leiria	290,692	0	0	NA
Viseu Dão Lafões	260,205	2	0.070	0.008-0.252
Beira Baixa	103,597	1	0.088	0.002-0.489
Beiras e Serra da Estrela	223,312	5	0.204	0.066-0.475
Oeste e Vale do Tejo	823,948	0	0	NA
Oeste	363,025	0	0	NA
Médio Tejo	219,266	0	0	NA
Lezíria do Tejo	241,657	0	0	NA
Grande Lisboa	2,052,392	13	0.058	0.031-0.098
Península de Setúbal	793,651	3	0.034	0.007-0.100
Alentejo	489,259	1	0.019	0.000-0.069
Alentejo Litoral	97,183	0	0	NA
Baixo Alentejo	120,777	0	0	NA
Alto Alentejo	111,714	1	0.081	0.002-0.453
Alentejo Central	159,585	0	0	NA
Algarve	459,174	2	0.040	0.005-0.143

*Arithmetic mean between the population size estimated in the National Census of 2011 and 2021.
**Number of new cases per 100,000 population, per year. Based on the following formula: Incidence = (New Cases) / (Population x Timeframe)
Abbreviations: CL - cutaneous leishmaniasis; ML - mucosal leishmaniasis; NA - not applicable; CI - confidence interval

3.2. Clinical Aspects of CL

Globally, the median time from onset of symptoms to presentation to healthcare services was 16 weeks (IQI 8–48), and the median time from presentation to diagnosis was 16 days (IQI 7–35). Hospital admission occurred in 60.5% of patients and was significantly more common in immunosuppressed patients (77.3% of cases; $p = 0.021$, $\chi 2 = 5.324$, df = 1).

The clinical aspects of autochthonous cases of CL ($n = 36$) are represented in Table 3. Approximately 40% of patients had multiple lesions; the median size of the largest lesion was 30 mm (IQI 10–40). The most common type of lesion was a papule/nodule (present in 62.1% of patients), followed by an ulcer (24.1%). The head was the most common anatomical location (48.3%), followed by the upper and lower limbs (each in 31.0%). Skin bacterial superinfection was reported in 11.1% of cases. The lesions were similar in immunosuppressed and non-immunosuppressed patients, except that they were more frequently localized in the trunk ($p = 0.002$, FET) and were painful ($p = 0.037$, FET) in the former group. Additionally, 54.5% of immunosuppressed patients had simultaneous VL, contrasting with 4.2% in the non-immunosuppressed group ($p < 0.001$, CST, $\chi 2 = 14.369$, df = 1). Disseminated CL was only seen in immunosuppressed patients, occurring in 22.2% of these cases ($n = 4$, all of them people living with HIV).

Table 3. Clinical presentation and management of autochthonous cutaneous leishmaniasis cases between 2010 and 2020.

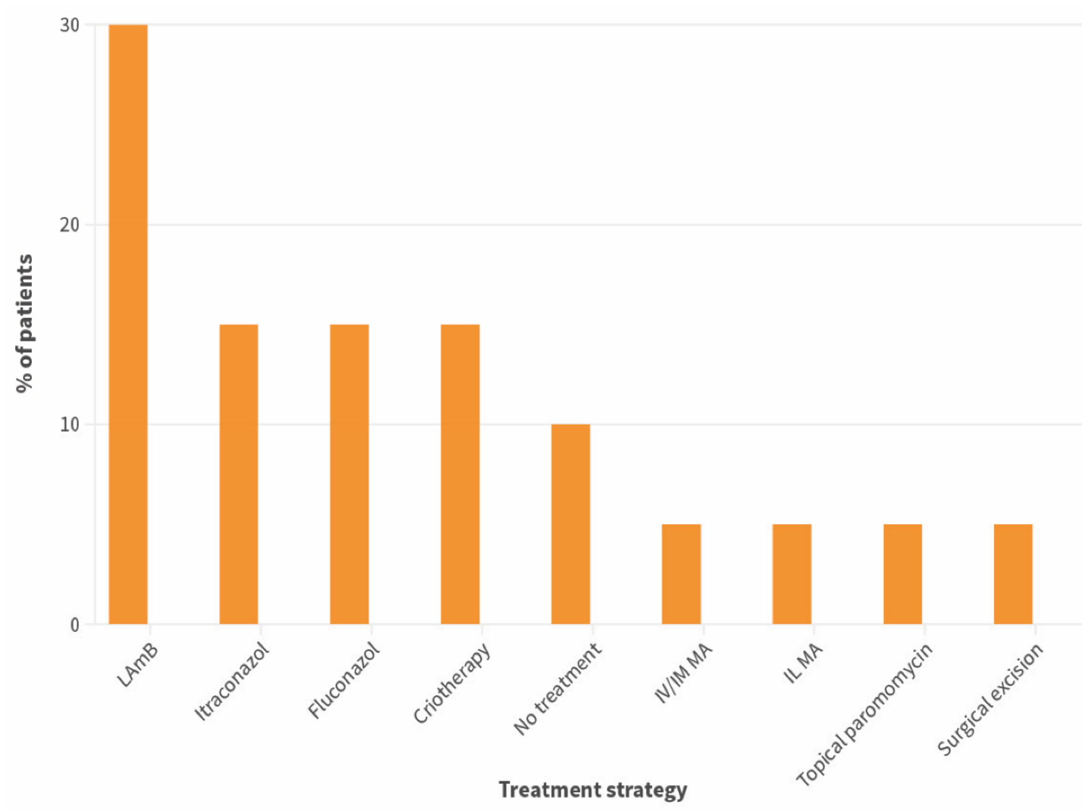
Type of lesion (%)	
Papule/Nodule	62.1 (18/29)
Ulcer	24.1 (7/29)
Macule/Plaque	20.7 (6/29)
Multiple lesions (%)	41.9 (13/31)
Median size of largest lesion, mm (IQI)	30 [10 - 40]
Location of lesions (%)	
Head	48.3 (14/29)
Upper limps	31.0 (9/29)
Lower limps	31.0 (9/29)
Trunk	20.7 (6/29)
Disseminated cutaneous leishmaniasis (%)	12.5 (4/32)
Local pain (%)	25.0 (5/20)
Skin superinfection [1] (%)	11.1 (3/27)
Simultaneous visceral leishmaniasis (%)	38.9 (14/36)
Technique used in skin/mucosa sample (%)	
Microscopy	100 (31/31)
Positive result	100 (30/30)
Polymerase chain reaction	28.6 (8/28)
Positive result	85.7 (6/7)
Identification of species [3] (%)	19.4 (6/31)
Serology (%)	
Yes [2]	34.6 (9/26)
% positive	37.5 (3/8)

Treatment of primary episode (%)	
Yes	93.8 (30/32)
Median time from diagnosis to treatment, days (IQI)	4 [0 - 31]
Monotherapy	93.3 (28/30)
Systemic	80.0 (24/30)
Topical	23.3 (7/30)
Side effects	15.4 (2/13)
Outcome of treatment (%)	
Improvement at 7 days	60.0 (9/15)
Improvement at 30 days	76.2 (16/21)
Switch of treatment/retreatment (non-improvement)	19.2 (5/26)
Relapse	5.6 (2/36)

[1] Methicillin-sensitive *Staphylococcus aureus* n = 1; Pseudomonas aeruginosa n = 1; non-identified n = 1
[2] Immunofluorescent antibody test n = 5; unknown n = 4
Abbreviations: IQI - interquartile interval

Skin samples of autochthonous CL cases were mostly obtained by biopsy, and by smear/scrapping in only in 2.4% of cases. Microscopy was used in all cases and was positive in 100% of these cases. PCR was used in 28.6% of cases and was positive in 85.7% of these. *Leishmania* species/complex was identified in 19.4% of cases (by molecular biology techniques); all belonged to the *L. donovani* complex (*n* = 6). Successful identification was available for 4/7 imported cases (*L. donovani* complex: *n* = 2, *L. Viannia* sp*.: n* = 2). Serology was used as part of the diagnostic workup in 34.6% of cases and was positive in 37.5% of these. Treatment for CL was introduced in 93.8% of patients, and the median time from diagnosis to treatment was four days (IQI 0–31). Most patients were treated with monotherapy (92.5%). Systemic treatment was used in 80.0% of patients and significantly more frequently in immunosuppressed patients (*p* = 0.033, FET). Detailed strategies used for primary treatment of patients who had exclusively CL (without simultaneous visceral involvement) (*n* = 20) are represented in Figure 2. Improvement by day 7 or 30 after starting therapy was mentioned in 60.0 and 76.2% of patients, respectively, and was not significantly different in immunosuppressed patients. Switching to a different regimen or retreatment due to non-improvement was implemented in 19.2% of patients. Relapse was reported in two patients, both immunosuppressed.

Figure 2. Strategies used for primary treatment of patients with autochthonous exclusively cutaneous leishmaniasis (no evidence of simultaneous visceral involvement) (n = 20).



Abbreviations: LAmB – liposomal amphotericin B; IL - Intralesional; IV/IM – Intravenous/intramuscular; MA – meglumine antimoniate

Time from presentation to diagnosis was the only variable that differed significantly between NUT2 regions ($p = 0.037$, KWT = 6.616, df = 2), being shorter in the Norte, followed by AML and Centro.

3.3. Clinical Aspects of ML

Leishmaniasis with mucosal involvement ($n = 7$) represented ML in all cases (no cases of MCL were identified). One patient was a person living with HIV, three were immunosuppressed for other reason, and two were non-immunosuppressed adults (one not defined). In five cases, only the nasal mucosa was involved (one with septal perforation), and in one case, both the oral and the nasal mucosa were involved. All cases were diagnosed by biopsy of the lesions and identification of amastigotes using microscopy, and PCR was used additionally in five cases. All cases were treated with liposomal amphotericin B except one, which was treated with IV meglumine antimoniate. One patient, who presented with simultaneously VL, died.

4. Discussion

The present study reinforces that the incidence of autochthonous CL cases seems to be lower than that of VL at a national level [24], although marked differences were noted between the NUTS2 and NUTS3 regions. Few data were previously available in the country and were mostly derived from case reports and case series, since no national reporting system is in place [25]. These data already suggested that the Beiras e Serra da Estrela region could be an important focus of CL [14], which was also pointed out in the present study. Recent cases revealed in this study in regions where CL has not been previously described in the literature, such as the Aveiro region, should be further investigated. Additionally, further studies could help elucidate whether or not this heterogeneity could be explained by an increased clinical awareness in certain regions or the presence of a distinct, particularly dermotropic *L. infantum* genotype. In support of the first hypothesis, it should be noted that in areas of neighboring Spain, the incidence of autochthonous CL was similar to that of VL [26]; however, CL notification is mandatory in Spain, as opposed to the protocol for Portugal [2] and in the present study, the underestimation of CL incidence could be due to inadequate coding at the hospital level and insufficient laboratory information.

Imported CL still represents a minority of cases in Portugal, as opposed to other European endemic countries, such as (metropolitan) France [27]; however, this could be expected to change in upcoming years due to increasing migration from CL endemic countries, such as Brazil [15]. Since no systematic clinical screening program is implemented in migrant populations in Portugal, leishmaniasis cases, especially (spontaneously resolving) CL, could go unnoticed and translate into an underestimation of imported cases.

In the autochthonous CL group, 48.0% were immunosuppressed, and in this group, 54.5% presented with simultaneously VL, which suggests that immunosuppressed people with CL benefit from a more intensive diagnostic workup in a setting where most CL is assumed to be caused by *L. infantum* [28]. Overall, time from onset of symptoms to presentation to healthcare and time from presentation to diagnosis were long, likely reflecting, on one hand, low concern of the patients for the lesions and, on the other hand, the low awareness of clinicians of the disease, or their unfamiliarity with the availability and performance of diagnostic techniques. In terms of lesion characteristics, the findings of the present study overlap those of other case series in the Mediterranean context, where *L. infantum* is endemic and includes frequent multiple lesions, a lower prevalence of ulcerated lesions compared to nodules/papules or plaques, and a predominance of head/neck lesions [3,29].

According to the present study, the diagnosis of CL in Portugal between 2010 and 2020 relied mostly on microscopy. As PCR was performed in only 42.1% of cases, *Leishmania* species was not identified in all imported cases, and therapy was commonly selected based on the probable geographical location of the infection. However, species identification in New World CL could have implications for individual management, especially in areas where multiple species co-circulate, since species in the Viannia subgenus, especially *L. braziliensis*, have been more frequently associated with MCL, requiring initial screening for this form and longer follow-up time [30]. On the other hand, species identification in Old World CL could be of public health interest, in the context of surveillance and assessment of risk of introduction of species such as dermotropic *L. tropica*. This anthroponotic species has been reported as the most imported species from refugees [31], and *Phlebotomus sergenti*, a specific vector of *L. tropica*, is widely distributed in Southern Europe, including in Portugal [32].

Treatment of CL cases was very heterogeneous but often relied on systemic therapy, especially IV/IM in immunosuppressed patients, which is in accordance with European recommendations [21]. Overall, rates of non-improvement (or failure) at 30 days after starting treatment (23.8%) were similar to those reported in previous studies in settings where *L. infantum* is endemic [3].

Although mucosal involvement is more commonly associated with *L. (Viannia)* sp., ML is increasingly recognized in the Old World in the context of *L. infantum* infection and especially in immunosuppression [33]. In the present study, seven cases were identified, all were autochthonous, and two were not immunosuppressed. Nose and throat physicians in endemic regions should be alert to the presentation, diagnostic approach, and management specificities of ML, particularly in regards to non-immunosuppressed patients and lesions located in the oral, pharyngeal, and laryngeal mucosa.

Finally, this study presents some limitations, beginning with the fact that in some hospitals, not all information was collected due to a lack of collaboration or the absence of patient consent. The coding of the diagnosis for inpatients was not uniformly performed and digitalized in every hospital for the whole duration of the study period, and coding for outpatients was irregularly performed in hospitals, so cases were screened via laboratory results, whenever feasible. Some hospitals required internal personnel to access information, so in some cases, interpretation of variables could be different, despite using the same database. Regarding the literature review of CL cases diagnosed in Portugal, a notable limitation may arise from the possibility that further cases might have been reported solely in national journals not indexed in PubMed or exclusively presented at conferences or congresses.

5. Conclusions

This study sheds light on the epidemiological and clinical landscape of CL and ML in Portugal between 2010 and 2020. While the incidence of autochthonous CL was low, CL was more common than previously reported, but still less common than in neighboring countries, possibly translating into significant underdiagnosis. Regional disparities highlight the importance of localized surveillance efforts. Programs to control leishmaniasis should focus not only on reducing underreporting, but also on raising awareness for the disease's different clinical forms among healthcare practitioners and providing tools for earlier diagnosis. Clinical suspicion should be particularly heightened for immunosuppressed people, who are disproportionately affected.

The present findings also underscore the potential for the underestimation of imported CL cases, particularly in the context of increasing migration from endemic regions, in the absence of systematic clinical screening programs. Given the implications for individual management and public health surveillance, there is a need for greater emphasis on species identification in imported cases.

Systematically combining clinical and national surveillance data could allow for a more detailed assessment of the epidemiologic situation and an evaluation of the progress in clinical practice, uncovering gaps that need to be addressed in the near future. In order to improve the overall outcome for leishmaniasis patients, human data should also be integrated with data from vectors and mammal hosts to produce holistic strategies to control the disease in several stages of the life cycle, following a One Health approach.

Ethics statement

This study received a favorable opinion of the Ethics Committees of all the involved institutions, namely: Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (reference 1.22); Centro Hospitalar do Baixo Vouga (16-01-2022/CES); Centro Hospitalar Barreiro Montijo; Centro Hospitalar de Entre o Douro e Vouga (29/2022); Centro Hospitalar de Leiria (34/2023); Centro Hospitalar de Lisboa Ocidental (2293); Centro Hospitalar do Médio Tejo (080/2022); Centro Hospitalar do Oeste; Centro Hospitalar de Setúbal (018/2022F); Centro Hospitalar do Tâmega e Sousa (63/2021); Centro Hospitalar Tondela Viseu (08/19/11/2021); Centro Hospitalar de Trás-os-Montes e Alto Douro; Centro Hospitalar de Vila Nova de Gaia e Espinho (65/2022); Centro Hospitalar Universitário do Algarve (172/2021); Centro Hospitalar Universitário de Coimbra (OBS.SF.083-2022); Centro Hospitalar Universitário Cova da Beira (03/2022); Centro Hospitalar Universitário de Lisboa Central (1287/2022); Centro Hospitalar Universitário Lisboa Norte; Centro Hospitalar Universitário de Santo António (2022.060); Centro Hospitalar Universitário de São João (06/2022); Hospital Beatriz Ângelo (4018/2022); Hospital de Braga (29_2022);

Hospital de Cascais Dr. José de Almeida (1/CE); Hospital Distrital de Santarém (31/2022); Hospital do Espírito Santo de Évora; Hospital Garcia de Orta; Hospital Professor Doutor Fernando Fonseca; Hospital da Senhora da Oliveira Guimarães (07/2022); Hospital de Vila Franca de Xira; Instituto Português de Oncologia de Coimbra Francisco Gentil; Instituto Português de Oncologia de Lisboa Francisco Gentil (73/2022); Unidade Local de Saúde do Alto Minho (30/2022); Unidade Local de Saúde do Baixo Alentejo (10/2021); Unidade Local de Saúde de Castelo Branco; Unidade Local de Saúde da Guarda (112/2022); Unidade Local de Saúde do Nordeste (59/2022); Unidade Local de Saúde do Norte Alentejano. Additionally, the study was authorized by the administration council of all the involved hospitals. In the three hospitals where informed consent was required, collection of data was only performed for patients who provided written consent.

6. References

1. Farrar J., Garcia P., Hotez P., Junghanss T., Kang G., Lalloo D., White N. *Manson's Tropical Diseases.* 24th ed. Elsevier; Amsterdam, The Netherlands: 2024.

2. ECDC Surveillance, Prevention and Control of Leishmaniases in the European Union and Its Neighbouring Countries. Stockholm. 2022. [(accessed on 3 March 2024)]. Available online: https://www.ecdc.europa.eu/sites/default/files/documents/leishmaniasis-surveillance-eu.pdf

3. Gaspari V., Gritti T., Ortalli M., Santi A., Galletti G., Rossi A., Rugna G., Mattivi A., Matteo G., Belloli G.L., et al. Tegumentary Leishmaniasis in Northeastern Italy from 2017 to 2020: A Neglected Public Health Issue. *Int. J. Environ. Res. Public Health.* 2022;19:16047. doi: 10.3390/ijerph192316047.

4. Phlebotomine sandflies maps. European Centre for Disease Prevention and Control and European Food Safety Authority. Stockholm: ECDC. [(accessed on 3 March 2024)]. Available online: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps

5. Maia C., Dantas-Torres F., Campino L. *The Leishmaniases: Old Neglected Tropical Diseases.* Springer International Publishing; Cham, Germany: 2018. Parasite Biology: The Reservoir Hosts; pp. 79–106.

6. Campino L., Abranches P. Leishmaniose cutânea: Uma doença rara em Portugal? *Acta. Med. Port.* 2002;15:387–390.

7. Borges-Costa J., Pacheco D., Soares-Almeida L., Sacramento-Marques M. Pápulas marrones y violáceas localizadas en la espalda de un paciente con infección por el virus de la inmunodeficiencia humana. *Enferm. Infecc. Microbiol. Clin.* 2013;31:410–411. doi: 10.1016/j.eimc.2012.12.003.

8. Costin A., Bonito F., Alves J., Barreiros H. Tratamiento de la leishmaniasis localizada mediante el antimoniato de meglumina intralesional y la terapia fotodinámica. *Actas Dermosifiliogr.* 2020;111:897–899. doi: 10.1016/j.ad.2020.02.004.

9. Vasconcelos J., Torres J., Granado J., Baptista T., Mansinho K. Cutaneous leishmaniasis in non-endemic countries: An emerging yet neglected problem. *IDCases.* 2019;17:e00570. doi: 10.1016/j.idcr.2019.e00570.

10. Marques N., Bustorff M., Cordeiro Da Silva A., Pinto A.I., Santarém N., Ferreira F., Nunes A., Cerqueira A., Rocha A., Ferreira I., et al. Visceral Dissemination of Mucocutaneous Leishmaniasis in a Kidney Transplant Recipient. *Pathogens.* 2020;10:18. doi: 10.3390/pathogens10010018.

11. Lopes L., Vasconcelos P., Borges-Costa J., Soares-Almeida L., Campino L., Filipe P. An atypical case of cutaneous leishmaniasis caused by *Leishmania infantum* in Portugal. *Dermatol. Online J.* 2013;19:20407. doi: 10.5070/D31911020407.

12. Matos A.L., Figueiredo C., Alves F., Pereira N., Gonçalo M. Mucocutaneous leishmaniasis complicating systemic lupus erythematosus and responding to high dose amphotericin B. *J. Eur. Acad. Dermatol. Venereol.* 2021;35:pe756. doi: 10.1111/jdv.17423.

13. Van der Auwera G., Davidsson L., Buffet P., Ruf M.T., Gramiccia M., Varani S., Chicharro C., Bart A., Harms G., Chiodini P.L., et al. Surveillance of leishmaniasis cases from 15 European centres, 2014 to 2019: A retrospective analysis. *Eurosurveillance.* 2022;27:2002028. doi: 10.2807/1560-7917.ES.2022.27.4.2002028.

14. Azevedo N. *Master's Thesis.* Universidade da Beira Interior; Covilhã, Portugal: 2009. Leishmaniose cutânea na Beira Interior: Um Diagnóstico Diferencial Obrigatório.

15. Fundação Francisco Manuel dos Santos PORDATA Base de Dados de Portugal Contemporâneo. População Residente Estrangeira Segundo os Censos: Total e por País de Nacionalidade. 2023. [(accessed on 3 March 2024)]. Available online: https://www.pordata.pt/portugal/populacao+residente+estrangeira+segundo+os+censos+total+e+por+pais+de+nacionalidade-3786

16. Fundação Francisco Manuel dos Santos PORDATA. O que são NUTS? [(accessed on 3 March 2024)]. Available online: https://www.pordata.pt/o+que+sao+nuts

17. Instituto Nacional de Estatística . *Censos 2021 Resultados Definitivos—Portugal, Lisboa.* Instituto Nacional de Estatística; Lisbon, Portugal: 2022.

18. Fundação Francisco Manuel dos Santos PORDATA Base de Dados de Portugal Contemporâneo. *SNS: Hospitais por Tipo—Continente.* 2022. [(accessed on 3 March 2024)]. Available online: https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=66196836&PUBLICACOESmodo=2

19. World Health Organisation Status of Endemicity of Cutaneous Leishmaniasis: 2022. 2023. [(accessed on 8 October 2023)]. Available online: https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html

20. Nemhauser J. *CDC Yellow Book 2024.* Oxford University Press; New York, NY, USA: 2023. Travelers with Additional Considerations; pp. 139–180.

21. Gradoni L., López-Vélez R., Mokni M. *Manual on Case Management and Surveillance of the Leishmaniasis in the WHO European Region.* World Health Organization; Geneva, Switzerland: 2017.

22. Daly L.E. Confidence Limits Made Easy: Interval Estimation Using a Substitution Method. *Am. J. Epidemiol.* 1998;147:783–790. doi: 10.1093/oxfordjournals.aje.a009523.

23. Antunes J., da Costa J.B., Guimarães M., Vaz-Pinto I., Soares-Almeida L., Filipe P. Post-Kala-azar dermal leishmaniasis due to *Leishmania donovani* in Europe—Case report. *Int. J. Dermatol.* 2013;52:1584–1586. doi: 10.1111/j.1365-4632.2011.05354.x.

24. Maia C., Conceição C., Pereira A., Rocha R., Ortuño M., Muñoz C., Jumakanova Z., Pérez-Cutillas P., Özbel Y., Töz S., et al. The estimated distribution of autochthonous leishmaniasis by *Leishmania infantum* in Europe in 2005–2020. *PLoS Negl. Trop. Dis.* 2023;17:e0011497. doi: 10.1371/journal.pntd.0011497.

25. Maia C., Campino L. Leishmaniose em Portugal no início do século XXI. *An. Inst. Hig. Med. Trop.* 2014;13:25–28.

26. Humanes-Navarro A.M., Herrador Z., Redondo L., Cruz I., Fernández-Martínez B. Estimating human leishmaniasis burden in Spain using the capture-recapture method, 2016–2017. *PLoS ONE.* 2021;16:e0259225. doi: 10.1371/journal.pone.0259225.

27. Pasquier G., Demar M., Lami P., Zribi A., Marty P., Buffet P., Desbois-Nogard N., Gangneux J.P., Simon S., Blaizot R., et al. Leishmaniasis epidemiology in endemic areas of metropolitan France and its overseas territories from 1998 to 2020. *PLoS Negl. Trop. Dis.* 2022;16:e0010745. doi: 10.1371/journal.pntd.0010745.

28. Campino L., Pratlong F., Abranches P., Rioux J.A., Santos-Gomes G., Alves-Pires C., Cortes S., Ramada J., Cristovão J.M., Afonso M.O., et al. Leishmaniasis in Portugal: Enzyme polymorphism of *Leishmania infantum* based on the identification of 213 strains. *Trop. Med. Int. Health.* 2006;11:1708–1714. doi: 10.1111/j.1365-3156.2006.01728.x.

29. Giavedoni P., Iranzo P., Fuertes I., Estrach T., Alsina Gibert M. Leishmaniasis cutánea. Experiencia de 20 años en un hospital español de tercer nivel. *Actas Dermosifiliogr.* 2015;106:310–316. doi: 10.1016/j.ad.2014.12.003.

30. Pan American Health Organization . *Guideline for the Treatment of Leishmaniasis in the Americas.* 2nd ed. Pan American Health Organization; Washington, DC, USA: 2022.

31. Rocha R., Pereira A., Maia C. Non-Endemic Leishmaniases Reported Globally in Humans between 2000 and 2021—A Comprehensive Review. *Pathogens.* 2022;11:921. doi: 10.3390/pathogens11080921.

32. European Centre for Disease Prevention and Control (ECDC) Phlebotomus sergenti-Current Known Distribution: August 2023. 2023. [(accessed on 13 October 2023)]. Available online: https://www.ecdc.europa.eu/en/publications-data/phlebotomus-sergenti-current-known-distribution-august-2023

33. Faucher B., Pomares C., Fourcade S., Benyamine A., Marty P., Pratlong L., Faraut F., Mary C., Piarroux R., Dedet J.P., et al. Mucosal *Leishmania infantum* leishmaniasis: Specific pattern in a multicentre survey and historical cases. *J. Infect.* 2011;63:76–82. doi: 10.1016/j.jinf.2011.03.012

CHAPTER 6

Epidemiological and Clinical Aspects of Visceral Leishmaniasis in Portugal.

This chapter is a transcription of the research article:

Rocha, R., Conceição, C., Gonçalves, L., LeishPT group, Maia, C. Epidemiological and clinical trends of visceral leishmaniasis in Portugal: retrospective analysis of cases diagnosed in public hospitals between 2010 and 2020. Infect Dis Poverty 13, 41 (2024). https://doi.org/10.1186/s40249-024-01204-5

Abstract

Background. *Leishmania infantum* is endemic in the Mediterranean region, presenting mostly as visceral leishmaniasis (VL). In Portugal, reporting of VL cases to public health authorities is mandatory, but significant underreporting is likely. This study aimed to describe the epidemiological and clinical aspects of the VL cases diagnosed in hospitals of the Portuguese National Health Service (NHS), between 2010 and 2020.

Methods. Collaboration was requested to every hospital of the Portuguese NHS in Mainland Portugal. Cases were screened through a search of diagnostic discharge codes or, if not available, by a search of positive laboratory results for *Leishmania* infection. Sociodemographic and clinical data was retrieved from medical records. Simultaneously, the National Health authority was contacted to request access to data of notified cases of VL between 2010 and 2020. Descriptive, hypothesis testing and multiple binary logistic regression models were performed.

Results. A total of 221 VL cases were identified. A significant increase in estimated national incidence was seen in the years after 2016 ($P = 0.030$). VL was predominantly diagnosed in people living with HIV (PLWH) and in children (representing around 60% of the new cases), but the outcome was generally poorer in non-HIV patients with associated immunosuppression, with significantly lower rates of clinical improvement at 7 ($P = 0.003$) and 30 days ($P = 0.008$) after treatment. Atypical presentations, with gastrointestinal and/or respiratory involvement, were seen in 8.5% of VL cases. Hemophagocytic lymphohistiocytosis was diagnosed in 40.0% of children under 5 years of age. Only 49.7% of incident VL cases were reported. Simultaneous involvement of the skin was confirmed in 5.9% of patients.

Conclusions. VL presents a continuing threat in Portugal, especially to PLWH and children, and an increasing threat to other immunosuppressed groups. Recent increases in incidence should be closely monitored to allow prompt interventions. Programs to control the disease should focus on providing tools for earlier diagnosis and on reducing underreporting and promoting an integrated surveillance of human and animal disease. These data should be

combined with asymptomatic infection and vector information, following a One Health approach.

Keywords: *Leishmania*, Leishmaniasis, Visceral, People living with HIV, Children, Portugal

1. Background

Leishmaniases are a group of diseases caused by protozoan parasites of the genus *Leishmania*. These parasites are transmitted by phlebotomine sand flies, and the disease is zoonotic in most settings [1]. Clinical spectrum of symptomatic disease is usually grouped into two main syndromes, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) [1], both of which are endemic and geographically widespread in the Mediterranean region. In this region, *L. infantum*, which belongs to the *L. donovani* complex, is the etiologic species of most autochthonous human leishmaniasis cases [2]. Infection with *L. infantum*, when symptomatic, usually presents as VL, although cases of simultaneous or independent CL and mucosal leishmaniasis caused by this species are increasingly recognized [3].

In the western Mediterranean regions where *L. infantum* is endemic, including in Portugal, *Phlebotomus perniciosus* is the main vector [4], and dogs are considered to be the main reservoir for human infection [5]. Increasing evidence suggests that cats [6] and some wild animals (such as leporids [7]) may also play a relevant epidemiological role.

An important share of symptomatic *L. infantum* infection in Southern Europe has been described in people living with human immunodeficiency virus (HIV) and children [8]. However, cases in the context of non-HIV related immunosuppression have been recently increasingly described, including solid organ transplant recipients and patients with autoimmune and inflammatory diseases chronically medicated with immunosuppressive drugs [9].

In the period from 2005 to 2020, 5813 VL cases were reported to the WHO in the European region [10]. The cumulative incidence in this period per 100,000 population of VL was highest in Albania (2.15 cases), followed by Montenegro, Malta, Greece, Spain and North Macedonia (0.53–0.42), Italy (0.16), Portugal (0.09). However, for several countries, incidence estimates according to hospital discharges were significantly higher than calculated using WHO reported cases [10].

In Portugal, reporting of VL cases to central public health authorities is mandatory, as part of a passive surveillance system. The most recent findings from this system showed that 6 to 14 cases were reported per year between 2014 and 2018 [11], although this likely represents a significant underreporting of cases, as revealed in a previous study where, between 1999 and 2009, only 38.6% of cases diagnosed in public hospitals were notified to central public health authorities [12].

This study aimed to describe the epidemiological and clinical aspects of the cases of VL diagnosed in hospitals of the Portuguese National Health Service, between 2010 and 2020, as well as those reported to public health authorities over the same period.

2. Methods

2.1. Study population

This multicenter retrospective study targeted all cases of leishmaniasis diagnosed in public hospitals in Mainland Portugal, between 2010 and 2020. Mainland Portugal is located in Southwest Europe, bordering Spain and the Atlantic Ocean. According to the 2021 national census, the population of mainland Portugal was 9,857,593 inhabitants [13], of which 542,165 (5.2%) were born abroad [14]. Mainland Portugal is divided into five NUTS2 (from the French *Nomenclature des Unités Territoriales Statistiques*, Nomenclature of Territorial Units for Statistics) regions, 23 NUTS3 regions (Supplementary Fig. 1 and Supplementary Table 1), 278 municipalities and 2882 parishes. Between 2010 and 2020, hospital-based healthcare services were provided by the Portuguese National Health Service in 102 to 111 general and specialized hospitals in Mainland Portugal, according to data from the Directorate-General for Health (DGS) of Portugal [15]. Some of these hospitals are grouped in Hospital Centers. Every episode of admission to these hospitals as an emergency or inpatient is given a code on discharge for primary and secondary diagnoses, following the International Classification of Diseases (ICD). Mandatory notifications of VL cases to central health authorities, initially done in paper format, have, since 2014, been submitted through an electronic platform, the National Epidemiologic Surveillance System (SINAVE) [16].

Individuals diagnosed with VL in one of the hospitals of the Portuguese National Health Service, in Mainland Portugal, were included in this study. No age restrictions were considered, and both inward and outpatient settings were accepted. Only laboratory confirmed cases were included. This consisted of the presence of a compatible clinical picture and meeting at least one of the following criteria: (i) Detection of antibodies against *Leishmania* in serum; (ii) Detection of *Leishmania* DNA in any biological sample; (iii) Visualization of intracellular organisms in macrophages, compatible with *Leishmania* amastigotes in biopsy material or cytological examination; (iv) Growth of *Leishmania* from any clinical sample inoculated in a specific culture medium.

2.2. Data collection

Every hospital or hospital center was contacted and collaboration in this study was requested. Cases in each included hospital were screened through a search of diagnostic discharge codes: 085, 085.0, 085.9 (ICD-9); B55, B55.0, B55.9 (ICD-10). In hospitals where codification of diagnosis was incomplete or unavailable for the whole or parts of the period of analysis, listing of cases was complemented by searching positive *Leishmania* serology results and positive *Leishmania* DNA detection by Polymerase Chain Reaction (PCR) in the database of the Pathology laboratory. Additionally, cytology and histopathology reports (all types of samples) were screened for the keyword "*Leishmania*". Reports where the word was identified were thoroughly read and selected for analysis if they mentioned observation of *Leishmania* amastigotes. Sociodemographic and clinical data of the cases identified (including clinical presentation, underlying conditions/comorbidities, diagnosis, management, and outcome) was extracted from the medical records of each episode, codified, and inserted into a digital database. Data extraction was carried out by different professionals; a common database was used and a protocol for filling in the required information was provided to every collaborator.

Simultaneously, the DGS was contacted and access to notified cases of VL between 2010 and 2020 was requested. Sociodemographic and clinical data of these cases was provided by the DGS in a codified database. Cases of VL obtained from the two sources (hospitals and notifications) were matched, considering the following individual details: age and sex of patient, municipality of residence at the time of diagnosis, date of notification or admission to hospital. For duplicated cases, data from both sources was merged into a single entry in the final database.

Categorical variables extracted from the clinical records or notifications were analyzed mostly using the original categories provided as options in the standardized database, but regrouping was performed in some cases. Non-improvement was defined as persistence or worsening of signs/symptoms or laboratory changes, despite appropriate therapy, and was assessed at seven and thirty days after starting treatment. These two timeframes were defined by the authors to allow homogeneous data collection regarding outcome in the different hospitals involved. Clinical improvement in VL (with resolution of fever) is usually evident at seven days, according to previous knowledge [1]. In addition, European guidelines propose a definition of non-response for VL as no clinical improvement at four weeks after start of therapy [17]. Relapses were defined as recurrence of signs/symptoms and positive culture/PCR/microscopy in blood or other biological sample after completing primary treatment with clinical improvement at 30 days. Other definitions, classifications or categories used for data collection and presentation in this study are presented in Supplementary Table 2.

2.3. Statistical analysis

Annual mean incidence of VL was estimated based on the following formula: Incidence = (New Cases) / (Population × Timeframe), considering a timeframe of 11 years and an at-risk population, for each region, consisting of the average value between the number of inhabitants estimated in the census of 2011 and the census of 2021, according to the National Institute of Statistics [13]. The corresponding 95% confidence intervals (CIs) for the incidence rate were obtained using a substitution method [18].

Descriptive statistics and hypothesis testing were performed using IBM® SPSS® Statistics (Version 29.0, IBM Corp, Armonk, United States of America - USA). Bar charts were built using Microsoft® Excel® (Version Office 365, Microsoft Corp, Redmond, USA). Geographical representation and analysis of results was obtained using QGIS® (Version 3.22, Open Source Geospatial Foundation, Beaverton, USA).

For categorical variables, absolute frequencies and percentages were calculated. Symmetric continuous variables were summarized by means with standard deviations and asymmetric continuous variables (e.g., age, analytical values) by medians with interquartile intervals (IQIs). Missing or unknown data were excluded from denominators, unless stated otherwise.

For analysis of clinical variables, VL patients were distributed in four groups: children 5 years old or younger; non-immunosuppressed individuals over 5 years old; people living with HIV (PLWH); and non-HIV infected immunosuppressed individuals. Comparisons between these groups were performed using Pearson Chi-Square test (CST) for categorical variables; or Fisher's exact test (FET) in case of failure of the assumptions of the CST. For continuous variables, after checking the assumptions of normality and homogeneity of the variances, the Mann-Whitney U test (MWT) or the Kruskal-Wallis test (KWT) were used, for comparing two or more independent groups, respectively. To compare survival distributions between two or more groups, the logrank test was used. A value of $P < 0.05$ was considered statistically significant.

To identify sociodemographic and clinical factors associated with non-improvement at 7 days after starting treatment and non-reporting of VL cases, multiple binary logistic regression models were explored, analyzing variables with statistical meaning in the univariate analysis ($P < 0.20$) and some biologically relevant or potentially confounding variables. For those variables that remained significant, crude odds ratio (*OR*) were updated to adjusted odds ratio (a*OR*) with 95% *CI*. The Hosmer–Lemeshow test was used for assessing goodness of fit in each multiple logistic regression model [19]. The reference categories used for each independent variable are specified in each results table.

3. Results

3.1. Sociodemographic characteristics and comorbidities

Data from 42 of the 45 hospitals or hospital centers in Mainland Portugal was available for analysis.

Sociodemographic characteristics of VL cases are represented in Table 1. A total of 221 cases of VL were diagnosed between 2010 and 2020 in the hospitals included: 201 as primary (or incident) cases and 20 as relapsing cases (first episode diagnosed before 2010). Of the 114 cases provided by the DGS, notified during this period, all but 13 were also identified through the hospital searches.

Table 1. Sociodemographic characteristics of visceral leishmaniasis cases diagnosed in public hospitals in Mainland Portugal in 2010–2020.

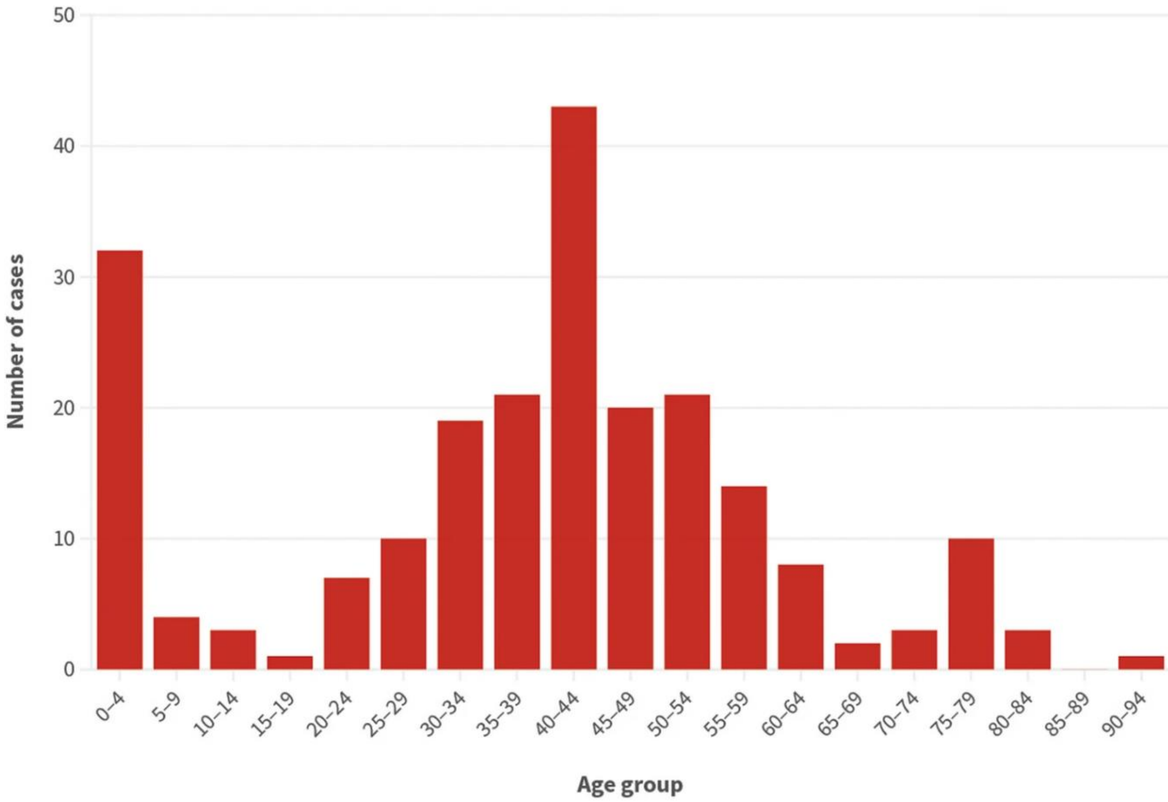
Number	221
Median age, years (IQI)	41 [28–50]
Male sex, % (*n*)	74.2 (164/221)
Region of diagnosis (NUTS2), % (*n*)	
Norte	17.2 (38/221)
Centro	15.8 (35/221)
Área Metropolitana de Lisboa	49.8 (110/221)
Alentejo	5.9 (13/221)
Algarve	11.3 (25/221)
Country of birth, % (*n*)	
Native	80.9 (157/194)
Migrant [1]	19.1 (37/194)
Origin of infection, % (*n*)	
Autochthonous	98.6 (214/217)
Imported	1.4 (3/217) [2]
Travel/residence abroad in the previous 12 months, % (*n*)	
Yes	8.9 (9/101)

Occupation, % (*n*)	
Unemployed	24.2 (23/95)
Retired	17.9 (17/95)
Service and sales, craft and industry	29.5 (28/95)
Agriculture and elementary	17.9 (17/95)
Professionals, technicians and clerical support	8.4 (8/95)
Type of home, % (*n*)	
Detached house	58.3 (35/60)
Apartment	18.3 (11/60)
Other [3]	23.3 (14/60)
Regular contact with domestic animals, % (*n*)	
Yes	73.4 (58/79)
Dogs	98.0 (50/51)
Cats	13.7 (7/51)
Other(s) [4]	17.6 (9/51)

[1] Angola *n* = 9; Cape Verde *n* = 6; Guinea-Bissau *n* = 5; Brazil *n* = 4; São Tomé e Príncipe *n* = 3; Mozambique *n* = 2; Senegal *n* = 1; Eritrea *n* = 1; Sweden *n* = 1; Ukraine *n* = 1
[2] Brazil *n* = 2; East Africa *n* = 1
[3] Homeless *n* = 7; Shelter or nursing home *n* = 6; Prison *n* = 1
[4] Cattle/sheep/goat *n* = 3; birds *n* = 7; rabbit *n* = 2;
Abbreviations: IQI - Interquartile interval

Median age was 41 years old (IQI: 28–50) and male sex was predominant. Age distribution of cases of VL is represented in Fig. 1. Approximately half of the cases were diagnosed in hospitals in the Lisbon Metropolitan Area (Área Metropolitana de Lisboa - AML) region. Only three cases (1.4%) were imported (from Brazil $n = 2$ and East Africa $n = 1$). Migrants represented approximately 20% of patients diagnosed, most of them born in sub-Saharan Africa (27/33) or Brazil (4/33). The two most common occupation status reported, accounting for around half of all patients, were unemployment (24.2%) or working in commerce/industry (29.5%). Patients reported living in a detached house (58.3%), apartment (18.3%) or other (23.3%, including shelter, nursing home, prison or homeless). Contact with domestic animals was common (73.4%), especially dogs. Moreover, close contact with animals with leishmaniasis was described for 9/57 of patients. No clear seasonality was seen in respect to month of presentation of autochthonous primary episodes to healthcare, although March and June accounted for the most admissions or first consultations (12.0% and 11.5% of total, respectively).

Figure 1. Age distribution (in years) of cases of visceral leishmaniasis diagnosed in 2010–2020 (n = 221).



Immunosuppressing conditions were present in 60.6% of patients. HIV infection/AIDS was reported in 53.5% of patients. Median CD4 cell count at time of diagnosis was 59.0/μlL (85.1% of patients had counts < 200/μl). Chronic pharmacologic immunosuppression for inflammatory diseases was reported in 10.8% of patients and other causes of immunosuppression included: solid organ transplant (*n* = 4), hematopoietic stem cell transplant (*n* = 1), solid organ malignancy (*n* = 4) and hematologic malignancy (*n* = 2). Immunosuppressing conditions and comorbidities of leishmaniasis patients are represented in Table 2.

Table 2. Immunosuppressing conditions and comorbidities of visceral leishmaniasis patients diagnosed in public hospitals in Mainland Portugal in 2010–2020.

Immunosuppression, % (*n*)	
Yes	60.6 (134/221)
Unknown/Not reported	8.1 (18/221)
HIV infection/AIDS	
Yes, % (*n*)	53.5 (108/202)
Median CD4 cell count, /µL (IQI)	59.0 [21.5–127.0]
CD4 cell count <200/µL, % (*n*)	85.1 (86/101)
Detectable viral load, % (*n*)	65.6 (63/96)
Median viral load, cp/mL (IQI)	80000 [220–631400]
Chronic pharmacologic immunosuppression, % (*n*)	
Inflammatory/autoimmune diseases	10.8 (21/194)
Anti-TNFα containing regimen	11.8 (2/17)
Methotrexate ± corticosteroid	58.8 (10/17)
Isolated corticosteroid	23.5 (4/17)
Other [1]	5.9 (1/17)
Solid organ transplant [2]	2.3 (4/173)
Chronic dysfunction/condition, % (*n*)	
Diabetes mellitus	7.9 (14/178)
Chronic kidney disease	12.5 (22/178)
Chronic liver disease	13.3 (24/181)
Chronic pulmonary disease	5.1 (9/178)
Chronic heart failure	3.9 (7/181)

[1] Azathioprine+corticosteroid *n* = 1; mycophenolate mofetil+corticosteroid *n* = 1
[2] Kidney *n* = 3; liver *n* = 1

Abbreviations: IQI – Interquartile interval; HIV - Human immunodeficiency virus; AIDS - Acquired Immunodeficiency syndrome; TNF - Tumor Necrosis Factor

The estimated incidence of VL by year and by NUTS2 region is represented in Fig. 2. Globally, there was a significant decrease in incidence from 2010 to 2015–2016 ($P = 0.001$, CST); however, incidence subsequently increased and, in 2019–2020, it was significantly higher than in 2015–2016 ($P = 0.030$, CST). The Alentejo, Algarve and Centro regions presented increasing incidence in the 2017–2020 period. Figure 3 shows the incidence of VL by NUTS3 and municipality. The number of cases of VL diagnosed between 2010 and 2020, inclusively, and the incidence in this period by NUTS2 and NUTS3 region are also provided in Table 3. In the study period, the estimated incidence was highest in the Algarve (0.495 cases /100,000 population /year) and lowest in the Norte NUTS2 region (0.095 cases /100,000 population /year).

Figure 2. Yearly incidence of visceral leishmaniasis between 2010 and 2020 per 100,000 population, in Mainland Portugal and in each NUTS (Nomenclature of Territorial Units for Statistics) 2 region.

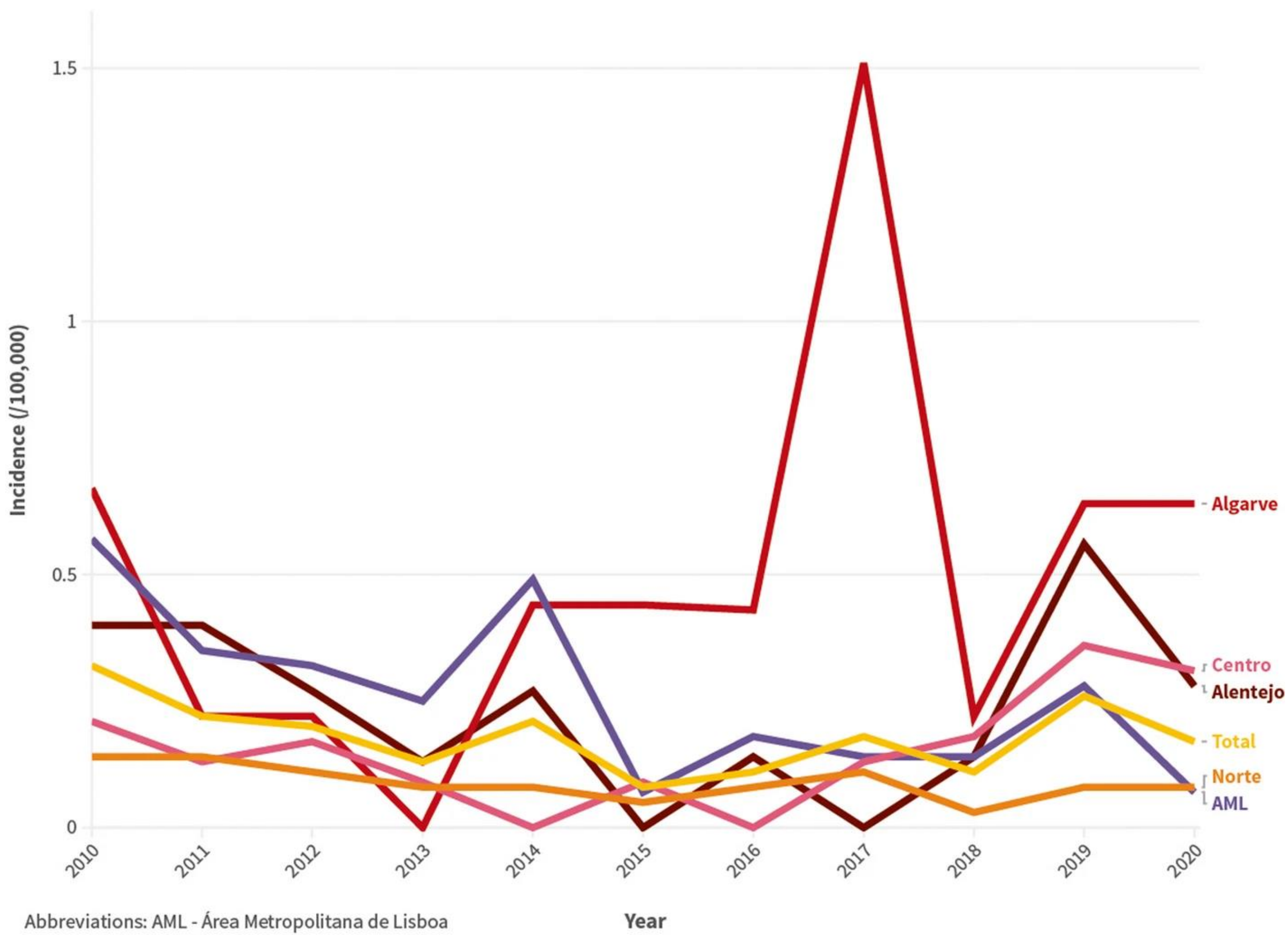
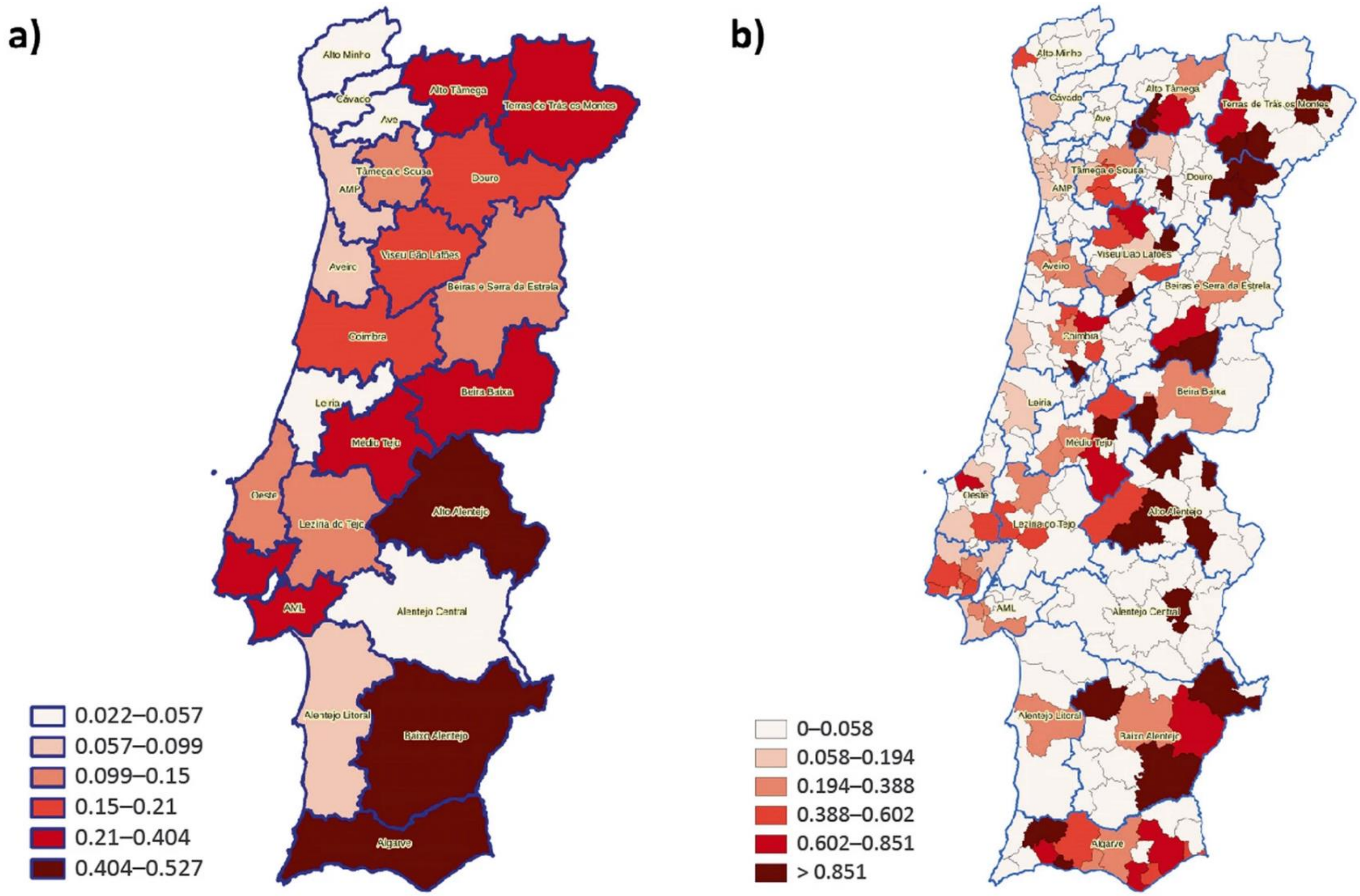


Figure 3. Mean annual incidence between 2010 and 2020, per 100,000 population, of visceral leishmaniasis by: a) NUTS (Nomenclature of Territorial Units for Statistics) 3 region; b) municipality.



Abbreviations: AML – Área Metropolitana de Lisboa; AMP – Área Metropolitana do Porto

Table 3. Number of cases of visceral leishmaniasis diagnosed in public hospitals in Mainland Portugal between 2010 and 2020, inclusively, and estimated mean annual incidence in this period, per 100,000 population, by NUTS (Nomenclature of Territorial Units for Statistics) 2 and NUTS3 region.

Region	Average population in 2011-2021*	Number of VL cases	VL mean annual incidence**	95% *CI*
Mainland Portugal	9,951,765	201	0.184	0.159–0.211
Norte	3,638,134	38	0.095	0.067–0.130
Alto Minho	238,051	1	0.038	0.001–0.213
Cávado	413,387	1	0.022	0.001–0.120
Ave	421,933	2	0.043	0.005–0.156
Área Metropolitana do Porto	1,747,876	15	0.078	0.044–0.129
Alto Tâmega	89,195	3	0.306	0.063–0.894
Tâmega e Sousa	420,776	6	0.130	0.048–0.282
Douro	194,516	4	0.187	0.051–0.479
Terras de Trás-os-Montes	112,399	5	0.404	0.131–0.944
Centro	2,277,497	38	0.152	0.107–0.208
Oeste	363,025	5	0.125	0.041–0.292
Região de Aveiro	368,898	4	0.099	0.027–0.252
Região de Coimbra	448,500	9	0.182	0.083–0.346
Região de Leiria	290,692	1	0.031	0.008–0.174
Viseu Dão Lafões	260,205	6	0.210	0.077–0.456
Beira Baixa	84,907	3	0.321	0.066–0.939
Médio Tejo	237,956	7	0.267	0.108–0.551
Beiras e Serra da Estrela	223,312	3	0.122	0.025–0.357
Área Metropolitana de Lisboa	2,846,042	81	0.259	0.206–0.322
Alentejo	730,917	19	0.236	0.142–0.369
Alentejo Litoral	97,183	1	0.094	0.002–0.521
Baixo Alentejo	120,777	7	0.527	0.212–1.086
Lezíria do Tejo	241,657	4	0.150	0.041–0.385
Alto Alentejo	111,714	6	0.488	0.179–1.063
Alentejo Central	159,585	1	0.057	0.001–0.317
Algarve	459,174	25	0.495	0.320–0.731

*Arithmetic mean between the population size estimated in the National Census of 2011 and 2021.
**Number of new cases per 100,000 population, per year. Based on the following formula: Incidence = (New Cases) / (Population x Timeframe)
Abbreviations: VL - visceral leishmaniasis; *CI* - Confidence interval

3.2. Clinical aspects

3.2.1. Clinical manifestations and laboratory alterations

Clinical presentation aspects of incident VL primary episodes are summarized in Table 4 globally and by group: children 5 years of age or younger (CU5), non-immunosuppressed adults and children over 5 years old (NISA), people living with HIV (PLWH) and non-HIV infected immunosuppressed adults (ISA).

Table 4. Clinical presentation characteristics of visceral leishmaniasis primary episodes, diagnosed in public hospitals in Mainland Portugal, and incident between 2010 and 2020, globally and by group.

	Global	CU5	NISA	PLWH	ISA	*P*-value
Number	194	34	35	82	25	
Median time from onset to presentation, weeks (IQI)	4 [1.5–11]	2 [1–3.5]	5 [1–19]	4 [2–14]	4 [1.75–9]	0.01 (H = 11.326, df = 3)
Department of consultation/ward, % (*n*)						
Infectious Diseases	40.9 (67/164)	0 (0/29)	18.2 (6/33)	66.7 (52/78)	37.5 (9/24)	< 0.001 (FET) (excluding CU5)
Internal Medicine	34.8 (57/164)	0 (0/29)	69.7 (23/33)	32.1 (25/78)	37.5 (9/24)	
Pediatrics	20.1 (33/164)	100 (29/29)	9.1 (3/33)	0 (0/78)	4.2 (1/24)	
Other	6.7 (11/164)	0 (0/29)	6.1 (2/33)	1.3 (1/78)	33.3 (8/24)	
Hospital admission, % (*n*)	94.5 (155/164)	100 (29/29)	96.9 (31/32)	91.1 (72/79)	95.8 (23/24)	0.363 (FET)
Median duration, days (IQI)	20 [12–36]	13.5 [9.0–17.75]	22 [11–49.5]	22 [12–45.75]	28 [17–34.5]	0.04 (H = 13.247, df = 3)
Critical care, % (*n*)	8.8 (14/160)	0 (0/27)	0 (0/32)	13.0 (10/77)	16.7 (4/24)	0.012 (FET)
Median duration, days (IQI)	8.5 [4.25–13.75]	N/A	N/A	8.5 [4.5–17.25]	9.0 [2.75–13.75]	0.734 (U = 0.116)
Signs/Symptoms, % (*n*)						
Splenomegaly	90.0 (153/170)	100 (30/30)	87.9 (29/33)	93.2 (69/74)	75.0 (18/24)	0.011 (FET)
Fever	71.9 (115/160)	96.6 (28/29)	69.7 (23/33)	59.7 (40/67)	70.8 (17/24)	0.004 (χ^2 = 13.254, df = 3)
Median highest value, ºC (IQI)	39.0 [38.6–40.0]	39.5 [39.0–40.0]	39.0 [38.75–40.0]	39.0 [38.2–39.2]	39.1 [39.0–40.0]	0.029 (H = 9.046, df = 3)
Hepatomegaly	71.8 (122/170)	63.3 (19/30)	60.6 (20/33)	88.0 (66/75)	56.5 (13/23)	0.001 (χ^2 = 15.826, df = 3)
Fatigue	69.8 (113/162)	29.6 (8/27)	81.3 (26/32)	75.0 (54/72)	76.2 (16/21)	<0.001 (χ^2 = 23.27, df = 3)
Anorexia	52.5 (84/160)	29.6 (8/27)	51.6 (16/31)	54.3 (38/70)	59.1 (13/22)	0.124 (χ^2 = 5.755, df = 3)
Weight loss	49.7 (75/151)	13.0 (3/23)	56.7 (17/30)	57.6 (38/66)	36.4 (8/22)	0.001 (χ^2 = 15.74, df = 3)
Gastrointestinal signs/symptoms	44.3 (66/149)	50.0 (14/28)	48.4 (15/31)	44.8 (30/67)	30.4 (7/23)	0.498 (χ^2 = 2.377, df = 3)

Respiratory signs/symptoms	28.3 (43/152)	25.9 (7/27)	25.8 (8/31)	29.6 (21/71)	30.4 (7/23)	0.964 (χ^2 = 0.279, *df* = 3)
Lymphadenopathy	23.0 (37/161)	14.3 (4/28)	29.0 (9/31)	27.5 (19/69)	13.0 (3/23)	0.272 (χ^2 = 3.902, *df* = 3)
Skin/mucosal hemorrage [1]	22.7 (35/154)	10.7 (3/28)	15.6 (5/32)	27.1 (19/70)	33.3 (8/24)	0.135 (χ^2 = 5.534, *df* = 3)
Neurological signs/symptoms	14.3 (22/154)	7.1 (2/28)	12.9 (4/31)	15.3 (11/72)	21.7 (5/23)	0.519 (FET)
Peripheral edema	12.8 (19/149)	0 (0/27)	12.5 (4/32)	16.4 (11/67)	17.4 (4/23)	0.097 (FET)
Abdominal image obtained, % (*n*) [2]	96.9 (155/160)	96.6 (28/29)	97.0 (32/33)	96.0 (72/75)	100 (23/23)	1 (FET)
Analytical changes, % (*n*)						
Anemia	98.9 (173/175)	100 (30/30)	97 (32/33)	100 (77/77)	100 (25/25)	0.533 (FET)
Median lowest value, g/dL (IQI)	7.6 [6.6–8.5]	6.7 [6.3–7.3]	8.1 [7.3–8.7]	7.7 [6.65–8.45]	7.6 [6.7–8.8]	0.267 (*H* = 2.642, *df* = 3)
Thrombocytopenia	90.2 (157/174)	90.0 (27/30)	87.5 (28/32)	90.9 (70/77)	88.0 (22/25)	0.930 (FET)
Median lowest value, /µl (IQI)	64000 [36500–112000]	76000 [47500–120250]	54000 [31000–106500]	69000 [44000–116500]	39000 [16000–92000]	0.026 (*H* = 7.271, *df* = 2)
Leukopenia	88.3 (143/162)	65.5 (19/29)	87.1 (27/31)	94.8 (73/77)	96 (24/25)	<0.001 (FET)
Median lowest value, /µl (IQI)	2000 [1272–2670]	3450 [2597–4850]	1685 [1025–2255]	1765 [1200–2300]	1300 [1077–1925]	0.250 (*H* = 2.769, *df* = 2)
C-reactive protein elevation	93.1 (148/159)	92.9 (26/28)	96.9 (31/32)	90.8 (69/76)	95.7 (22/23)	0.744 (FET)
Median highest value, mg/L (IQI)	88.0 [44.6–136.2]	88.0 [49.0–115.6]	113.0 [65.55–136.8]	68.9 [31.05–132.0]	125.7 [60.0–229.0]	0.027 (*H* = 7.254, *df* = 2)
Acute kidney failure	14.6 (23/157)	0 (0/27)	16.1 (5/31)	13.2 (10/76)	34.8 (8/23)	0.004 (FET)
Liver failure/decompensated chronic liver disease	6.2 (10/161)	3.4 (1/29)	12.9 (4/31)	5.2 (4/77)	4.2 (1/24)	0.464 (FET)
Hemophagocytic lymphohistiocystosis, % (*n*)	9.5 (14/147)	40.0 (10/25)	0 (0/31)	0 (0/67)	16.7 (4/24)	< 0.001 (FET)
Coinfection/superinfection, % (*n*)	14.6 (23/157)	34.5 (10/29)	29.0 (9/31)	50.7 (38/75)	43.5 (10/23)	0.163 (χ^2 = 5.122, *df* = 3)

[1] Lower gastrointestinal tract *n* = 10; ecchymosis/hematoma/petechiae *n* = 8; epistaxis *n* = 7; hemoptysis *n* = 7; gingival *n* = 3; upper gastrointestinal tract *n* = 1; vaginal *n* = 1
[2] Ultrasonography 72.3% (112/155); computed tomography scan 53.5% (83/155)

Abbreviations: IQI - Interquartile interval; FET - Fisher's exact test; CU5 - children 5 years of age or younger; NISA - non-immunosuppressed adults and children over 5 years old; ISA - non-HIV infected immunosuppressed adults; PLWH - people living with HIV

Median time from onset of signs/symptoms to first presentation to healthcare was 4 weeks globally (IQI: 2–11) and was significantly different between groups (shorter in children under 5 years old, $P = 0.010$, $H = 11.326$, $df = 3$). In ISA, median time from start of immunosuppressive therapy to onset of signs/symptoms was 16 weeks (IQI: 12–66). Over 90% of patients in all groups were admitted as inpatients. Median duration of hospitalization was 20 days (IQI: 12–36) and was significantly different between groups: shortest in children

[14] and longest in ISA [27] ($P \equiv 0.040$, KWT, $H = 13.247$, $df = 3$). Admission to critical care was only observed in ISA (16.7%) or in PLWH (13.0%). Fever was the most common presenting symptom (71.9%), followed by fatigue (69.8%), anorexia (52.5%) and weight loss (49.7%). Compared to NISA, fever was significantly less common in PLWH and more common in CU5, and the highest temperature was lower in PLWH and higher in CU5. Splenomegaly was detected in 90.0%, hepatomegaly in 71.8% and lymphadenopathy in 23.0%. Frequent laboratory abnormalities included: anemia (98.9%), thrombocytopenia (90.2%), leukopenia (88.3%), C-reactive protein (CRP) elevation (93.1%) and hepatic cytolysis or cholestasis (55.7%). Acute kidney failure was detected on admission in 14.6% of patients and was more common in ISA ($P \equiv 0.004$, FET). Criteria for HLH were met in 14 patients: 10 CU5 (40.0% of cases) and 4 in ISA (16.7%). Considering primary episodes and relapses, atypical presentations were diagnosed in 14 patients (8.5%), representing 12.8% of PLWH and 16.7% of ISA. Involvement was: colorectal ($n = 6$), duodenal/ileal ($n = 7$), gastric ($n = 4$), peritoneal ($n = 1$), pleural ($n = 1$), and bronchial ($n = 1$). Simultaneous involvement of the skin (with CL) was confirmed in 5.9% of patients. Coinfection/superinfection was detected in 42.4% of patients, without significant differences between groups, and was caused by the microbiological agents described in Supplementary Fig. 2. Respiratory and oropharyngeal/esophageal infections were the most common and *Candida* sp. and *Escherichia coli* were the most implicated microorganisms.

3.2.2. Diagnosis

Diagnosis, treatment, and outcome aspects of incident VL primary episodes are summarized in Table 5, globally and by group. Median time from presentation to diagnosis was 10 days (4.5–19.5) and was significantly different between groups: shortest in CU5 (5.5 days) and longest in ISA (17.5 days) ($P \equiv 0.011$, KWT, $H = 11.192$, $df = 3$). Samples most frequently used for direct diagnosis were: bone marrow (94.1%) and blood (25.0%). Techniques most often used in bone marrow samples were: microscopy (95.6%), PCR (41.6%), and culture (22.7%). Positivity rate was similar for PCR, microscopy, and culture (81.7%, 80.9% and 75.0%, respectively) and was not significantly different between groups. In blood samples, PCR was the technique most used for direct diagnosis (70.3%) and was positive in 73.1% of cases. In all cases in which *Leishmania* species identification was attempted and successful (n = 59), *L. donovani* complex was identified (by molecular biology techniques). Serologic techniques were used in 52.5% of patients, most commonly immunofluorescent antibody test (73.3%) and enzyme-linked immunosorbent assay (17.8%). Serology was positive in 82.9% of patients, ranging from 72.4% in PLWH to 92.3% in CU5, although this difference was not statistically significant (P = 0.482, FET).

Table 5. Diagnosis, treatment, and outcome aspects of visceral leishmaniasis primary episodes, diagnosed in public hospitals in Mainland Portugal, and incident in the period between 2010 and 2020, globally and by group.

	Global	CU5	NISA	PLWH	ISA	*P*-value
Median time from presentation to diagnosis, days (IQI)	10 [4.5–19.5	5.5 [2.25–12.5]	11.0 [5.5–30.0]	10 [4.0–17.5]	17.5 [9.5–30.0]	0.011 (H = 11.192, df = 3)
Samples used (direct diagnosis), % (*n*)						
Bone marrow	94.1 (160/170)	96.4 (27/28)	100 (34/34)	93.4 (71/76)	82.6 (19/23)	0.058 (FET)
Aspirate	91.4 (139/152)	81.5 (22/27)	97.1 (33/34)	93.0 (66/71)	89.5 (17/19)	0.158 (FET)
Biopsy	50.7 (77/152)	59.3 (16/27)	50.0 (17/34)	46.5 (33/71)	57.9 (11/19)	0.639 (χ^2 = 1.692, df = 3)
Blood	25.0 (37/148)	34.8 (8/23)	17.9 (5/28)	13.7 (10/73)	13.6 (3/22)	0.166 (FET)
Technique used in bone marrow sample, % (*n*)						
Microscopy	95.6 (152/159)	92.3 (24/26)	96.7 (29/30)	97.3 (71/73)	95.5 (21/22)	0.609 (FET)
Positive result	80.9 (123/152)	66.7 (16/24)	75.9 (22/29)	83.1 (59/71)	90.5 (19/21)	0.193 (FET)
Polymerase chain reaction	41.6 (62/149)	60.9 (14/23)	32.3 (10/31)	38.2 (26/68)	47.4 (9/19)	0.158 (χ^2 = 5.194, df = 3)
Positive result	81.7 (49/60)	78.6 (11/14)	60.0 (6/10)	87.5 (21/24)	88.9 (8/9)	0.315 (FET)
Culture	22.7 (32/141)	9.5 (2/21)	20.0 (6/30)	29.2 (19/65)	11.8 (2/17)	0.200 (FET)
Technique used in blood sample, % (*n*)						
Polymerase chain reaction	17.8 (26/146)	34.8 (8/23)	17.9 (5/28)	13.7 (10/73)	13.6 (3/22)	0.166 (FET)
Positive result	73.1 (19/26)	87.5 (7/8)	60.0 (3/5)	80.0 (8/10)	33.3 (1/3)	0.321 (FET)
Serology, % (*n*)						
Yes [1]	52.5 (83/158)	54.2 (13/24)	69.7 (23/33)	41.4 (29/70)	50.0 (11/22)	0.063 (χ^2 = 7.288, df = 3)
Positive result	82.9 (68/82)	92.3 (12/13)	86.4 (19/22)	72.4 (21/29)	81.8 (9/11)	0.482 (FET)
Samples sent to reference laboratory, % (*n*)	40.0 (50/125)	55.0 (11/20)	44.0 (11/25)	31.1 (19/61)	47.4 (9/19)	0.216 (χ^2 = 4.463, df = 3)

Treatment of primary episode, % (*n*)						
Yes	99.4 (161/162)	100 (27/27)	100 (32/32)	98.7 (78/79)	100 (24/24)	1 (FET)
Median time from diagnosis to treatment, days (IQI)	0 [0–1]	0 [0–1]	0 [0–0.2]	0 [0–0.2]	0 [0–4.75]	0.787 (H = 1.060, df = 3)
Median duration of treatment, days (IQI)	21 [10–38]	21 [7.75–21.0]	21 [10.0–21.0]	21 [10.0–38.0]	24 [11.0–38.0]	0.031 (H = 9.894, df = 3)
Liposomal amphotericin B monotherapy	98.8 (158/160)	92.6 (25/27)	100 (32/32)	100 (77/77)	100 (24/24)	1 (FET)
Side effects	30.5 (40/131)	9.5 (2/21)	42.3 (11/26)	25.0 (15/60)	45.5 (10/22)	0.025 (χ^2 = 9.365, df = 3)
Outcome of treatment, % (*n*)						
Median time to defervescence, days (IQI)	3 [1.75–5]	2 [1.0–3.0]	3 [1.0–3.0]	3 [2.0–5.5]	6 [2.5–10.5]	0.008 (H = 11.823, df = 3)
Improvement at 7 days	88.6 (132/149)	100 (27/27)	96.6 (28/29)	87.1 (61/70)	69.6 (16/23)	0.003 (FET)
Improvement at 30 days	96.4 (135/140)	100 (25/25)	100 (27/27)	98.5 (64/65)	82.6 (19/23)	0.008 (FET)
Switch of treatment/retreatment [2]	3.9 (6/153)	3.6 (1/28)	3.2 (1/31)	2.7 (2/73)	8.3 (2/24)	0.583 (FET)
Death in current episode	4.3 (7/163)	0 (0/30)	0 (0/33)	5.2 (4/77)	13.0 (3/23)	0.066 (FET)
Relapse						
Rate (episodes/patient-year)	0.112	0	0	0.175	0.147	0.578
Median time to first relapse, months (IQI)	12 [7.25–33.5]	N/A	N/A	15 [9.5–36.0]	6 [5.0–11.0]	0.009 (U = 48.0)
Secondary profilaxis initiated [3], % (*n*)	29.7 (44/148)	4.3 (1/23)	0 (0/30)	54.9 (39/71)	16.7 (4/24)	< 0.001 (χ^2 = 43.327, df = 3)
Follow-up in consultation, % (*n*)						
Yes	93.3 (140/150)	92.3 (24/26)	96.9 (31/32)	91.5 (65/71)	95.2 (20/21)	0.813 (FET)
Cure test performed [4]	16.9 (23/136)	13.6 (3/22)	3.4 (1/29)	24.2 (16/66)	15.8 (3/19)	0.080 (FET)
Notification of case to SINAVE, % (*n*)	49.7 (92/185)	75.8 (25/33)	50.0 (18/36)	40.0 (36/90)	50.0 (13/26)	0.006 (χ^2 = 12.353, df = 3)

[1] Immunofluorescent antibody test (33/45); Enzyme-linked immunosorbent assay (8/45); Western Blot (5/45); k39 rapid diagnostic test (2/45)
[2] Due to side effects n = 3; Due to non improval n = 3
[3] Liposomal amphotericin B n = 42; Miltefosine n = 1; unknown n = 1
[4] Bone marrow microscopy n = 12; PCR in blood n = 10
Abbreviations: IQI - Interquartile interval; FET - Fisher's exact test; CU5 - children 5 years of age or younger; NISA - non-immunosuppressed adults and children over 5 years old; ISA - non-HIV infected immunosuppressed adults; PLWH - people living with HIV; SINAVE - Sistema Nacional de Vigilância Epidemiológica

3.2.3. Treatment and outcome

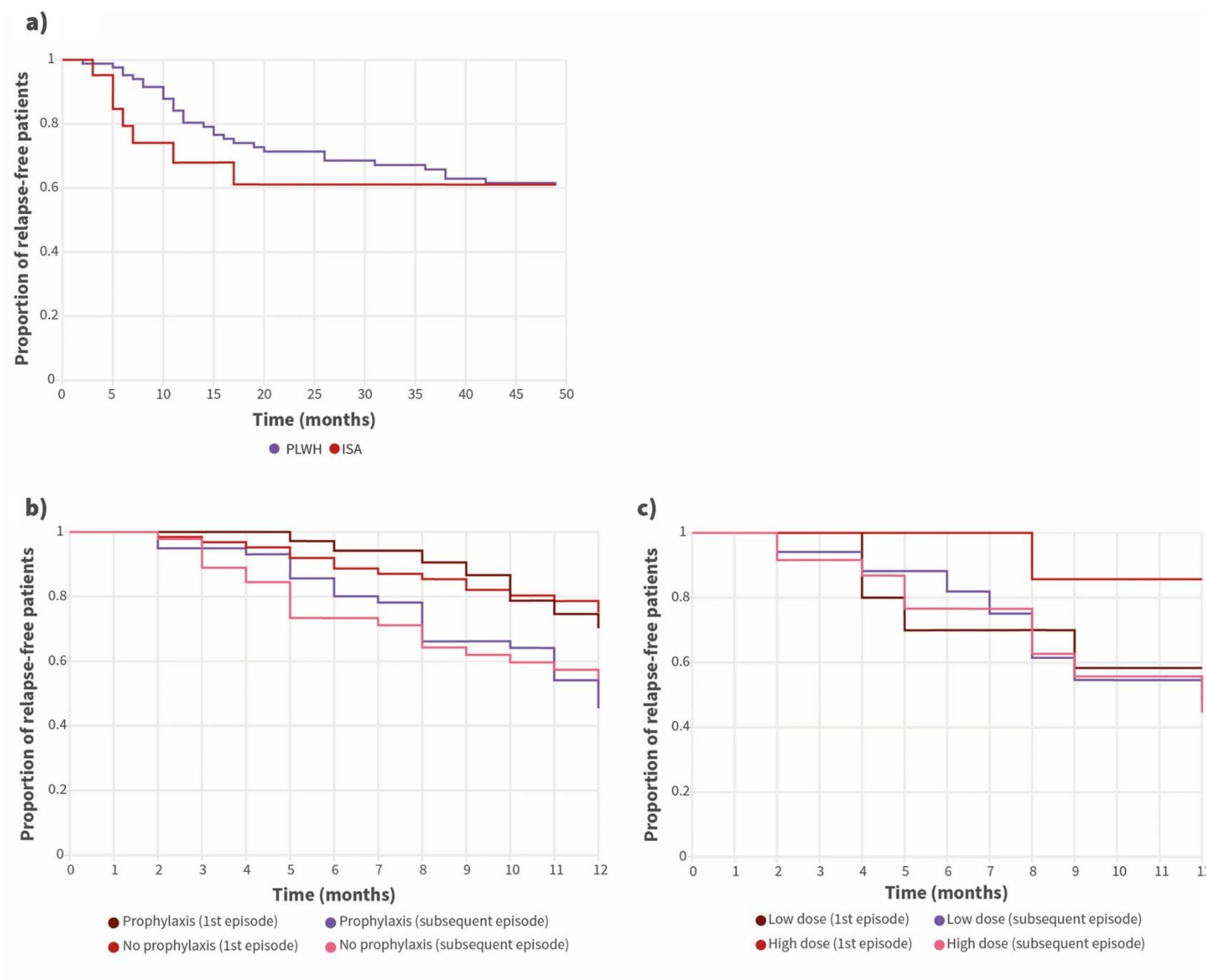
In most cases, treatment was initiated on the same day of diagnosis (median time 0 days, IQI: 0–1). Liposomal amphotericin B (LAmB) was used for primary treatment in 98.8% of cases and meglumine antimoniate for the rest (n = 2, both CU5). Side effects were reported globally in 30.5% of patients (n = 40) and were significantly less common in CU5 ($P \equiv 0.025$, CST, $\chi^2 = 9.365$, $df = 3$). The reported side effects included: acute kidney injury and/or hypokalemia (19.8%, n = 26), hepatotoxicity (n = 4), vomiting and/or diarrhea (n = 4), fever/shivering ($n = 3$), myalgia ($n = 2$), anaphylaxis ($n = 1$). In PLWH, antiretroviral therapy was initiated or reinitiated in 48.6% of patients; one case of paradoxical immune reconstitution inflammatory syndrome was documented. In ISA, withdrawal of immunosuppressive drugs or reduction of dose was done in 66.7% of cases. Median time to defervescence after initiation of anti-*Leishmania* therapy was 3.0 days (IQI: 1.75–5) and was significantly shorter for CU5 and longer for ISA ($P \equiv 0.008$, KWT, $H = 11.823$, $df = 3$). Improvement by day 7 after initiation of anti-*Leishmania* therapy was documented in 88.6% of cases, ranging from 69.6% in ISA to 87.1% in PLWH and 100% in CU5 ($P \equiv 0.003$, FET). Improvement by day 30 after initiation of therapy was documented in 96.4% of patients and was over 95% in all groups except ISA (82.6%, $P \equiv 0.008$, FET). Death occurred in seven cases (4.3%): four PLWH (5.2%) and three ISA (13.0%). Secondary prophylaxis was implemented in 54.9% of PLWH, but in only 16.7% of ISA ($P = 0.001$, $\chi^2 = 10.599$, $df = 1$); drugs used for prophylaxis were LAmB (97.7%) and miltefosine (2.3%). Cure tests were performed for 16.9% of patients, especially PLWH, and median time to cure test was 6.1 weeks after completing primary treatment (IQI: 3.25–23.5).

3.2.4. Relapses

In total, there were 151 episodes of relapse in the study period, affecting 61 patients. The number of relapses per patient ranged from 1 to 9. Relapses were documented only in PLWH and in ISA, at a similar rate: 0.175 and 0.147 episodes per patient-year, respectively ($P \equiv 0.578$, CST). Relapse-free survival was significantly higher for PLWH than for ISA at nine months follow-up ($P \equiv 0.023$, $\chi^2 = 5.203$, $df = 1$) but not at 48 months ($P \equiv 0.453$, $\chi^2 = 0.562$, df $= 1$) (Fig. 4a). Relapse-free survival was slightly higher for patients on any prophylaxis in the first 10 months after a primary VL episode or a relapse (Fig. 4b), but this difference did not reach statistical significance ($P \equiv 0.396$, $\chi^2 = 0.720$, $df = 1$ for primary episodes; $P \equiv 0.674$, $\chi^2 = 0.177$, $df = 1$, for relapses). Rate of relapse was significantly higher in the 12 months after a relapse than after a primary episode, either with or without prophylaxis ($P \equiv 0.023$, $\chi^2 = 5.195$, $df = 1$; $P \equiv 0.012$, $\chi^2 = 6.364$, $df = 1$, respectively). Use of LAmB for prophylaxis at doses of 4–5 mg/kg every 2–3 weeks was associated with significantly higher relapse-free survival at 12 months than doses of 3–

4 mg/kg every 4 weeks, for primary episodes ($P \equiv 0.048$, $\chi^2 = 3.893$, $df = 1$), but not for relapses ($P \equiv 0.862$, $\chi^2 = 0.030$, $df = 1$) (Fig. 4c).

Figure 4. Relapse-free survival of visceral leishmaniasis patients: a) according to group; b) according to use of prophylaxis and primary/subsequent episode; c) according to dose of liposomal amphotericin B used and primary/subsequent episode.



Abbreviations: ISA - HIV non-infected immunosuppressed adults; PLWH - people living with HIV
Note: High dose prophylactic LAmB - 4-5mg/kg every 2-3 weeks; Low dose - 3-4mg/kg every 4 weeks

In relapse cases, compared to primary episodes (in PLWH or ISA), time from onset to presentation was significantly shorter (median 3.0 vs. 4.0 weeks, $P \equiv 0.030$, $U = 3377.0$). Drugs used for secondary prophylaxis after a relapse included LAmB (80.5%), miltefosine (6.5%), and LAmB + miltefosine (6.5%). Outcome of treatment of VL relapses according to drug used is represented in Table 6. The percentage of episodes with improvement was higher for combination therapy at 7 (90.0 vs. 80.8%) and 30 days (93.3 vs. 83.5%) after initiation of therapy, but this difference was not statistically significant ($P \equiv 0.238$, $\chi^2 = 1.395$, $df = 1$; $P \equiv 0.235$, FET, respectively). Subsequent relapse-free survival after a relapse was not significantly different for patients treated with monotherapy or combination therapy ($P \equiv 0.816$, $\chi^2 = 0.054$, $df = 1$). Side effects were less commonly reported for LAmB (39.4%), compared to miltefosine (55.6%) or meglumine antimoniate (71.4%).

Table 6. Outcome of treatment of episodes of relapse of visceral leishmaniasis, diagnosed in public hospitals in Mainland Portugal in 2010–2020, according to drug or combination of drugs used.

	Total	Monotherapy	LAmB	Miltefosine	Meglumine antimoniate	Combination	LAmB + Miltefosine	LAmB + Paromomycin	Others	*P*-value (monotherapy vs combination)	*P*-value (LAmB vs LAmB+miltefosine)	*P*-value (LAmB vs miltefosine vs meglumine)
Number, % (*n*)	100 (141/141)	77.3 (109/141)	63.1 (89/141)	6.4 (9/141)	7.8 (11/141)	22.7 (32/141)	17.7 (25/141)	2.1 (3/141)	2.8 (4/141) [1]			
Side effects, % (*n*)	44.0 (51/116)	43.7 (38/87)	39.4 (28/71) [2]	55.6 (5/9) [3]	71.4 (5/7) [4]	44.8 (13/29)	37.5 (9/24) [5]	100 (3/3) [6]	50.0 (1/2)	0.914 ($\chi^2 = 0.012$, $df = 1$)	0.866 ($\chi^2 = 0.028$, $df = 1$)	0.169 (FET)
Result, % (*n*)												
Improvement at 7 days	82.8 (111/134)	80.8 (84/104)	81.4 (70/86)	77.8 (7/9)	77.8 (7/9)	90.0 (27/30)	87.5 (21/24)	100 (3/3)	100 (3/3)	0.238 ($\chi^2 = 1.395$, $df = 1$)	0.760 (FET)	0.798 (FET)
Improvement at 30 days	86.2 (106/123)	83.5 (76/91)	84.0 (63/75)	75.0 (6/8)	87.5 (7/8)	93.3 (28/30)	91.7 (22/24)	100 (3/3)	100 (3/3)	0.235 (FET)	0.507 (FET)	0.852 (FET)
Subsequent relapse												
Median time to relapse, months (IQI)	11.0 [7.0–19.0]	11.0 [6.5–19.0]	11.0 [5.0–19.5]	8.5 [4.75–14.0]	11.0 [8.25–17.5]	11.0 [6.5–15.0]	11.0 [5.0–13.5]	14.5 [11.0–18.0]	12.0 [8.0–16.0]	0.789 ($U = 696.5$)	0.519 ($U = 403,5$)	0.536 ($H = 1.248$, $df = 2$)

[1] LAmB + Meglumine + Voriconazol *n* = 1; LAmB + Paromomycin + Miltefosine *n* = 1; Meglumine + Miltefosine *n* = 1; Meglumine + Paromomycin *n* = 1
[2] Renal dysfunction *n* = 20; Fever/shivering *n* = 2
[3] Diarrhea *n* = 5
[4] Pancreatitis *n* = 5; cardiac toxicity *n* = 1
[5] Renal dysfunction *n* = 6; diarrhea/vomiting *n* = 3
[6] Renal dysfunction *n* = 2; Ototoxicity *n* =1
Abbreviations: IQI - Interquartile interval; LAmB - liposomal amphotericin B

3.3. Notification of cases and regional differences

Only 49.7% of incident VL cases in 2010–2020 were notified to the National Epidemiologic Surveillance System; cases in CU5 were significantly more notified (75.8%, $P \equiv 0.006$, $\chi^2 = 12.353$, $df = 3$). The percentage of cases notified was significantly different according to the region of the hospital: Norte 45.7%, Centro 69.7%, AML 44.0%, Alentejo 81.8%, and Algarve 70.8% ($P = 0.007$, $\chi^2 = 14.106$, $df = 4$); notification was not significantly different for patients admitted to Internal Medicine (45.6%) or Infectious Diseases departments (46.3%, $P = 0.942$, $\chi^2 = 0.005$, $df = 1$).

The main regional differences in presentation and management of VL are summarized in Table 7. The Alentejo was the region with a lower percentage of cases in PLWH/ISA and a higher percentage in CU5. In the Algarve and the Alentejo regions more patients were admitted to Internal Medicine vs. Infectious Diseases departments and time from presentation to diagnosis was longer in these regions. Use of serology for diagnosis was more common in the Alentejo, and less common in the Algarve.

Table 7. Presentation and management of incident visceral leishmaniasis cases diagnosed in public hospitals in Mainland Portugal in 2010–2020, by NUTS2 region of residence: Norte, Centro, Área Metropolitana de Lisboa, Alentejo and Algarve.

	Global	Norte	Centro	AML	Alentejo	Algarve	*P*-value
Number, *n*	194	37	36	79	18	24	
Children under 5 years old, % (*n*)	18.9 (33/175)	20.6 (7/34)	17.4 (4/23)	16.7 (13/78)	35.3 (6/17)	13 (3/23)	0.449 (FET)
Immunosuppressed patients, % (*n*)	61.1 (107/175)	50.0 (17/34)	65.2 (15/23)	69.2 (54/78)	35.3 (6/17)	65.2 (15/23)	0.060 (χ^2 = 9.027, *df* = 4)
PLWH, % (*n*)	46.9 (82/175)	29.4 (10/34)	47.8 (11/23)	61.5 (48/78)	17.6 (3/17)	43.5 (10/23)	0.002 (χ^2 = 16.846, *df* = 4)
Median time from onset to presentation, weeks (IQI)	4.0 [1.5–11.0]	3.0 [1.0–12.0]	8.0 [2.5–17.0]	4.0 [2.0–10.0]	2.0 [1.0–4.0]	2.0 [1.0–5.5]	0.086 (*H* = 8.152, *df* = 4)
Department of consultation/yard, % (*n*)							
Internal Medicine	43.5 (57/131)	34.6 (9/26)	35.3 (6/17)	33.3 (20/60)	62.5 (5/8)	85.0 (17/20)	< 0.001 (FET)
Infectious Diseases	51.1 (67/131)	46.2 (12/26)	58.8 (10/17)	66.7 (40/60)	25.0 (2/8)	15.0 (3/20)	
Hospital admission, % (*n*)	94.5 (155/164)	87.1 (27/31)	89.5 (17/19)	97.4 (74/76)	93.3 (14/15)	100 (23/23)	0.094 (FET)
Median duration, days (IQI)	20.0 [12.0–36.0]	16.5 [7.0–35.25]	29.0 [13.0–41.0]	20.0 [11.75–32.75]	16.0 [11.0–23.0]	29.0 [14.0–56.0]	0.290 (*H* = 4.973, *df* = 4)
Median time from presentation to diagnosis, days (IQI)	10.0 [4.5–19.5]	12.0 [2.5–26.5]	8.0 [2.25–20.75]	8.0 [4.0–15.0]	15.0 [7.0–50.0]	20.0 [8.0–60.0]	0.004 (*H* = 15.413, *df* = 4)
Samples used (direct diagnosis), % (*n*)							
Bone marrow	94.1 (160/170)	87.9 (29/33)	88.0 (22/25)	98.7 (74/75)	92.9 (13/14)	95.7 (22/23)	0.054 (FET)
Blood	17.8 (26/146)	27.6 (8/29)	5.9 (1/17)	13.6 (9/66)	41.7 (5/12)	13.6 (3/22)	0.066 (FET)
Technique used in bone marrow sample, % (*n*)							
Microscopy	95.6 (152/159)	92.6 (25/27)	91.7 (22/24)	95.9 (70/73)	100 (13/13)	100 (22/22)	0.609 (FET)
PCR	41.6 (62/149)	39.3 (11/28)	43.5 (10/23)	44.6 (29/65)	76.9 (10/13)	10.0 (2/20)	0.004 (χ^2 = 15.234, *df* = 4)
Culture	22.7 (32/141)	46.4 (13/28)	22.7 (5/22)	35.5 (22/62)	22.2 (2/9)	0 (0/20)	0.002 (FET)
Serology, % (*n*)	52.5 (83/158)	46.9 (15/32)	60.0 (15/25)	59.1 (39/66)	69.2 (9/13)	22.7 (5/22)	0.022 (χ^2 = 11.400, *df* = 4)
Samples sent to reference laboratory, % (*n*)	40.0 (50/125)	33.3 (10/30)	11.1 (1/9)	46.3 (25/54)	80.0 (8/10)	27.3 (6/22)	0.014 (FET)
Treatment of primary episode, % (*n*)							
Median tme from diagnosis to treatment, days (IQI)	0.0 [0.0–1.0]	0.0 [0.0–3.0]	1.0 [0.0–2.5]	0.0 [0.0–5.0]	0.0 [0.0–1.75]	0.0 [0.0–0.0]	0.001 (H = 18.384, *df* = 4)
Median duration of treatment, days (IQI)	21.0 [10.0–38.0]	21.0 [10.0–38.0]	21.0 [18.0–28.0]	21.0 [10.0–38.0]	21.0 [11.0–38.0]	21.0 [10.0–38.0]	0.954 (H = 0.681, *df* = 4)
Outcome of treatment, % (*n*)							
Improvement at 7 days	88.6 (132/149)	80.8 (21/26)	76.5 (13/17)	91.4 (64/70)	100 (13/13)	91.3 (21/23)	0.176 (FET)
Improvement at 30 days	96.4 (135/140)	96.2 (25/26)	92.9 (13/14)	98.5 (65/66)	90.9 (10/11)	95.7 (22/23)	0.306 (FET)
Follow-up in consultation, % (*n*)							
Yes	93.3 (140/150)	100 (26/26)	81.3 (13/16)	91.5 (65/71)	92.9 (13/14)	100 (23/23)	0.880 (FET)
Median time to first consultation, days (IQI)	15.5 [7.0–30.0]	12.0 [7.0–28]	21.5 [7.75–43.75]	12.0 [5.5–27.0]	13.0 [7.0–16.0]	30.0 [18.0–55.0]	0.004 (*H* = 15.318, *df* = 4)
Cure test performed	16.9 (23/136)	28.6 (8/28)	0 (0/13)	16.1 (10/62)	10.0 (1/10)	17.4 (4/23)	0.236 (FET)

Abbreviations: AML – Área Metropolitana de Lisboa; IQI – Interquartile interval; FET – Fisher's exact test; PCR – Polymerase chain reaction; PLWH – people living with HIV

3.4. Associations in VL

In univariate analysis, non-improvement at day 7 after initiation of anti-*Leishmania* therapy for primary treatment of VL was associated with male sex, immunosuppression, chronic organ dysfunction, renal failure at admission, severe leukopenia (< 1500/µl), coinfection/superinfection and CRP level over 100 (Table 8a). However, in multivariate analysis, CRP level over 100 was the only statistically significant factor.

Non-reporting of a VL case was associated in univariate analysis with age over 5 years old, immunosuppressed status, admission to a hospital located in the Norte or AML region and admission to a secondary center. In multivariate analysis, age over 5 years old, admission to a hospital located in the Norte or AML region and admission to a secondary center remained significant (Table 8b).

Table 8. Potential factors for non-improvement at 7 days after starting treatment and for non-reporting of primary cases newly diagnosed between 2010 and 2020 in public hospitals in Mainland Portugal, according to logistic regression models to estimate crude and adjusted odds ratio values.

a)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude *OR*	95% *CI*	Adjusted *OR*	95% *CI*	*P*-value
Non-improvement at 7 days	Male sex	68.5	3.88	[0.85–17.88]	2.66	[0.49–14.41]	0.257
	Immunosuppressed	62.4	11.36	[1.47–90.91]	5.71	[0.65–50.0]	0.115
	Chronic organ dysfunction	24.6	2.72	[0.93–7.94]	2.79	[0.65–12.05]	0.168
	Acute kidney injury	15.2	3.82	[1.24–11.76]	1.37	[0.32–5.92]	0.670
	Leucocyte count <1500/µl	37.7	3.86	[1.24–12.01]	2.47	[0.62–9.76]	0.199
	CRP >100 mg/L	43.6	2.69	[0.93–7.75]	5.18	[1.19–22.22]	**0.028**
	Coinfection/superinfection	41.5	3.17	[1.03–9.80]	1.62	[0.32–5.95]	0.468
	Constant				77.713		<0.001
	Hosmer and Lemeshow Test				0.758		

b)

	Potential Risk Factor	Univariate			Multivariate		
		% in Sample	Crude *OR*	95% *CI*	Adjusted *OR*	95% *CI*	*P*-value
Non-reporting	Age >5 years old	17.5	2.78	[1.22–6.32]	4.07	[1.19–13.93]	**0.026**
	Immunosuppressed	60.8	2.11	[1.14–3.92]	1.15	[0.46–2.87]	0.758
	Region of hospital AML or Norte	64.9	3.22	[1.71–6.09]	3.91	[1.74–8.75]	**<0.001**
	Secondary center	50.3	2.02	[1.13–3.60]	2.09	[1.03–4.27]	**0.042**
	Non-improvement at 7 days	11.4	1.66	[0.60–4.63]	1.56	[0.50–4.86]	0.446
	Constant				0.345		0.310
	Hosmer and Lemeshow Test				0.354		

Abbreviations: *OR* - odds ratio; *CI* - confidence interval; CRP - C-reactive protein; AML - Área Metropolitana de Lisboa

4. Discussion

The present study raises the attention to the ongoing burden of VL in Portugal, especially in children and in PLWH and other immunosuppressed patients. Until 2015–16, the calculated national incidence seems to follow the decreasing trend observed in a study respective to the 1999–2009 period [12], but in more recent years incidence seems to be increasing, driven mostly by increasing regional incidence in the Centro, Algarve, and Alentejo NUTS2 regions. An increasing absolute number of cases in the Algarve region had already been noted in previous reports [20]. Some NUTS3 regions with highest incidence in the 2010–2020 period largely overlap the districts where canine seroprevalence was estimated as highest in a recent study [21], namely Terras de Trás-os-Montes, Beiras e Serra da Estrela, Beira Baixa, and Alto Alentejo. Similarly, the Ave, Cávado and Alto Minho regions were expected to be lower incidence areas, according to canine seroprevalence [20] and previous human case report data [11]. Regions such as the AML and the Douro region, traditionally recognized as endemic foci of disease [22] remain so, despite showing an intermediate incidence in the present study. PLWH continue to represent the major group of VL cases, other immunosuppressed patients represent an increasing percentage compared to the 1999–2009 period (12.3 vs. 6.5%) [12]. Children 10 years old or younger, in contrast, represented only 16.3% of cases vs. 30.4% in the previous decade [12]. This shift in affected populations could represent a reduced risk of ongoing active primary transmission of *Leishmania* and an increased contribution of reactivating infection in a growing population of immunosuppressed adults. Increasing incidence in more recent years could be related to changing environmental conditions favoring prolonged vector survival and geographical expansion, as seen in other areas in Europe, including in Northern Spain [23] and as expected by modelling [24]. Additionally, the changing epidemiology of the HIV pandemic could help explain the evolution of VL incidence since 2000, taking into consideration progressive decreasing incidence of new diagnosis of HIV infection and of AIDS in Portugal [25]. The Algarve is the region with the second highest HIV infection incidence in recent years, after the AML [25]; approximately 85% of cases of VL diagnosed in PLWH occurred in the setting of CD4 cell counts $< 200/\mu l$ and, according to the most current data, 37.9% of PLWH are still diagnosed at this stage [25]. In summary, these findings suggest that, in the Mediterranean context, control of HIV infection, including early diagnosis and prevention of transmission is a cornerstone in controlling VL. Predominance of male sex has been described previously in Portugal and in other Mediterranean countries [10] and has been attributed to biological factors, besides sociocultural determinants [26].

Imported disease still represents a minority of cases in Portugal, opposed to other European endemic countries such as (metropolitan) France [27]; however, this could be expected to change in upcoming years, in relation with increasing migration from VL endemic countries such as Brazil, India and Nepal [28]. Since no systematic clinical screening program is implemented in migrant populations in Portugal, leishmaniasis cases could go unnoticed and translate into an underestimation of imported cases. On the other hand, even though migrants represent only 5.2% of the Portuguese national population [28], they represented 19.1% of VL cases; these cases were mostly autochthonous, in people born in non-endemic sub-Saharan African countries. This disproportionally high burden in migrant populations suggests their increased vulnerability to locally acquired infections, besides the risk for imported disease. Additionally, a higher percentage of homelessness and of unemployment was seen in VL cases compared to the national value (22.2 vs. 6.6%) [29], reinforcing leishmaniasis as a disease of neglect and of deprived settings.

The fact that immunosuppressed patients represent an increasing share of VL cases, including in the setting of use of methotrexate, and/or anti-TNFα drugs, raises the attention to the role that screening prior to starting these therapies could have in preventing symptomatic primary *Leishmania* infection or reactivation. Currently, there is no consensus on the indications for screening, nor on the techniques that should be used and how to define asymptomatic infection [30]. Management of asymptomatic infection, when detected, is currently based on clinical monitoring and no treatment strategies have been adequately studied [31]. These gaps should be addressed.

Regarding VL, even though the clinical findings have largely been already described in the Mediterranean context, including in Portugal [12], the present study contributes to reinforce dissimilarities in presentation in the different groups included. In children five years of age or younger, compared to older patients, the presentation was more abrupt, and time from onset of signs/symptoms to presentation to healthcare was shorter. Fever was more commonly reported, and the median highest temperature was higher. Splenomegaly was present in all cases, but weight loss, anorexia and fatigue were less frequent. Criteria for HLH were met in 40% of children – this percentage is somewhat higher than observed in studies performed in the Mediterranean [32] and Brazilian [33] contexts (possibly representing statistical variation associated with the small sample size in all of these studies) and highlights the need to rule out VL in all children presenting with HLH in endemic settings.

In PLWH, compared to non-immunosuppressed adults, fever was less common, and lower grade, and hepatomegaly was more common. Maximal CRP elevation was lower. Bacterial, fungal and/or viral coinfection was most common.

ISA were more frequently admitted to Internal Medicine wards, but also to other specialties according to their underlying conditions, reinforcing the growing need to recognize leishmaniasis in transplant, oncology, and hematology settings. Diagnostic delay was especially pronounced in ISA, in whom VL is less commonly considered in the differential diagnostic list. This group presented more severe disease, with longer hospital stays, more frequent admission to critical care, more frequent kidney failure, lower median platelet counts, more frequent skin/mucosal hemorrhage, and higher percentage of deaths. Of note 4/24 patients presented HLH, which has been rarely reported in adults with VL; however, VL still represents a considerable share of all adult HLH cases [34].

A high rate (8.5%) of atypical presentations was documented, especially with involvement of the gastrointestinal tract. Current knowledge of these forms of disease is limited to case reports and small series [35, 36], and suggests that they do not have a poorer prognosis or response to treatment than classic VL (in patients with similar immune status), but they could pose a diagnostic challenge in patients in whom other findings such as pancytopenia, splenomegaly or fever are absent.

Regarding diagnostic techniques, microscopy of bone marrow was preferred, even though European guidelines suggest a first approach using serology [17], probably reflecting unavailability in many centers and, on the other hand, the fact that bone marrow biopsy/aspirate allows investigation of alternative diagnoses. Use of PCR increased compared to the previous 10-year period (41.6 vs. 25.1%) [12]; in particular, use of PCR in blood has emerged as a less invasive alternative, with a reasonable positivity rate, both in the present and in previous studies [37]. In all cases of VL in which identification of complex was performed, *L. donovani* complex was identified; efforts for identification to the species level should be intensified, taking into account the increasing migrant population from South Asia [35] and the risk of introduction of anthroponotic and clinically distinct *L. donovani* sensu stricto (s.s.). Phlebotomine vectors for *L. infantum*, widely distributed across Portugal, are also permissive for *L. donovani* s.s [38]. This species has already been documented in Cyprus in humans and dogs [39] and hybrids between *L. infantum* and *L. donovani* have been demonstrated in Turkey [40].

Contrasting with the 1999–2009 period, when meglumine antimoniate was frequently used [12], in the present study LAmB was almost the only drug used to treat primary episodes of VL, in accordance with European guidelines [36]. There are no randomized clinical trials to support the use of combination therapy (LamB + miltefosine) in the Mediterranean setting, although this strategy has been studied in PLWH in South Asia, where *L. donovani* s.s. is endemic, revealing significantly higher relapse-free survival at day 210, compared to LAmB monotherapy [41]. In the present study, clinical response was comparable to described in the literature in Europe (cure rates > 90%) [42], but differed among groups: faster and greater in CU5, evidenced by shorter interval to defervesce and higher percentage of patients with clinical improvement by days 7 and 30 after initiating LAmB. ISA showed a slower response and lower improvement rates. In the present study, in multivariate analysis, CRP level over 100 mg/L was the only factor associated with non-improvement at day 7 after initiation of anti-*Leishmania* therapy for primary treatment of VL. High CRP has not been consistently suggested as a worse prognosis factor in previous studies; in a meta-analysis from East Africa [43] and a historical cohort from Brazil [44], prognostic factors for mortality among patients with VL included jaundice, edema, bleeding, splenomegaly, older age and *Leishmania*–HIV coinfection. However, findings in these populations may not be translatable to the Mediterranean context, considering baseline differences in sociodemographic aspects such as nutritional status and access to healthcare.

Secondary prophylaxis is common practice and endorsed by regional guidelines for PLWH [45]; for ISA, there is no consensus on indication, drug, frequency, and dosing and in the present study it was infrequently implemented; rate of relapse was similar between groups and in PLWH with or without prophylaxis. However, this possibly reflects the fact that in many cases prophylaxis could not be sustained until immunological recovery due to side effects, non-compliance, or dropout of patients. Even so, a longer time to relapse was documented in PLWH (compared to ISA) and especially in those on prophylaxis.

In most cases, treatment of relapses consisted of the use of the same or higher total dose of LAmB, but other regimens were used in selected cases. The results of the present study seem to suggest that improvement at 7 days could be higher with combination therapy, but a larger cohort would be needed. Randomized controlled trials could help understand if combination therapy is associated with better outcomes and whether there is any impact on subsequent relapse.

Although notification of VL cases increased compared to the previous period (49.7 vs. 38.6%) [12], approximately half of cases are still not reported, especially in the Norte and the AML regions, which could hamper public health efforts to control leishmaniasis in these regions. Incomplete and inconsistent reporting of VL increases the risk of bias in official data. Further studies should investigate causes for non-reporting, to better define strategies to tackle this gap in information.

Finally, this study presents some limitations, beginning with the fact that in some hospitals information was not collected due to lack of collaboration or due to absence of patient consent. In addition, coding of diagnosis for inpatients was not uniformly performed and digitalized in every hospital for the whole duration of the study period, and coding for outpatients was irregularly performed in hospitals, so cases were screened via laboratory results, whenever feasible. Some hospitals required internal personnel to access information, so in some cases interpretation of variables could be different, despite using the same database.

5. Conclusions

Although globally in Portugal the incidence of VL decreased compared to the previous 10 years, the disease remains an individual, public and One Health problem and a marker of neglect. Rising incidence in the more recent years could be related to climate change, increased mobility and/or increase in susceptible groups. These factors could also favor a future geographic expansion of endemic *L. infantum* and the introduction of new *Leishmania* species.

Leishmaniasis presents a continuing threat in Portugal to PLWH and children and an increasing threat to other immunosuppressed groups. Disease in the latter poses specific problems in relation to diagnosis and treatment as a consequence of different clinical presentation, worse outcome, and general lack of scientific knowledge. Multicentric research efforts could provide evidence to optimize treatment strategies for these patients in the European context, especially concerning the use of secondary prophylaxis and treatment of relapses. Programs to control leishmaniasis should focus not only on reducing underreporting, but also on raising awareness for the disease among healthcare practitioners and providing tools for earlier diagnosis.

Systematically combining clinical and national surveillance data could allow a more detailed assessment of the epidemiologic situation and an evaluation of the progress in clinical practice, uncovering gaps that need to be addressed in the near future. In order to improve the overall outcome for leishmaniasis patients, human data should also be integrated with data from vectors and mammal hosts, to produce holistic strategies to control the disease in several parts of the life cycle, following a One Health approach.

Ethics statement

This study received a favorable opinion of the Ethics Committees of all the involved institutions, namely: Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (reference 1.22); Centro Hospitalar do Baixo Vouga (16-01-2022/CES); Centro Hospitalar Barreiro Montijo; Centro Hospitalar de Entre o Douro e Vouga (29/2022); Centro Hospitalar de Leiria (34/2023); Centro Hospitalar de Lisboa Ocidental (2293); Centro Hospitalar do

Médio Tejo (080/2022); Centro Hospitalar do Oeste; Centro Hospitalar de Setúbal (018/2022F); Centro Hospitalar do Tâmega e Sousa (63/2021); Centro Hospitalar Tondela Viseu (08/19/11/2021); Centro Hospitalar de Trás-os-Montes e Alto Douro; Centro Hospitalar de Vila Nova de Gaia e Espinho (65/2022); Centro Hospitalar Universitário do Algarve (172/2021); Centro Hospitalar Universitário de Coimbra (OBS.SF.083-2022); Centro Hospitalar Universitário Cova da Beira (03/2022); Centro Hospitalar Universitário de Lisboa Central (1287/2022); Centro Hospitalar Universitário Lisboa Norte; Centro Hospitalar Universitário de Santo António (2022.060); Centro Hospitalar Universitário de São João (06/2022); Hospital Beatriz Ângelo (4018/2022); Hospital de Braga (29_2022); Hospital de Cascais Dr. José de Almeida (1/CE); Hospital Distrital de Santarém (31/2022); Hospital do Espírito Santo de Évora; Hospital Garcia de Orta; Hospital Professor Doutor Fernando Fonseca; Hospital da Senhora da Oliveira Guimarães (07/2022); Hospital de Vila Franca de Xira; Instituto Português de Oncologia de Coimbra Francisco Gentil; Instituto Português de Oncologia de Lisboa Francisco Gentil (73/2022); Unidade Local de Saúde do Alto Minho (30/2022); Unidade Local de Saúde do Baixo Alentejo (10/2021); Unidade Local de Saúde de Castelo Branco; Unidade Local de Saúde da Guarda (112/2022); Unidade Local de Saúde do Nordeste (59/2022); Unidade Local de Saúde do Norte Alentejano.

Additionally, the study was authorized by the Administration Council of all the involved Hospitals. In the three hospitals where informed consent was required, collection of data was only performed for patients who consented and after signing the form.

6. References

1. Manson's. Tropical diseases. Elsevier; 2024.

2. ECDC. Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. Stockholm; 2022.

3. Gaspari V, Gritti T, Ortalli M, Santi A, Galletti G, Rossi A, et al. Tegumentary leishmaniasis in Northeastern Italy from 2017 to 2020: a neglected Public Health Issue. Int J Environ Res Public Health. 2022;19(23):16047.

4. European Centre for Disease Prevention and Control and European Food Safety Authority. Phlebotomine sandflies maps. Stockholm: ECDC; 2023. Available from:

https://ecdc.europa.eu/en/disease-vecto rs/surveillance-and-disease-data/phlebotomine-maps. Accessed 3 Mar 2024

5. Maia C, Dantas-Torres F, Campino L. Parasite Biology: the Reservoir hosts. The leishmaniases: Old Neglected Tropical diseases. Cham: Springer International Publishing; 2018. pp. 79–106.

6. Asfaram S, Fakhar M, Teshnizi SH. Is the cat an important reservoir host for visceral leishmaniasis? A systematic review with meta-analysis. J Venom Anim Toxins incl Trop Dis. 2019;25:e20190012.

7. Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012;190(1–2):268–71.

8. Herrador Z, Gherasim A, Jimenez BC, Granados M, San Martín JV, Aparicio P. Epidemiological Changes in Leishmaniasis in Spain According to Hospitalization-Based Records, 1997–2011: Raising Awareness towards Leishmaniasis in Non-HIV Patients. Carvalho EM, editor. PLoS Negl Trop Dis. 2015;9(3):e0003594.

9. Akufo H, Costa C, van Griensven J, Burza S, Moreno J, Herrero M. New insights into leishmaniasis in the immunosuppressed. Rafati S, editor. PLoS Negl Trop Dis. 2018;12(5):e0006375.

10. Maia C, Conceição C, Pereira A, Rocha R, Ortuño M, Muñoz C, et al. The estimated distribution of autochthonous leishmaniasis by Leishmania infantum in Europe in 2005–2020. PLoS Negl Trop Dis. 2023;17(7):e0011497.

11. SPMS. Portal da Transparência. Doenças de Declaração Obrigatória. 2018 https://transparencia.sns.gov.pt/explore/dataset/doencas-de-declaracaoobrigatoria/table/?disjunctive.regiao_de_saude_de_notificacao&disjunctive.residencia_nut_iii&disjunctive.doenca_de_declaracao_obrigatoria&disjunctive.sexo&disjunctive.grupo_etario&sort.

12. Serrada E. A leishmaniose visceral em Portugal Continental: 1999–2009. Universidade Nova de Lisboa; 2010. (in Portuguese).

13. Instituto Nacional de Estatística. Censos 2021 Resultados Defnitivos - Portugal. Lisboa; 2022. (in Portuguese).

14. Instituto Nacional de Estatística. O que nos dizem os Censos sobre a população de nacionalidade estrangeira residente em Portugal. 2023. (in Portuguese).

15. Fundação Francisco Manuel dos Santos. PORDATA Base de Dados de Portugal Contemporâneo. SNS: hospitais por tipo - Continente [Internet]. 2022. https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=66196836&PUBLICACOESmodo=2. Accessed 3 Mar 2024. (in Portuguese).

16. Portugal Mar. Direção Geral da Saúde. SINAVE (Sistema Nacional de Vigilância Epidemiológica). 2024 https://www.dgs.pt/servicos-on-line1/ sinave-sistema-nacional-de-vigilancia-epidemiologica.aspx. Accessed 3 Mar 2024. (in Portuguese)

17. Gradoni L, López-Vélez RMM. Manual on case management and surveillance of the leishmaniasis in the WHO European Region. World Health Organization; 2017.

18. Daly LE. Confdence limits made Easy: interval estimation using a substitution method. Am J Epidemiol. 1998;147(8):783–90.

19. Fagerland MW, Hosmer DW. A generalized Hosmer–lemeshow goodness-offt test for Multinomial Logistic Regression models. Stata J. 2012;12(3):447–53.

20. Guerreiro AR, Mingo A, Proenca A, Pereira R, Pedro L, Faria D. Visceral leishmaniasis in southern Portugal: relapse of an old disease. In Madrid: 28th ECCMID. 2018.

21. Almeida M, Maia C, Cristóvão JM, Morgado C, Barbosa I, Ibars RF, et al. Seroprevalence and risk factors Associated with Leishmania Infection in dogs from Portugal. Microorganisms. 2022;10(11):2262.

22. Campino L, Maia C. Epidemiology of leishmaniases in Portugal | Epidemiologia Das leishmanioses em Portugal. Acta Med Port. 2010. (in Portuguese).

23. Gálvez R, Montoya A, Cruz I, Fernández C, Martín O, Checa R, et al. Latest trends in Leishmania infantum infection in dogs in Spain, Part I: mapped seroprevalence and sand fy distributions. Parasit Vectors. 2020;13(1):204.

24. Koch LK, Kochmann J, Klimpel S, Cunze S. Modeling the climatic suitability of leishmaniasis vector species in Europe. Sci Rep. 2017;7(1):13325.

25. Portugal. Direção Geral Da Saúde, Instituto Nacional De Saúde Doutor Ricardo Jorge. Infeção VIH E SIDA em Portugal – 2022. Lisboa; 2022. (in Portuguese).

26. Cloots K, Burza S, Malaviya P, Hasker E, Kansal S, Mollett G, et al. Male predominance in reported Visceral Leishmaniasis cases: Nature or nurture? A comparison of population-based with health facility-reported data. Werneck GL, editor. PLoS Negl Trop Dis. 2020;14(1):e0007995.

27. Pasquier G, Demar M, Lami P, Zribi A, Marty P, Bufet P, et al. Leishmaniasis epidemiology in endemic areas of metropolitan France and its overseas territories from 1998 to 2020. Barbosa DS, editor. PLoS Negl Trop Dis. 2022;16(10):e0010745.

28. Fundação Francisco Manuel dos Santos. PORDATA Base de Dados de Portugal Contemporâneo. População residente estrangeira segundo os Censos: total e por país de nacionalidade [Internet]. 2023. https://www.
pordata.pt/portugal/populacao+residente+estrangeira+segundo+os+
censos+total+e+por+pais+de+nacionalidade-3786. Accessed 3 Mar 2024. (in Portuguese).

29. Fundação Francisco Manuel dos Santos. PORDATA Base de Dados de Portugal Contemporâneo. População desempregada segundo os Censos: total e por grupo etário [Internet]. 2022. https://www.pordata.pt/portu
gal/populacao+desempregada+segundo+os+censos+total+e+por+ grupo+etario-3721. Accessed 3 Mar 2024. (in Portuguese).

30. Ibarra-Meneses AV, Corbeil A, Wagner V, Onwuchekwa C, FernandezPrada C. Identifcation of asymptomatic Leishmania infections: a scoping review. Parasit Vectors. 2022;15(1):5.

31. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic Leishmania infection: a New Challenge for Leishmania Control. Clin Infect Dis. 2014;58(10):1424–9.

32. Blázquez-Gamero D, Domínguez-Pinilla N, Chicharro C, Negreira S, Galán P, Pérez-Gorricho B, et al. Hemophagocytic Lymphohistiocytosis in children with visceral leishmaniasis. Pediatr Infect Dis J. 2015;34(6):667–9.

33. Daher EF, Lima LLL, Vieira APF, Nascimento LS, Soares DS, Abreu KLS, et al. Hemophagocytic syndrome in children with visceral leishmaniasis. Pediatr Infect Dis J. 2015;34(12):1311–4.

34. Badiola J, Muñoz-Medina L, Callejas JL, Delgado-García A, Jurado M, Hernández-Quero J. Hemophagocytic lymphohistiocytosis associated with Leishmania: a hidden passenger in endemic areas. Enferm Infecc Microbiol Clin. 2021;39(4):188–91.

35. Soria López E, Olalla Sierra J, del Arco Jiménez A, Pereda Salguero T, de la Abitei C. Torre Lima J. Colonic leishmaniasis in a patient with HIV: a case report. Rev Esp Enferm Dig. 2016;108.

36. Gimeno-Pitarch L, Almela P, Nos P. Infección Por Leishmania en pacientes con enfermedad infamatoria intestinal: Serie De casos y revisión de la literatura. Gastroenterol Hepatol. 2023 Apr. (in Spanish).

37. da Costa Lima MS, Zorzenon DCR, Dorval MEC, Pontes ERJC, Oshiro ET, Cunha R, et al. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pac J Trop Dis. 2013;3(1):10–5.

38. Myskova J, Svobodova M, Beverley SM, Volf P. A lipophosphoglycan-inde - pendent development of Leishmania in permissive sand fies. Microbes Infect. 2007;9(3):317–24.

39. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, Soteri - adou K. Leishmania Donovani leishmaniasis in Cyprus. Lancet Infect Dis. 2008;8(1):6–7.

40. Rogers MB, Downing T, Smith BA, Imamura H, Sanders M, Svobodova M, et al. Genomic Confrmation of Hybridisation and Recent Inbreeding in a Vector-Isolated Leishmania Population. Didelot X, editor. PLoS Genet. 2014;10(1):e1004092.

41. Burza S, Mahajan R, Kazmi S, Alexander N, Kumar D, Kumar V et al. AmBi - some Monotherapy and Combination AmBisome-Miltefosine Therapy for the treatment of visceral leishmaniasis in patients coinfected with Human Immunodefciency Virus in India: a randomized Open-Label, Parallel-Arm, phase 3 trial. Clin Infect Dis. 2022.

42. Rosenthal E, Delaunay P, Jeandel PY, Haas H, Pomares-Estran C, Marty P. Le Traitement De La Leishmaniose viscérale en Europe en 2009. Place De l'amphotéricine B liposomale. Med Mal Infect. 2009;39(10):741–4. (in French).

43. Abongomera C, van Henten S, Vogt F, Buyze J, Verdonck K, van Griensven J. Prognostic factors for mortality among patients with visceral leishma - niasis in East Africa: systematic review and meta-analysis. Werneck GL, editor. PLoS Negl Trop Dis. 2020;14(5):e0008319.

44. Coura-Vital W, de Araújo VEM, Reis IA, Amancio FF, Reis AB, Carneiro M. Prognostic factors and Scoring System for death from visceral leishmania - sis: an historical cohort study in Brazil. Santiago H Da C, editor. PLoS Negl Trop Dis. 2014;8(12):e3374.

45. EACS. EACS Guidelines version 11.1.

7. Supplementary data

Available at: https://idpjournal.biomedcentral.com/articles/10.1186/s40249-024-01204-5

Additional file 1: Supplementary figure 1. Maps showing the location of Mainland Portugal in Western Europe and the territorial division in NUTS (Nomenclature of Territorial Units for Statistics) 2 and NUTS3 regions.

Additional file 2: Supplementary table 1. List of NUTS (Nomenclature of Territorial Units for Statistics) 2 and NUTS3 regions in Mainland Portugal and sociodemographic characteristics.

Additional file 3: Supplementary table 2. Definitions, classifications or categories used for data collection and presentation in this study.

Additional file 4: Supplementary figure 2. Location (a) and microbiological agents (b) of coinfection/superinfection in primary visceral leishmaniasis episodes diagnosed between 2010 and 2020 (n=194).

CHAPTER 7

General discussion and conclusions

1. General discussion and conclusions

This doctoral project was developed around three focal questions: "What is the burden of *Leishmania* infection and disease in Portugal? How is that burden distributed across geographical areas and social sectors? What is the perception of the general population and of health professionals and students regarding that burden?". These three questions were developed over three distinct studies, which provided data regarding different, but complementary, faces of *Leishmania* infection. The resulting combined information allows for a more detailed and updated perspective of the infection in mainland Portugal and could help design interventions to control the disease in the country.

1.1. Global epidemiology of *Leishmania* infection in mainland Portugal

Combining the results from human seroprevalence (in blood donors) and incidence produced in this work and the most recent data from canine seroprevalence, some considerations can be drawn regarding the current epidemiology of *Leishmania* infection. The national incidence of leishmaniasis in humans declined compared to the 1999-2009 period (1). The estimated VL mean annual incidence of 0.184/100,000 population is comparable to other South European countries, such as Spain or Italy (2); however, an increase in incidence was detected in the last four years analyzed in this work (after 2016). Serial seroprevalence studies in dogs (with sampling performed 12 years apart, in 2009 and 2021 (3,4)), also showed increasing global canine seroprevalence. These studies were performed on dogs in selected veterinary clinics.

This trend could be explained by several factors, including changes in suitability for vectors in mainland Portugal, possibly related to climate change. Increasing temperatures could allow for: an increased geographical dispersion of vectors; an increased survival time and activity; and a decreased time to develop in the intermediate host (extrinsic incubation time). Modelling studies in the European region support this conclusion, suggesting an increase in the number of climatically suitable regions for leishmaniasis, especially in southern and eastern countries, coupled with a northward expansion towards central Europe (5,6). Indeed, outbreaks of leishmaniasis and detection of seropositive dogs have been reported in previously non-endemic regions of Spain (Galicia, Asturias (7–9)). And, in the present work, autochthonous symptomatic and asymptomatic human infections were detected in regions of northern coastal Portugal, which were considered as non-endemic. In dogs, in the two serial studies mentioned above, pronounced increases in seroprevalence were noted in areas of previous low endemicity (for example, in the Porto district the seroprevalence increased to similar levels as in the Lisboa district) (3,4).

Additionally, the increasing trend in *Leishmania* prevalence/incidence could be related to a low adherence to general and specific individual preventive measures for reservoir hosts: although a high percentage of blood donors reported the use of insecticide or arthropod-repellent products in pet dogs in the present work, other studies have shown that the type of product used and the frequency of application were often inappropriate (10,11). Furthermore, many donors reported not using repellents throughout all the phlebotomine season (in Portugal, mostly from May to October) (12).

An increase in human incidence could also be explained by the increase in population at risk for symptomatic disease due to host-related factors, including secondary immunosuppression related to pharmacological treatment for solid organ or hematopoietic stem cell transplant, solid organ or hematological malignancy, autoimmune and inflammatory conditions. In addition, in a steadily aging country, reactivation of latent infection in the setting of immunosenescence could also represent an increasing percentage of cases. This is illustrated in the present study, where the percentage of VL cases who were non-HIV immunosuppressed increased (from 6.5% in 2000-2009 (1) to 13.1% in the present study) and the median age of patients also increased (from 31 to 41 years old). Moreover, in Portugal, HIV infection, an important contributor to symptomatic *Leishmania* infection, is still often diagnosed at late stages (in 2022, 37.9% of newly diagnosed cases had CD4 cell counts below 200/µL at time of diagnosis (13)). As such, prevention of HIV transmission and early diagnosis are still cornerstones for controlling the public health impact of *L. infantum* infection.

Increased internal and international human and animal mobility could also play a role in the increasing *Leishmania* incidence/prevalence. Although only 4.0% of human cases in

Portugal were imported in the present study, travel to and migration from endemic areas represent a much higher percentage of cases in other (endemic) European countries, such as France (14). Migrants represent an increasing fraction of the Portuguese population (5.2% in the last census (15)). Besides the risk of imported infection in migrants from other endemic areas, migrants from non-endemic areas could be particularly vulnerable to *Leishmania* infection in Portugal, possibly due to increased exposure compared to the non-migrant population and to barriers in access to healthcare.

1.2. Regional and local epidemiology of *Leishmania* infection in mainland Portugal

At a regional and local level, some conclusions concerning *Leishmania* epidemiology could be pointed out. Table 1 summarizes seroprevalence and incidence data generated from the present and previous works. To allow this comparison, results are shown at a district level (as opposed to the NUTS3 regions used in the previous chapters). It should be noted that the estimates of human seroprevalence in the present study were obtained from blood donors. Although they could represent the population of blood donors in mainland Portugal, these estimates may not be transposable to the general population, considering differences in age and health status, among other factors. Blood donors were selected as the target population due to the expected easier sampling of the required number of healthy adults, with a representative geographical dispersion, in this population.

Table 1. Estimated seroprevalence of *Leishmania* (%) in human (blood donors) and canine populations and mean annual incidence of visceral leishmaniasis (number of cases/100,000 population/year) in different periods in Mainland Portugal, by region and district, according to data generated from the present and previous works.

		Seroprevalence			Incidence (VL)	
Region	District	Human 2022	Canine 2009	Canine 2021	1999-2009	2010-2020
Norte		6.3	3.8	9.6	0.194	0.093
	Braga	4.4	2.1	6.9		0.021
	Bragança	3.1	8.0	15.7		0.421
	Porto	7.2	3.2	9.2		0.101
	Viana do Castelo	8.5	0.9	0		0.038
	Vila Real	3.6	8.4	13.4		0.185
Centro		6.5	7.8	12.5	0.321	0.135
	Aveiro	7.9	1.4	1.2		0.064
	Castelo Branco	5.4	17.4	29.9		0.340
	Coimbra	4.6	6.4	16.4		0.173
	Guarda	10.0	6.8	19.3		0.179
	Leiria	5.8	3.1	3.9		0.059
	Viseu	5.6	7.1	13.8		0.200
LVT		4.9	6.0	11.2	0.530	0.240
	Lisboa	5.2	5.9	9.2		0.289
	Santarém	5.0	7.9	11.4		0.166
	Setúbal	4.5	5.0	15.5		0.147
Alentejo		1.9	10.4	16.1	0.337	0.303
	Beja	0.6	12.1	14.3		0.428
	Évora	2.7	2.5	10.6		0.057
	Portalegre	1.4	12.5	30.5		0.488
Algarve		4.2	4.7	17.2	0.108	0.495
	Faro	4.2	4.7	17.2		0.495
Reference		This work	(3)	(4)	(1)	This work

Abbreviations: LVT, Lisboa e Vale do Tejo; VL, visceral leishmaniasis.

Note: original data from this work was reanalyzed to be displayed in this table by district, to allow comparison with previous studies which presented their data using these territorial divisions. In other chapters of this work, NUTS (Nomenclature of Territorial Units for Statistics) are used. The correspondence between districts and NUTS3 regions in mainland Portugal is approximately as follows: Braga – Cávado and Ave; Bragança – Terras de Trás-os-Montes; Porto – Área Metropolitana do Porto and Tâmega e Sousa; Viana do Castelo – Alto Minho; Vila Real – Alto Tâmega and Douro; Aveiro – Região de Aveiro; Coimbra – Região de Coimbra; Guarda – Beiras e Serra da Estrela; Castelo Branco – Beira Baixa; Leiria – Região de Leiria and Oeste; Viseu – Viseu Dão-Lafões and Douro; Lisboa – Área Metropolitana de Lisboa (North of Tagus river); Santarém – Lezíria do Tejo and Médio Tejo; Setúbal – Área Metropolitana de Lisboa (South of Tagus river) and Alentejo Litoral; Beja – Baixo Alentejo; Évora – Alentejo Central; Portalegre – Alto Alentejo; Faro – Algarve.

In the Norte region, human incidence was highest in the Terras de Trás-os-Montes, Alto Tâmega and Douro subregions, which nearly correspond to the two districts with highest canine seroprevalence (Bragança and Vila Real) and to the long recognized Alto Douro endemic region. Unexpectedly high human seroprevalence in Viana do Castelo/Alto Minho could represent emerging foci of parasite circulation, as discussed above, while the high human seroprevalence in the Porto/Área Metropolitana do Porto region is consistent with increasing seroprevalence in dogs in this region.

In the Centro region, Beira Baixa and Beiras e Serra da Estrela (BSE) (approximately corresponding to Guarda and Castelo Branco districts) presented the highest human incidence; additionally, BSE seems to represent a significant focus of CL, as suggested presently and in a previous work (16); although Aveiro/Região de Aveiro was suggested as an area of low parasite circulation by canine seroprevalence studies, both human incidence and seroprevalence studies contradict this idea, with recent cases described in the region, including CL. Possible explanations for these findings include an increasing awareness of medical professionals in the region and/or the circulation of a distinct, more dermotropic, *L. infantum* genotype.

In the Área Metropolitana de Lisboa (AML) region, a marked decline in human incidence was noted, compared to the 1999-2009 period (1). The municipalities of Odivelas, Lisboa, Sintra and Cascais registered the highest incidences within the region.

In the Alentejo region, human incidence was stable and was the highest in Alto Alentejo and Baixo Alentejo. This finding seems inconsistent with the estimated low human seroprevalence in these subregions. Small sample sizes and sampling difficulties (with only half of the municipalities in Alto Alentejo sampled) may have affected representativeness (especially considering the localized nature of *Leishmania* foci), potentially leading to these discrepancies that might not have a real significance.

Finally, in the Algarve region, estimated human incidence has sharply increased after 2013 – this is consistent with the increased canine seroprevalence (4) and with the detection of sand fly vectors in several municipalities of the region, some of which were infected with *Leishmania* (in the Faro municipality), in a more recent study (17).

1.3. Host-related factors associated with leishmaniasis

The series of leishmaniasis cases described in the present work represents one of the largest current series of VL in the Mediterranean, where clinical differences between groups were analyzed. Similarly, this is the first work to describe a temporal series of CL and ML cases in Portugal, which is relevant considering their noncompulsory notification status in the country. Regarding host-related factors associated with leishmaniasis, some findings should be highlighted.

A predominance of male sex in clinical cases of VL and CL is consistent with male sex as a potential risk factor for positive serology, as demonstrated in other endemic contexts (18). This sex bias could be explained in part by gender-related behavior (e.g., practice of outdoors activities), but increasing evidence suggests a significant role of biological factors (such as sex-dependent differences in macrophage activity, granuloma formation and cytokine responses) (19).

Children under five years of age remained one of the most affected groups by VL in the present work, although less prominently than in the 2000-2009 period (1). Cases in children are epidemiologically relevant, as they are more likely to represent primary infections, indicating foci of active transmission, which should be the primary targets of public health interventions.

HIV remains an important factor for symptomatic disease, especially when associated with CD4 cell counts lower than 200/µL; other forms of immunosuppression contribute to an increasing percentage of cases, especially secondary to drugs, such as methotrexate and anti-TNFα; this observation poses questions regarding the introduction of screening practices in this population.

1.4. Clinical presentation and management of leishmaniasis

Concerning clinical aspects of VL, signs and symptoms described in the present work are globally similar to those reported in previous series (1). However, the present work clearly shows differences in presentation between groups, revealing a more abrupt onset in children under five years of age and faster response to treatment; in non-HIV immunosuppressed patients, higher rates of non-improvement, longer hospital stays, and higher fatality were noted, possibly reflecting lower health professional awareness and delayed diagnosis in this group.

In Portugal, diagnosis of leishmaniasis mostly relies on observation of amastigotes. This technique does not allow for species differentiation, which could be relevant for epidemiological surveillance purposes, as detailed below. Additionally, confusion with intracellular forms of *Histoplasma capsulatum* could occur, an important differential diagnosis in people migrating from or travelling to endemic areas in South America. Use of nucleic acid amplification tests, such as PCR can avoid this uncertainty and has increased in the country but is still not widely available in many of the diagnostic laboratories. In addition, PCR in blood allows less invasive, yet sensitive diagnosis, especially in immunosuppressed patients (20); it can also be used (and is currently being used) to monitor response to treatment in PLWH and detect recurrence (21). Despite its indication as a first line tool for diagnosis of VL (22), serology is less commonly used in Portugal, compared to microscopy, perhaps reflecting access issues.

Treatment of primary VL episodes in Portugal follows current recommendations in Europe (22), where LAmB is largely available. In the present study, use of LAmB for primary treatment showed a good efficacy overall; relapsing disease seemed to occur at similar rates in PLWH and HIV non-infected patients, although earlier in the latter. Randomized clinical trials have shown superior efficacy (parasite clearance or relapse-free survival) of combination therapy (LAmB+miltefosine) for primary treatment in PLWH in South Asia and East Africa (23,24). However, studies to assess the use of this strategy are lacking in the European context. Additionally, in the heterogeneous group of HIV non-infected patients, studies are still needed to evaluate which particular patients benefit from secondary prophylaxis, which strategies (drug, dose and frequency) are the most effective and when can prophylaxis be safely interrupted.

Concerning CL, since most cases were considered autochthonous, the present work gives essentially a picture of CL by *L. infantum*. As shown in other Mediterranean countries (25), lesions caused by this species are predominantly located in the face and are less often ulcerated. Diagnosis in Portugal mostly relied on skin biopsy, which allows studying alternative diagnoses. Treatment was very heterogeneous, probably reflecting lack of access to drugs/therapeutic strategies within health services and lack of experience of most professionals. In this series, a significant proportion of CL cases had immunosuppressing factors and, in this group, simultaneous VL was common, justifying an active search for this clinical form.

Although the present work has shed light on autochthonous CL, it likely represents an incomplete picture, considering the estimate of a much lower incidence of CL than VL, contrasting with Spain (26,27) and other European countries (28), where the two clinical forms show similar incidences. A possible explanation includes underdiagnosis due to cases not coming to medical attention (self-healing), lack of awareness of the disease by clinicians and/or assumption of the disease as an importation disease, as shown in the questionnaires.

ML caused by *L. infantum* is extremely rare, but can be disfiguring and debilitating, hence it should be suspected in endemic areas, especially in elderly people and immunosuppressed patients. Besides involvement of the nasal and oral mucous membranes, laryngeal involvement is increasingly recognized (29), underscoring the importance of involving and training a broad range of medical specialties in leishmaniasis, including ear, nose and throat physicians.

1.5. Knowledge, perceptions and practices of blood donors and health students and professionals

The present work also sheds light on the knowledge regarding leishmaniasis and the practices related to domestic animals in different groups, from professionals, to students and blood donors, allowing a comparison between them.

Awareness of leishmaniasis was high both in blood donors and in health students/professionals, although higher among the latter: over 95% of students and professionals had previously heard of leishmaniasis, compared to only around 70% of blood donors. Television advertisements were common non-academic sources of information for all groups. The role of veterinarians as providers of information regarding leishmaniasis was also highlighted by every group; in this sense, academic training and continuous education of veterinarians in this disease could be a decisive strategy for control of leishmaniasis in Portugal, as well as in other zoonotic settings.

Unawareness regarding mode of transmission of leishmaniasis was more common in blood donors; arthropod bite was the mode of transmission most often pointed by participants, especially health students/professionals; sand flies were identified as the vectors much more commonly by students/professionals than by blood donors, who preferentially selected mosquito bites. Insufficient recognition of phlebotomine sand flies as vectors and confusion between these two arthropods could lead to improper individual management of potential *Leishmania* vector breeding sites, such as animal burrows and shelters and leaf litter, which differ from mosquito breeding sites (small or large bodies of water). Additionally, belief of transmission via direct contact (excluding bites or scratches), reported in over 10% of participants in every group, could lead to inadequate isolation measures or rejection of diseased animals. Over 90% of participants in each group admitted leishmaniasis affected animals; however, only in the professional group over 90% of participants were aware of human leishmaniasis. Decreased recognition by blood donors of potential human infection with *Leishmania*, including in endemic areas in Portugal, could implicate a lower perception of individual or community risk, even in areas where canine cases are seen, and a low stimulus to implement animal protective measures.

Although systematic use of repellents in dogs was consistently reported by students/professionals and blood donors, the latter were less likely to apply them spanning all the phlebotomine season. Other practices that could be different between groups and impact prevention, such as type of repellent substance used, frequency and mode of application, were not assessed in the present study.

1.6. Integrated surveillance and control

Leishmaniases caused by *L. infantum* in the Mediterranean region pose a threat to domestic animals, mainly dogs, although the impact in other species, including cats, is increasingly recognized (30). As a zoonotic infection, the risk to humans persists while enzootic cycles exist. As such, integrated surveillance and control are necessary, following a One Health perspective, to achieve maximal health outcomes to humans and domestic and wild animals. A national structured plan for surveillance and control of leishmaniasis could overcome some of the current challenges, namely by combining systematic surveillance of vectors and integrated reporting of animal and human cases of disease and by investing in health education concerning the prevention of vector-borne infections.

Regarding vectors of medical and veterinary importance, systematic surveillance of ticks and mosquitoes has been implemented in Portugal for over ten years, through a national network (REVIVE - Rede de Vigilância de Vetores), coordinated by the National Health Institute (INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge) and devoted to specimen collection, species identification and detection of pathogens. However, systematic collection of sand flies has only been integrated in this network since 2023, with many gaps in geographical coverage (31).

Surveillance of circulating parasite species and genotypes is important for clinical purposes and for tailoring intervention strategies. However, this analysis is not possible when diagnosis of human or canine cases is based exclusively on microscopic observation of amastigotes, which remains a common practice in clinical laboratories in human leishmaniasis in Portugal. However, increased human and animal mobility creates new challenges in settings where molecular identification and speciation is still scarcely available, since it poses a risk of unrecognized introduction of new species. Detection of non-endemic *Leishmania* species has already been documented in Portugal (*L. major* hybrids (32)) and other areas of Europe, such as Cyprus (*L. donovani* (33)). In Portugal, introduction of *L. donovani* could be facilitated considering its mostly anthroponotic nature, the presence of competent vectors in many parts of the territory, and the considerable migrant population from areas of endemicity in the Indian subcontinent and East Africa (15).

Concerning reporting of cases of disease, notification of CL and canine leishmaniasis (CanL) cases is not mandatory in Portugal, unlike in other European countries (34), precluding quick and targeted control interventions. VL reporting is mandatory, using a national electronic platform, the SINAVE (Sistema Nacional de Vigilância Epidemiológica). However, underreporting of VL in the country is still significant, although it is decreasing (49.7% in the present study compared to 38.6% in 2000-2009 (1)). For diseases in which both human and animal cases are subject to compulsory reporting, distinct platforms for reporting are provided, and the resulting data are not presented combined in official national databases or reports. At a European level, however, One Health reports provide an overview of some zoonotic diseases across the human, animal and environmental domains (35); leishmaniasis is not, however, one of such diseases.

In terms of prevention in the animal reservoir host, regular use of topical repellent insecticides in dogs is highly effective in preventing phlebotomine sand fly bites and, therefore, has a central role in controlling *L. infantum* transmission, from a One Health perspective (36). However, this measure in not routinely promoted by Public Health authorities, nor are the products freely available to dog owners in Portugal. Vaccines may play a secondary role in *L. infantum* control, since they do not prevent infection, but they reduce the risk for development of clinical signs and disease progression in infected dogs (37). Systematic (yearly) use of vaccines against CanL is uncommon in Portugal, as demonstrated in the present and in previous studies (4); this could be possibly attributed to the price of the vaccines and lack of awareness. Lastly, immune therapy (e.g., domperidone) may lead to protection against *L. infantum* infection or improvement of the clinical condition of infected individuals, possibly reducing the risk of treated dogs transmitting *L. infantum* to vectors (38). In this sense, it may be an additional tool for *L. infantum* control.

Regarding the prevention of disease in humans, important scientific gaps remain in the identification and management of asymptomatic infection. No standardized and consensual criteria have been proposed regarding who should be tested and which technique(s) should be used. There are no clear indications concerning the frequency of clinical and/or analytical monitoring for people who test positive. There is no solid scientific evidence for adopting a preemptive treatment (for positive screening individuals) or a primary prophylaxis strategy (for negative screening individuals), as no drugs have been evaluated in randomized clinical trials for these purposes. Symptomatic cases could be avoided if cost-effective screening strategies were adopted and if markers of progression could be clearly identified. There is ongoing research in that area, with promising results – transcriptomic analyses comparing signatures showed the innate immune system pathway was strongly activated only in asymptomatic profiles, whereas pathways such as MHC Class II antigen presentation and NF-kB activation in B cells seemed to be specifically induced in symptomatic profiles (39,40). Besides the possible individual gain in detection of asymptomatic infection at risk for progression, there could be a Public Health gain in identifying asymptomatic people at risk for transmission of *Leishmania* to the vectors, perpetuating the parasite's life cycle, as has been demonstrated for some immunosuppressed individuals (41).

1.7. Future research

Although fundamental issues concerning the current epidemiology and clinical presentation of leishmaniases in Portugal were settled in this thesis, there are still some outstanding questions that will need to be addressed in the future:

- How is *Leishmania* infection distributed at a local (subregional) level? What are the specific foci and the ecological niches of the vectors?
- Which biogeographic and socioeconomic factors could help predict the current distribution of the disease and how could these be used to model future distribution in Portugal?
- What is the expected impact of climate change on the geographical distribution and activity of *Leishmania* vectors in Portugal?
- What is the estimated risk of introduction of anthroponotic *Leishmania* species in Portugal for which competent vectors are widely distributed?
- Which factors could explain the disproportionately high burden of leishmaniasis observed in migrants?
- Which factors could explain the geographical differences in CL and VL in Portugal?
- In *L. infantum* cases of CL, how cost effective could screening of simultaneous VL be? Should it be directed to specific groups such as immunosuppressed people?
- How can different prophylactic regimens lower the risk of VL relapse in HIV and non-HIV immunosuppressed patients? In which non-HIV cases could prophylaxis be beneficial? When should it be started and stopped?
- What is the role for new combination treatment strategies for VL in PLWH in the European context, for primary episodes and especially for relapses?
- When could screening of asymptomatic *Leishmania* infection be cost effective prior to starting immunosuppressive drugs? Is there any benefit of a preemptive treatment strategy? Which drug(s) could be effective?

- What measures targeting the general community could have evidence-based efficacy in reducing *Leishmania* infection prevalence in dogs? Are educational programs/interventions helpful?
- How can the health and economic impact of addressing leishmaniasis under a One Health lens be measured?

1.8. Conclusions

As a conclusion, leishmaniasis remains a significant individual and public health problem in Portugal and serves as an indicator of neglect. The increasing human incidence and canine seroprevalence in recent years may be linked to climate change, greater mobility, and/or the rise in susceptible groups. These factors could also facilitate the future geographic expansion of endemic *L. infantum* and the introduction of new *Leishmania* species. Gaps in knowledge in the general population could explain insufficient protective practices, such as insufficient adherence to adequate use of insecticides and repellents in dogs. Health professionals could play an important role in promoting health related to vector-borne infections. Animal health professionals, in particular, could have a greater role in disseminating knowledge. Stronger collaborations between different health groups and active input from the community are essential to optimize health for all. Control programs for leishmaniasis should focus not only on reducing underreporting but also on raising awareness of the disease among healthcare professionals and providing tools for earlier diagnosis. Multicentric research efforts can provide evidence to optimize treatment strategies for patients in the European context. Human data should be integrated with data from vectors and mammalian hosts to develop holistic strategies for disease control across various parts of the life cycle, promoting a One Health approach.

2. References

1. Serrada E. A leishmaniose visceral em Portugal Continental: 1999-2009 [Tese Saúde Pública]. Universidade Nova de Lisboa; 2010. Available from: http://hdl.handle.net/10362/5546

2. ECDC. Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. Stockholm; 2022. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/leishmaniasis-surveillance-eu.pdf

3. Cortes S, Vaz Y, Neves R, Maia C, Cardoso L, Campino L. Risk factors for canine leishmaniasis in an endemic Mediterranean region. Vet Parasitol. 2012;

4. Almeida M, Maia C, Cristóvão JM, Morgado C, Barbosa I, Ibars RF, et al. Seroprevalence and risk factors associated with *Leishmania* infection in dogs from Portugal. Microorganisms. 2022 Nov 15;10(11):2262.

5. Koch LK, Kochmann J, Klimpel S, Cunze S. Modeling the climatic suitability of leishmaniasis vector species in Europe. Sci Rep. 2017 Oct 17;7(1):13325. Available from: https://www.nature.com/articles/s41598-017-13822-1

6. Carvalho BM, Maia C, Courtenay O, Llabrés-Brustenga A, Lotto Batista M, Moirano G, et al. A climatic suitability indicator to support *Leishmania infantum* surveillance in Europe: a modelling study. The Lancet Regional Health - Europe. 2024 Aug;43:100971.

7. Amusategui I, Sainz A, Aguirre E, Tesouro MA. Seroprevalence of *Leishmania infantum* in northwestern Spain, an area traditionally considered free of leishmaniasis. In: Annals of the New York Academy of Sciences. 2004.

8. Díaz-Regañón D, Roura X, Suárez ML, León M, Sainz Á. Serological evaluation of selected vector-borne pathogens in owned dogs from northern Spain based on a multicenter study using a commercial test. Parasit Vectors. 2020.

9. Miró G, Checa R, Montoya A, Hernández L, Dado D, Gálvez R. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. Parasit Vectors. 2012 Dec 27;5(1):60. Available from: https://parasitesandvectors.biomedcentral.com/articles/10.1186/1756-3305-5-60

10. Pereira A, Martins Â, Brancal H, Vilhena H, Silva P, Pimenta P, et al. Parasitic zoonoses associated with dogs and cats: A survey of Portuguese pet owners' awareness and deworming practices. Parasites and Vectors. 2016.

11. Matos M, Alho AM, Owen SP, Nunes T, Madeira de Carvalho L. Parasite control practices and public perception of parasitic diseases: A survey of dog and cat owners. Prev Vet Med. 2015.

12. Zé-Zé L, Amaro F, Osório HC, Giovanetti M, Lourenço J, Alves MJ. Molecular identification and ecology of Portuguese wild-caught phlebotomine sandfly specimens. Zoonotic Diseases. 2022;

13. Portugal. Direção Geral da Saúde, Instituto Nacional de Saúde Doutor Ricardo Jorge. Infeção VIH e SIDA em Portugal – 2022. Lisboa; 2022.

14. Pasquier G, Demar M, Lami P, Zribi A, Marty P, Buffet P, et al. Leishmaniasis epidemiology in endemic areas of metropolitan France and its overseas territories from 1998 to 2020. Barbosa DS, editor. PLoS Negl Trop Dis. 2022 Oct 7;16(10):e0010745. Available from: https://dx.plos.org/10.1371/journal.pntd.0010745

15. Fundação Francisco Manuel dos Santos. PORDATA Base de Dados de Portugal Contemporâneo. População residente estrangeira segundo os Censos: total e por país de nacionalidade [Internet]. 2023. Available from: https://www.pordata.pt/portugal/populacao+residente+estrangeira+segundo+os+censos+total+e+por+pais+de+nacionalidade-3786

16. Azevedo N. Leishmaniose Cutânea na Beira Interior: Um Diagnóstico Diferencial Obrigatório [Master thesis]. Universidade da Beira Interior; 2009. Available from: https://ubibliorum.ubi.pt/handle/10400.6/888

17. Amaro F, Vilares A, Martins S, Reis T, Osório HC, Alves MJ, et al. Co-circulation of *Leishmania* parasites and phleboviruses in a population of sand flies collected in the South of Portugal. Trop Med Infect Dis. 2023 Dec 20;9(1):3.

18. Cloots K, Burza S, Malaviya P, Hasker E, Kansal S, Mollett G, et al. Male predominance in reported visceral leishmaniasis cases: Nature or nurture? A comparison of population-based with health facility-reported data. Werneck GL, editor. PLoS Negl Trop Dis. 2020 Jan 29;14(1):e0007995. Available from: https://dx.plos.org/10.1371/journal.pntd.0007995

19. Lockard RD, Wilson ME, Rodríguez NE. Sex-related differences in immune response and symptomatic manifestations to infection with *Leishmania* species. Journal of Immunology Research. 2019.

20. da Costa Lima MS, Zorzenon DCR, Dorval MEC, Pontes ERJC, Oshiro ET, Cunha R, et al. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pac J Trop Dis. 2013 Feb;3(1):10–5. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2222180813600031

21. Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, et al. Clinical Use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. Clinical Infectious Diseases. 2007 Jun 15;44(12):1602–10.

22. Gradoni, L. López-Vélez R MM. Manual on case management and surveillance of the leishmaniasis in the WHO European Region. World Health Organization. 2017.

23. Diro E, Blesson S, Edwards T, Ritmeijer K, Fikre H, Admassu H, et al. A randomized trial of AmBisome monotherapy and AmBisome and miltefosine combination to treat visceral leishmaniasis in HIV co-infected patients in Ethiopia. PLoS Negl Trop Dis. 2019 Jan 17;13(1):e0006988.

24. Burza S, Mahajan R, Kazmi S, Alexander N, Kumar D, Kumar V, et al. AmBisome monotherapy and combination AmBisome–miltefosine therapy for the treatment of visceral leishmaniasis in patients coinfected with human immunodeficiency virus in India: a randomized open-label, parallel-arm, phase 3 trial. Clinical Infectious Diseases. 2022 Oct 12;75(8):1423–32.

25. Gaspari V, Gritti T, Ortalli M, Santi A, Galletti G, Rossi A, et al. Tegumentary leishmaniasis in Northeastern Italy from 2017 to 2020: a neglected public health issue. Int J Environ Res Public Health. 2022 Nov 30;19(23):16047. Available from: https://www.mdpi.com/1660-4601/19/23/16047

26. Humanes-Navarro AM, Herrador Z, Redondo L, Cruz I, Fernández-Martínez B. Estimating human leishmaniasis burden in Spain using the capture-recapture method, 2016–2017. Yurchenko V, editor. PLoS One. 2021 Oct 29;16(10):e0259225. Available from: https://dx.plos.org/10.1371/journal.pone.0259225

27. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Eurosurveillance. 2013 Jul 25;18(30). Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES2013.18.30.20546

28. Mattivi A., Massimiliani E., Cagarelli R., Albieri A. Leishmaniosi in Emilia-Romagna, Aggiornamento Epidemiologico 1999–2015. Available online: https://salute.regione.emilia-

romagna.it/normativa-e-documentazione/rapporti/malattie-infettive/leishmaniosi-er-epidemiologia-1999-2015.

29. Faucher B, Pomares C, Fourcade S, Benyamine A, Marty P, Pratlong L, et al. Mucosal *Leishmania infantum* leishmaniasis: Specific pattern in a multicentre survey and historical cases. Journal of Infection. 2011 Jul;63(1):76–82.

30. Pereira A, Maia C. *Leishmania* infection in cats and feline leishmaniosis: An updated review with a proposal of a diagnosis algorithm and prevention guidelines. Current research in parasitology & vector-borne diseases. 2021;1:100035.

31. Centro de Estudos de Vetores e Doenças Infeciosas Doutor Francisco Cambournac. Relatório REVIVE 2023 - Culicídeos, Ixodídeos e Flebótomos: Rede de Vigilância de Vetores. Lisboa; 2024.

32. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*. Int J Parasitol. 2006 Nov;36(13):1383–8.

33. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, Soteriadou K. *Leishmania donovani* leishmaniasis in Cyprus. Lancet Infect Dis. 2008 Jan;8(1):6–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1473309907702979

34. Berriatua E, Maia C, Conceição C, Özbel Y, Töz S, Baneth G, et al. Leishmaniases in the European Union and neighboring countries. Emerg Infect Dis. 2021 Jun;27(6):1723–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34013857

35. The European Union One Health 2019 Zoonoses Report. EFSAn J. 2021 Feb;19(2):e06406.

36. Dantas-Torres F, Miró G, Baneth G, Bourdeau P, Breitschwerdt E, Capelli G, et al. Canine leishmaniasis control in the context of One Health. Emerg Infect Dis. 2019 Dec;25(12):1–4.

37. Dantas-Torres F, Nogueira F dos S, Menz I, Tabanez P, da Silva SM, Ribeiro VM, et al. Vaccination against canine leishmaniasis in Brazil. Int J Parasitol. 2020 Mar;50(3):171–6.

38. Travi BL, Miró G. Use of domperidone in canine visceral leishmaniasis: gaps in veterinary knowledge and epidemiological implications. Mem Inst Oswaldo Cruz. 2018 Oct 18;113(11).

39. Portela ÁSB, Costa LE, Salles BCS, Lima MP, Santos TTO, Ramos FF, et al. Identification of immune biomarkers related to disease progression and treatment efficacy in human visceral leishmaniasis. Immunobiology. 2018 Mar;223(3):303–9.

40. da Matta VLR, Gonçalves AN, Gomes CMC, Chouman IH, Ferreira FM, Campos MB, et al. Gene signatures of symptomatic and asymptomatic clinical-immunological profiles of human infection by *Leishmania (L.) chagasi* in Amazonian Brazil. Microorganisms. 2023 Mar 3;11(3):653.

41. Singh OP, Tiwary P, Kushwaha AK, Singh SK, Singh DK, Lawyer P, et al. Xenodiagnosis to evaluate the infectiousness of humans to sandflies in an area endemic for visceral leishmaniasis in Bihar, India: a transmission-dynamics study. Lancet Microbe. 2021.