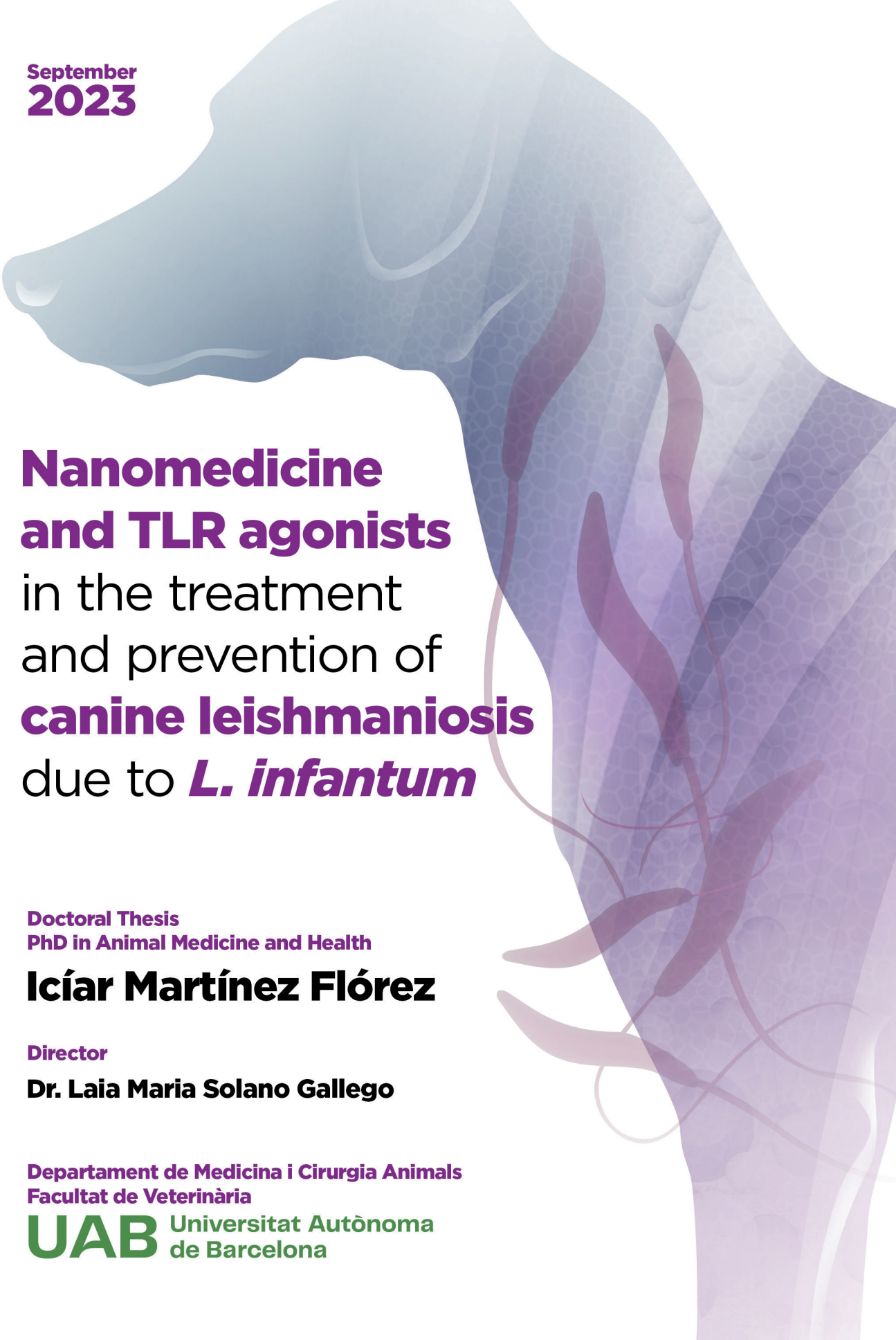
“Nanomedicine and TLR agonists in the treatment and prevention of canine leishmaniosis due to L. infantum”

Icíar Martínez Flórez

September
2023



Nanomedicine and TLR agonists in the treatment and prevention of **canine leishmaniosis** due to ***L. infantum***

Doctoral Thesis
PhD in Animal Medicine and Health

Icíar Martínez Flórez

Director

Dr. Laia Maria Solano Gallego

Departament de Medicina i Cirurgia Animals
Facultat de Veterinària
UAB Universitat Autònoma de Barcelona

UAB
Universitat Autònoma
de Barcelona

La Dra.

Laia Maria Solano Gallego

Catedràtica del Departament de Medicina i Cirurgia Animals
de la Universitat Autònoma de Barcelona.

INFORMA:

Que el treball de tesi doctoral titulat

"Nanomedicine and TLR agonists in the treatment and prevention of canine leishmaniosis due to *L. infantum*"

del que és autora la llicenciada en veterinària

Icíar Martínez Flórez

Ha estat realitzat sota la meva direcció
i compleix les condicions exigides per optar
al títol de Doctora per la Universitat Autònoma de Barcelona.

I per a què així consti, signo el present informe a Bellaterra, Setembre 2023.

"A scientist in their laboratory is not a mere technician:
they are also a child confronting natural phenomena
that impress them as though they were fairy tales"

Marie Curie

"Y una cosa puedo jurar:
yo, que me enamoré de tus alas,
jamás te las voy a querer cortar"

Frida Kahlo

Acknowledgements

Escribir una tesis y realizar el proceso de experimentación es un camino largo, cansado y, a veces, tedioso, por eso es tan importante rodearse de animales humanos y no humanos que te ayudan a sobrellevar los malos momentos.

En primer lugar, me gustaría agradecer a mi directora de tesis, Laia Solano la oportunidad brindada. Desde esas prácticas donde nos conocimos hasta ahora acabando la tesis, han pasado muchas cosas, algunas buenas y otras no tanto, pero Laia sin duda, ocupa un lugar en mi corazón. Gracias por creer en mí, por apoyarme y escucharme, gracias por tenerme en cuenta en este último año y gracias por ayudarme. No siempre ha sido fácil para ti ni para mí por eso creo que el lazo que hemos formado es fuerte y será duradero. Sin ti y tu comprensión, este año habría sido mucho más complicado. Te estaré eternamente agradecida.

A mis compañeras Ale, Marta, Andrea y Clara por todas las horas que hemos pasado juntas en el laboratorio y fuera de él. Por todas las conversaciones, las confesiones, las risas, las penas, las quejas y las ilusiones compartidas. Habéis sido excepcionales. Gracias por todo lo que me habéis enseñado en el mundo de la investigación y en la vida.

A Lourdes, que me ha ayudado y enseñado tanto. Me alegro de haber compartido contigo mis primeros pasos en el mundo de la investigación como doctoranda. Tenerte en el laboratorio fue un regalo. Mil gracias por las horas de más que echabas por nosotras. Y gracias también por tus risas, tu energía, tus ocurrencias, tus vídeos de baile y tus sonrisas. ¡Gracias!

A Magda, muchísimas gracias por todo. Por acogernos en tu maravillosa isla y mover cielo y tierra para encontrar a gente dispuesta a querer entrar en nuestros estudios. Por enseñarme el protocolo de la PCR con tanta paciencia, por los innumerables antígenos de *L.infantum*...y sobre todo por los consejos de oráculo que atesoro.

A Tamara, por toda la ayuda brindada en el laboratorio y por tener siempre mil historias que contar y echarnos unas risas.

Al Servei de Dermatologia del HCV, en concreto a Laura Ordeix y Maria Cabré que me han ayudado siempre que lo he necesitado y me han aportado tantísimas cosas.

A la gente del pasillo de Anatomia Patològica donde está nuestro despacho, en especial a Blanca, Mar, Aida, Jaume y Alberto porque siempre me habéis sacado una sonrisa cuando me encontraba con vosotros y eso lo valoro muchísimo. También a Ester por la paciencia enorme que siempre ha demostrado compartiendo despacho con nosotras, ha sido un gusto.

Al personal del Departament de Medicina i Cirurgia Animals por vuestra ayuda y por poner las cosas más fáciles en todo momento.

A todos los veterinarios y propietarios que participaron en los estudios. Gracias a vosotros, esta tesis es una realidad.

A los estudiantes que decidían realizar sus trabajos de fin de grado y máster con nosotras. Todos vosotros me habéis aportado algo. En especial a Ignasi que vino tantas veces a sacar a los beagles conmigo y me ayudó tantísimo.

A mi compi de doctorado, Miquel, por todas las veces que nos reímos y nos desesperamos juntos. Por nuestras visitas a *gosseres* y la de horas que pasamos allí. Por nuestras catas de vemut y hasta de *cocktails*.

A mis chicas de Muntaner Ale, Bea, Cris y Mouna por abrirme las puertas de su casa, pero también las de su corazón. Por haberme integrado en esa bonita familia, por todas las cenas, las risas, los lloros, los abrazos, los dramas, por mil cosas más. Gracias de corazón, os quiero.

A Ari, por ser la persona más maravillosa del mundo. Porque, aunque pasen mil años, sé que siempre vamos a estar juntas. Por todos los momentos que hemos vivido desde aquellas prácticas de anatomía en primero hasta que te convertiste en una persona imprescindible en mi vida. Todo ello son recuerdos guardados en mi corazón (y en mi archivador). Gracias por escucharme, aconsejarme con sinceridad, contar conmigo para todo, gracias por quererme. En definitiva, gracias por todo lo que haces por mí, eres increíble. Te quiero, pavish.

A Cris, por ser la mejor amiga que una pueda imaginar. Por enseñarme que abrirme contigo no significa rechazo ni miedo. Por instarme a mostrar mis debilidades y ver que sigo estando cómoda a tu lado. Por demostrarme lo que es el amor de verdad. Por estar siempre ahí. Por incluirme en tu vida en todos los aspectos. Por entrar en mi corazón y quedarte allí a vivir. Por esas "broncas" tan necesarias y por ser una fuente inagotable de sabiduría. Por sacarme siempre una sonrisa. Por presentarme a gente bonita que se ha convertido en una socia emocional, Aina, esa eres tú. Por las exposiciones, los edificios con historia, por el teatro, la poesía y los bailes en la calle. Porque eres increíble y una inspiración. Porque ahora mismo no imagino mi vida sin ti. Qué suerte tengo de tenerte en mi vida. Te quiero, mija papaya.

A Alba, mi hermana que, aunque me avisó del duro camino que comportaba un doctorado, nunca dejó de estar orgullosa de mí y de ayudarme en lo necesario. En especial, ahora por confiar en mí para el cargo más importante que tengo, ser madrina orgullosa de Nel.

A Gabriel, mi amigo del alma. Porque siempre me ves con buenos ojos. Siempre dispuesto a ayudarme, sean las 6 o las 2 de la mañana. Porque sé que contigo tengo un apoyo 24/7. Porque me encantaría descubrir Argentina contigo y

espero que podamos hacerlo. Porque la confianza que tengo en ti no creo que pueda romperse. Te quiero mucho y estoy muy orgullosa de todo lo que has conseguido en la vida. Aquí me tienes siempre.

A Mariana, por acompañarme en cada paso de mi vida desde hace ya unos años. Por descubrirme Colombia y hacer que me enamore de ella sin haberla pisado. Por todas las veces que quedamos "solo una horita" y volvimos a casa a las mil. Por tu lucha por los derechos humanos, en especial, de las mujeres en situaciones de riesgo. Gracias por los debates, los vinos, la confianza depositada cuando se trata de Noah o Tíbet. Gracias amiga, Barcelona está ligada a ti.

A mis Vets por el mundo, mis amigos y familia en Madrid. Por todos estos años juntos, que ya son unos cuantos. Por la cantidad de cosas vividas. Los recuerdos infinitos y los momentos vividos. Los días enteros en la biblioteca, las prácticas, las clases, las fiestas, las risas, los llantos, los nervios, el estrés, los viajes, los cumpleaños disfrazados de Halloween, las nuevas incorporaciones, las bodas, los nacimientos, por tantas cosas…Por los "vetencuentros" de cada año, por estar todo este tiempo a mi lado y porque siga así muchos más años. Os quiero.

A Núria y a María porque sin ellas, no estaría aquí. No tengo palabras para expresar tanta gratitud. Gracias de todo corazón por confiar en mí, por la paciencia y por ayudarme en los momentos en los que tanto lo necesitaba. Gracias de nuevo.

Als Botejara's, per ser la millor família que una pot triar. Per obrir-me els braços quan vaig arribar i per fer-me sentir una més cada dia que passa. Per tots els moments viscuts, els pampings, els reis, els aniversaris, les cançons, les camises i els jocs de taula. Per haver guanyat cosins, germans, tietes, tiets i fins i tot, una neboda. Volia agrair especialment a la Rosaura i en Joan que em donen suport en cada cosa que faig com si fos filla seva, sé que puc comptar amb vosaltres per al que sigui. Per deixar-me ser com soc i acceptar-me tal qual. Perquè sou la família més unida i especial que he conegut mai i perquè em sento orgullosa d'estar en ella. Moltes gràcies família, sou meravellosos, us estimo.

Al club de lectura feminista, El cuarto de atrás, de Crisi. Por enseñarme tantísimo en materia de feminismo. Por todas las veces que no voy muy convencida con el libro y salgo de allí enamorada. Por presentarme a Jess que cambió mi mirada y me atrapó por completo. Por hacerme sentir parte de un grupo de mujeres empoderadas, increíbles, inteligentes, sensibles, tiernas, fuertes y dueñas de sí mismas. Por compartir vuestras historias y empatizar con las mías. Por las cañas de después y los planes que se fraguan allí. Por ser TAN maravillosas, gracias.

Volia agrair al Club d'agility Ciutat Comtal per ensenyar-me que l'agility és molt més que un esport. Per tota la gent que he conegut allà, sou increïbles. Pel

vostre compromís amb els gossos i el seu benestar. Per proporcionar-me salut mental en aquests mesos tan durs i per creure en mi portant un chihuahua i un beagle. Per convidar-me aquest any a Rialp, heu estat el millor del meu estiu sens dubte. Pels riures, les abraçades, les jornades intenses, el cansament, els podis, els esmorzars amb energia (uns més que uns altres), pels balls i per ser com sou. Moltes gràcies per fer-me sentir part d'aquesta gran família.

A todos los perros que, de una u otra forma, participaron en estos estudios y gracias a los cuales hoy podemos conocer un poco más acerca de esta infección/enfermedad. Y en general, a todos los animales de experimentación, ojalá no fueseis necesarios. Mil gracias por todo.

A los adoptantes de mis queridos beagles, gracias a vosotros hoy tienen una familia que los quiere.

A todos los perros que me han acompañado en la vida. A Buck, por crecer a mi lado, marcarme el camino y ayudarme a decidir mi futuro profesional. A Tro, por aceptarme sin reservas y hacer que me enamorase de ti. A Curie y a Frida por ser mis apoyos emocionales, por compartir tantas cosas conmigo, por alegrarme cada día, por hacerme sonreír, por aceptar cada cosa que quiero hacer con vosotras y simplemente, por existir. Todos me habéis marcado. Os quiero.

Y, por último, a Carles. La persona más importante de mi vida. Gracias, gracias, gracias de corazón por todo lo que me has ayudado. Gracias por involucrarte tanto. Por decir que sí cuando estás agotado. Por hacerme reír, por tus caras, tus ocurrencias y tus canciones. Por todos los recuerdos que atesoramos juntos. Por brindarme una vida a tu lado. Por enseñarme que las relaciones pueden ser preciosas. Por nuestro baile del pasillo y la confianza que tienes en mí. Eres increíble. Te quiero.

Y muchas gracias a todos aquellos que habéis formado parte de mi doctorado de una u otra forma. Gracias, de verdad.

Contents

List of Abbreviations

A2 + CPI + CPII: Recombinant amastigote-specific antigen and cysteine proteinases of *Leishmania donovani*
Ab: Antibody
AHCC: Active hexose correlated compound
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AMPs: Antimicrobial peptides
ART: Artemisinin and its derivatives
AST: Aspartate aminotransferase
ATP: Adenosine triphosphate
BALB/c mice: Bagg Albino laboratory-bred strain mouse
BCG: Live bacillus Calmette-Guérin
BM: Bone marrow
BQ: Biochemistry panel
BT1: Biopterin transporter
CanL: Canine leishmaniosis
CAV1: Canine adenovirus type 1
CAV2: Canine adenovirus type 2
CBC: Complete blood count
CCL: Canine cutaneous leishmaniosis
CDV: Canine distemper virus
cELISA: Crude antigen ELISA
CI: Confidence interval
CKD: Chronic kidney disease
CL: Human cutaneous leishmaniosis
CMI: Cellular-mediated immunity
Con A: Concanavalin A
CpG-ODN: CpG-oligodeoxynucleotides
CPI: *Leishmania infantum* cysteine proteinase A
CPII: *L. infantum* cysteine proteinase B
CPIII: *L. infantum* cysteine proteinase type III
CPiV: Canine parainfluenza virus
CPV: Canine parvovirus
CPV2c: Canine parvovirus type 2c
Cq: Quantification cycle
CTLA: Crude total *L. infantum* antigen
DNA: Deoxyribonucleic acid
DPP-CVL: Dual-path platform® canine visceral leishmaniasis rapid test (BioManguinhos)
DTH: Delayed-type hypersensitivity
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent Assay
EMA: European Medicines Agency
EU: ELISA units
FDA: US food and drug administration
FML: Fucose-mannose ligand
FML-ELISA: Fucose-mannose ligand ELISA
GLA-SE: Glucopyranosyl lipid adjuvant-squalene
GM-CSF: Granulocyte macrophage colony-stimulating factor
GP63: Surface expressed glycoprotein GP63
H1: Histone H1
H2A: Histone H2A
HGPRT: Hypoxanthine-guanine phosphoribosyltransferase
HL: Human leishmaniosis
HSP: Heat shock protein
IFAT: Indirect fluorescent antibody test
IFN-γ: Interferon-gamma
Ig: Immunoglobulin
IL: Interleukin
iNOS: Inducible nitric oxide synthase
IRIS: Disease International Renal Interest Society
KD: Kalazar detectTM rapid test for visceral leishmaniasis (InBios)
KMP-11: kinetoplastid membrane protein-11
LACK: *Leishmania* homologue of receptors for activated C kinase
LBSap: *Leishmania braziliensis* antigens plus saponin
Ldccys1: Cysteine proteinase of 30 kDa from *Leishmania infantum*
Ldp27: *L. donovani* amastigote-specific protein p27
LeIF: *L. braziliensis* elongation and initiation factor
LiESP: Purified excreted-secreted proteins of *L. infantum*
LiP: *L. infantum* acidic ribosomal protein
LirCyP1: Cyclophilin protein 1 of *Leishmania infantum*
LmSTI1: *Leishmania major* stress inducible protein 1
LN: Lymph node
LPA: *Leishmania* promastigote antigen
LSA: Soluble *Leishmania* antigen
Max: Maximum

MDP: Muramyl dipeptide
MGG: May-Grünwald Giemsa
MHCI: Major histocompatibility complex class I
MHCII: Major histocompatibility complex class II
Min: Minimum
MPLA: Monophosphoryl lipid A
MPL-SE: Monophosphoryl lipid A plus squalene
MPL-TDM: Monophosphoryl lipid A-trehalose dicorynomycolate
MVA: Modified vaccinia virus Ankara
n: Number
N.D.: Not determined
N.S.: Not stated
NaCl: Sodium chloride
NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen
NBT: Nitroblue tetrazolium reduction test
NH36: Nucleoside hydrolase of *L. donovani*
NK: Natural killer cells
NO: Nitric oxide
NOD: Nucleotide oligomerization domain
Non-prod: Non-producers
NPs: Nanoparticles
OD: Optical density
ORFF: Open reading frame fragment
P.O.: Per oral
P0: Protein 0 of *L. infantum*
PAHO: Pan American Health Organization
PAMPs: Pathogen-associated molecular patterns
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase chain reaction
PD: Papular dermatitis
PD-1: Programmed cell death protein-1
PHMB: polyhexamethylene biguanide
PLGA: poly(D,L-lactide-co-glycolide)
P-MAPA: Protein aggregate of magnesium ammoniumphospholinoleate palmitoleate anhydride
PQ: Protein Q
PRRs: Pattern recognition receptors
PSA: Parasite surface antigen
pSP: Secretory *L. donovani* serine protease
QA-21: Purified extract of *Quillaja saponaria*
QuilA: Saponin adjuvant produced with *Q. saponaria*
R: Riedel de Haën saponin
rA2: Recombinant *L. infantum* and *L. donovani* amastigote-specific antigen
rLdccys1: Recombinant cysteine proteinase
rLdP1: Recombinant *L. donovani* ribosomal P1 gene
rLdp45: Recombinant *L. donovani* p45
rLdPDI: Recombinant *L. donovani* protein disulfide isomerase
rLeish-111f: Polyprotein derived by fusion of three antigens namely *L. major* homologue
rLelF-2: *L. donovani* elongation factor-2 protein
rLiHyp1: Hypothetical *Leishmania* amastigote-specific protein
ROS: Reactive oxygen species
rVV: Recombinant vaccinia virus
S.C.: Subcutaneous administration
Sb(V): Pentavalent antimonials
SD: Standard desviation
SDMA: Symmetric dimethylarginine
SIR2: Silent information regulatory 2
SMT: Sterol 24-c-methyltransferase
SS: Saponins
TC2: Chromane-derived hydrazone
TGF-β: Transforming growth factor-beta
Th: T helper
TLR: Toll-like receptor
TLR4a: Toll-like receptor 4 agonist
TLRa: Toll-like receptor agonist
TNF-α: Tumor necrosis factor-alpha
TP: Total proteins
TSA: Thiol-specific antioxidant
uGGT/UC: Urinary gamma-glutamyl transferase to urinary creatinine ratio
UPC: Urinary protein:creatine ratio
WBA: Whole blood assay
WHO: World Health Organization
WNL: Within normal limits
χ2: Chi-square

List of Figures

List of Tables

Summary

Leishmaniosis is a disease caused by parasitic protozoans of the *Leishmania* genus which are mainly transmitted through sandfly bites [1,2]. It is considered a zoonotic disease that affects millions of people mainly in tropical regions, impacting health and socioeconomic development, and dogs act as the main reservoir [3]. *Leishmania infantum* is the specie considered one of the most relevant infecting dogs, cats and other domestic and wild animals as well as humans [4]. Clinical canine leishmaniosis is manifested by a broad spectrum of clinical signs and clinicopathological abnormalities [5]. Cutaneous presentations are the most common, and papular dermatitis as the sole clinical presentation, is considered a typical presentation in endemic areas [5,6]. Nevertheless, there is a gap of knowledge regarding treatment options and management for these patients with papular dermatitis-mild disease (LeishVet stage I). *Leishmania*'s interaction with the host's immune system involves complex dynamics between innate and adaptive responses, influencing disease outcome [5]. Adaptive T-cell immunity play a significant role, with a cellular immune response linked to disease resistance and humoral response to clinical illness [1,7]. Innate immunity has recently shown to also play a crucial role controlling the infection and guiding the development of durable, pathogen-specific adaptive immune responses [8]. Within this, neutrophils are suspected to have a key role particularly in the early stages of infection, as they are the first immune cells to encounter the parasite and engulf it [9–13]. Disease progression depends on the interaction between parasite elimination and its mechanisms of evasion of immune cells [14–16]. Available treatment choices for *Leishmania* spp. infection are suboptimal, as despite often reducing parasite levels and improving clinical signs, they do not completely eradicate the infection and carry a moderate risk of side effects [17]. Additionally, treatment effectiveness is limited due to the emergence of resistance mechanisms in the parasite in endemic areas [18–20]. Consequently, preventive medicine is considered the cornerstone of infection control and epidemiologic management [1]. However, currently available preventative tools are deemed insufficient. The development of an effective vaccine is highly desired, although it has been proven to be challenging, and available vaccines have shown limited efficacy [21,22].

The general aim of this thesis was to investigate new tools for prevention and treatment as well as enhancing knowledge regarding innate and adaptive immunity for canine *L. infantum* infection. This thesis was divided in three main studies **(chapters 3, 4 and 5)**.

The first study **(chapter 3)** aimed to enhance our understanding of treatment options for dogs affected by papular dermatitis-stage I, and was designed with the hypothesis that local administration of either meglumine antimoniate or polyhexamethylene biguanide (PHMB) used alone or in combination with a TLR4 agonist in patients with papular dermatitis leads to a faster resolution of clinical

signs as well as no significant adverse effects. In addition, dogs with papular dermatitis show a strong cellular immune response and a weak humoral immune response with low parasitemia. Thus, its specific objectives were to evaluate the safety and clinical efficacy of meglumine antimoniate and topical administration of PHMB used alone or in combination with a TLR4 agonist in the resolution of cutaneous lesions in dogs with papular dermatitis due to *L. infantum* infection, and to evaluate parasite-specific humoral and cellular immunity as well as parasitemia at the time of diagnosis and during the follow-up period of these dogs.

In comparison to the placebo (n=9), the local application of PHMB (alone or combined with TLR4a, n=9) exhibited a stronger inclination towards resolving papular dermatitis. On the other hand, local administration of meglumine antimoniate demonstrated the fastest clinical resolution (n=10) after treatment. In conclusion, the localized use of meglumine antimoniate seems to be both safe and clinically effective in treating canine papular dermatitis due to *L. infantum* infection.

The second study **(chapter 4)** was conceived with the explicit goal of bridging the existing knowledge gap concerning the impact of neutrophils on disease progression. It was meticulously structured to achieve the following specific objectives: to assess the variations in peripheral blood neutrophil oxidative activity by means of the nitroblue tetrazolium reduction test (NBT) rate and to investigate the potential association between neutrophil oxidative activity and IFN-γ concentrations and antibody levels in dogs in different states of *L. infantum* infection. Two hypotheses were made, the first being that healthy seropositive infected dogs or dogs with mild disease (stage I-papular dermatitis) show a higher proportion of activated neutrophils in peripheral blood than dogs in moderate to severe diseases (stages II-III), and the second that IFN-γ is positively associated to activated neutrophils in peripheral blood of dogs with *L. infantum* infection as well as inversely related to antibody levels.

The findings from the NBT revealed significantly higher activation of neutrophils in sick dogs in stage I-papular dermatitis (n=25) compared to the healthy-seronegative group (n=25), healthy-seropositive group (n=21), stage II group (n=41), and stage III-IV group (n=16). Healthy-seropositive dogs also displayed higher values than all groups except stage I.

Concerning IFN-γ, dogs in stage I exhibited elevated concentrations compared to healthy-seronegative dogs, stage II dogs, and stage III-IV dogs. However, no significant differences were observed between the concentrations of IFN-γ in healthy seropositive dogs and those in stage I. In conclusion, neutrophil activation was more pronounced in dogs with mild disease and healthy-seropositive dogs, and a correlation between neutrophil activation and IFN-γ production was observed.

Resumen

La leishmaniosis es una enfermedad causada por protozoos parasitarios del género *Leishmania* que se transmiten principalmente a través de las picaduras de flebótomos [1,2]. Se considera una enfermedad zoonótica que afecta a millones de personas, principalmente en regiones tropicales, con un impacto en la salud y el desarrollo socioeconómico, y los perros actúan como su principal reservorio [3]. *Leishmania infantum* una de las especies más relevantes en cuanto a la infección de perros, gatos y otros animales domésticos y salvajes, así como, humanos [4]. La leishmaniosis canina clínica se manifiesta con un amplio espectro de signos clínicos y anormalidades clinicopatológicas que se pueden clasificar en cuatro estadios según LeishVet. Las manifestaciones cutáneas son las más comunes siendo la dermatitis papular, como única forma de presentación clínica, considerada una presentación típica en áreas endémicas. Sin embargo, no existe una recomendación clara respecto a las opciones de tratamiento y manejo de estos pacientes con dermatitis papular-enfermedad leve (LeishVet estadio I). La interacción de *Leishmania* con el sistema inmunitario del huésped implica una dinámica compleja entre las respuestas inmunitarias innatas y adaptativas que influyen en el resultado de la enfermedad [5]. Se ha estudiado ampliamente la inmunidad adaptativa de los linfocitos T y se ha demostrado que desempeña un papel importante, con una respuesta inmunitaria celular asociada a la resistencia a la enfermedad y una respuesta humoral relacionada con la enfermedad clínica [1,7]. La inmunidad innata también parece desempeñar un papel crucial en el control de la infección y guiar el desarrollo de respuestas inmunitarias adaptativas duraderas [8]. Dentro de esto, se sospecha que los neutrófilos tienen un papel clave, particularmente en las primeras etapas de la infección, ya que son las primeras células inmunológicas en reconocer el parásito y fagocitarlo [9–13]. La progresión de la enfermedad depende de la interacción entre la eliminación del parásito y sus mecanismos de evasión de las células inmunes [14–16]. Las opciones de tratamiento disponibles para la infección por *Leishmania* spp. no son óptimas, ya que, a pesar de reducir frecuentemente los niveles de parásitos y mejorar los signos clínicos, no erradican completamente la infección y conllevan un riesgo moderado de efectos adversos secundarios [17]. Además, la eficacia del tratamiento se ve limitada por la aparición de mecanismos de resistencia en el parásito en áreas endémicas [18–20]. En consecuencia, la medicina preventiva se considera el pilar del control de la infección y la gestión epidemiológica [1]. Sin embargo, las herramientas de prevención disponibles actualmente se consideran insuficientes. Por ello, el desarrollo de una vacuna eficaz es muy deseado, aunque ha demostrado ser un desafío, y las vacunas disponibles han mostrado una eficacia limitada [21,22].

El objetivo general de la tesis fue investigar nuevas herramientas para la prevención y el tratamiento, así como, mejorar el conocimiento sobre la inmunidad innata y

adaptativa de la infección por *L. infantum* en el perro. Esta tesis se dividió en tres estudios principales **(capítulos 3, 4 y 5)**.

El primer estudio **(capítulo 3)** tuvo como objetivo mejorar nuestra comprensión sobre las opciones de tratamiento en perros afectados por dermatitis papular-estadio I, y se diseñó con las hipótesis de que la administración local de antimoniato de meglumina o biguanida de polihexametileno (PHMB) solo o en combinación con un agonista de TLR4 en pacientes con dermatitis papular conducía a una resolución más rápida de los signos clínicos, sin efectos adversos significativos. Además, los perros con dermatitis papular mostraban una fuerte respuesta inmunitaria celular y una respuesta inmunitaria humoral débil con baja parasitemia. Por lo tanto, sus objetivos específicos eran evaluar la seguridad y eficacia clínica del antimoniato de meglumina y la administración local de (PHMB) solo o en combinación con un agonista de TLR4 en la resolución de lesiones cutáneas en perros con dermatitis papular debida a la infección por *L. infantum*, y evaluar la inmunidad celular y humoral específica del parásito, así como la parasitemia en el momento del diagnóstico y durante el período de seguimiento de estos perros.

En comparación con el placebo (n=9), la aplicación local de PHMB (solo o combinado con TLR4a, n=9)) mostró una inclinación más fuerte hacia la resolución de la dermatitis papular. Por otro lado, la administración local de antimoniato de meglumina (n=10) demostró la resolución clínica más rápida después del tratamiento. En conclusión, el uso local de antimoniato de meglumina parece ser seguro y clínicamente eficaz en el tratamiento de la dermatitis papular canina debido a la infección por *L. infantum*.

El segundo estudio **(capítulo 4)** se concibió con el objetivo explícito de cerrar la brecha de conocimiento existente con respecto al impacto de los neutrófilos en la progresión de la enfermedad. Se estructuró meticulosamente para lograr los siguientes objetivos específicos: evaluar las variaciones en la actividad oxidativa de los neutrófilos en la sangre periférica mediante la tasa de *nitroblue tetrazolium test* (NBT) e investigar la asociación potencial entre la actividad oxidativa de los neutrófilos y las concentraciones de IFN-γ y los niveles de anticuerpos en perros en diferentes estados de infección por *L. infantum*. Se plantearon dos hipótesis, la primera de las cuales era que los perros infectados seropositivos sanos o los perros con enfermedad leve (estadio I-dermatitis papular) mostraban una mayor proporción de neutrófilos activados en la sangre periférica que los perros con enfermedad moderada a grave (estadios II-III), y la segunda era que el IFN-γ estaba positivamente asociado con los neutrófilos activados en la sangre periférica de los perros con infección por *L. infantum*, así como, inversamente relacionado con los niveles de anticuerpos.

Los resultados del NBT revelaron una activación significativamente mayor de los neutrófilos en los perros enfermos en estadio I-dermatitis papular (n=25) en

comparación con el grupo sano-seronegativo (n=25), el grupo sano-seropositivo (n=21), el grupo en estadio II (n=41) y el grupo en estadio III-IV (n=16). Los perros sanos-seropositivos también mostraron valores más altos que todos los grupos excepto los del estadio I.

En cuanto al IFN-γ, los perros en el estadio I exhibieron concentraciones elevadas en comparación con los perros sanos-seronegativos, los perros en el estadio II y los perros en los estadios III-IV. Sin embargo, no se observaron diferencias significativas entre las concentraciones de IFN- γ de los perros sanos seropositivos y los del estadio I. En conclusión, la activación de los neutrófilos fue más pronunciada en los perros con enfermedad leve y los perros sanos-seropositivos, y se observó una correlación entre la activación de los neutrófilos y la producción de IFN-γ.

Resum

La leishmaniosi és una malaltia causada per protozous parasitaris del gènere *Leishmania* que es transmeten principalment a través de les picades de flebotomins [1,2]. Es considera una malaltia zoonòtica que afecta milions de persones, principalment a regions tropicals, amb un impacte en la salut i el desenvolupament socioeconòmic, i els gossos actuen com el seu principal reservori [3]. *Leishmania infantum* és una de les espècies més rellevants pel que fa a la infecció de gossos, gats i altres animals domèstics i salvatges, així com humans [4]. La leishmaniosi canina clínica es manifesta amb un ampli espectre de signes clínics i anormalitats clinicopatològiques que es poden classificar en quatre estadis clínics segons la classificació LeishVet. Les manifestacions cutànies són les més comunes, sent la dermatitis papular, com a única forma de presentació clínica, considerada una presentació típica en àrees endèmiques. No obstant això, no existeix una recomanació clara respecte a les opcions de tractament i maneig d'aquests pacients amb dermatitis papular-malaltia lleu (LeishVet estadi I). La interacció de *Leishmania* amb el sistema immunitari de l'hoste implica una dinàmica complexa entre les respostes immunitàries innates i adaptatives que influeixen en el resultat de la malaltia [5]. La immunitat adaptativa dels limfòcits T ha estat àmpliament estudiada i s'ha demostrat que juga un paper important. S'ha trobat una associació de la resposta immunitària cel·lular a la resistència a la malaltia, així com una associació de la resposta humoral amb la malaltia clínica [1,7]. La immunitat innata també ha demostrat tenir un paper crucial en el control de la infecció, així com en guiar el desenvolupament de respostes immunitàries adaptatives duradores [8]. Dins d'aquesta, es sospita que els neutròfils tenen un paper clau, particularment en les primeres etapes de la infecció, ja que són les primeres cèl·lules immunològiques a trobar el paràsit i fagocitar-lo [9–13]. La progressió de la malaltia depèn de la interacció entre els sistemes d'eliminació del paràsit i els seus mecanismes d'evasió de les cèl·lules de la immunitat [14–16]. Les opcions de tractament disponibles per a la infecció per *Leishmania* spp. no són òptimes, ja que, malgrat reduir sovint els nivells de paràsits i millorar els signes clínics, no erradiquen completament la infecció i comporten un risc moderat d'efectes secundaris [17]. A més, l'eficàcia del tractament es veu limitada per l'aparició de mecanismes de resistència en el paràsit en àrees endèmiques [18–20]. En conseqüència, la medicina preventiva es considera el pilar del control de la infecció i la gestió epidemiològica [1]. No obstant això, les eines de prevenció disponibles actualment es consideren insuficients. Per això, el desenvolupament d'una vacuna eficaç és molt desitjat, encara que ha demostrat ser un repte, i les vacunes disponibles han mostrat una eficàcia limitada [21,22].

L'objectiu general de la tesi va ser investigar noves eines per a la prevenció i el tractament, així com millorar el coneixement sobre la immunitat innata i adaptativa de la infecció per *L. infantum* en el gos. Aquesta tesi es va dividir en tres estudis principals **(capítols 3, 4 i 5)**.

El primer estudi **(capítol 3)** va tenir com a objectiu millorar la nostra comprensió sobre les opcions de tractament en gossos afectats per dermatitis papular - estadi I, i es va dissenyar amb les hipòtesis que l'administració local d'antimoniat de meglumina o biguanida de polihexametilè (PHMB) sol o en combinació amb un agonista de TLR4 en pacients amb dermatitis papular porta a una resolució més ràpida dels símptomes clínics sense induir efectes adversos significatius. A més, els gossos amb dermatitis papular mostren una forta resposta immunitària cel·lular i una resposta immunitària humoral feble amb baixa parasitèmia. Per tant, els seus objectius específics eren avaluar la seguretat i eficàcia clínica de l'antimoniat de meglumina i l'administració local de (PHMB) sol o en combinació amb un agonista de TLR4 en la resolució de lesions cutànies en gossos amb dermatitis papular deguda a la infecció per *L. infantum*, i avaluar la immunitat cel·lular i humoral específica del paràsit, així com la parasitèmia en el moment del diagnòstic i durant el període de seguiment d'aquests gossos.

En comparació amb el placebo (n=9), l'aplicació local de PHMB (sol o combinat amb TLR4a, n=9) va demostrar una inclinació més forta cap a la resolució de la dermatitis papular. D'altra banda, l'administració local d'antimoniat de meglumina (n=10) va demostrar la resolució clínica més ràpida després del tractament. En conclusió, l'ús local d'antimoniat de meglumina sembla ser segur i clínicament eficaç en el tractament de la dermatitis papular canina deguda a la infecció per *L. infantum*.

El segon estudi **(capítol 4)** es va concebre amb l'objectiu explícit de tancar la bretxa de coneixement existent pel que fa a l'impacte dels neutròfils en la progressió de la malaltia. Es va estructurar meticulosament per aconseguir els següents objectius específics: avaluar les variacions en l'activitat oxidativa dels neutròfils en la sang perifèrica mitjançant la taxa de *nitroblue tetrazolium test* (NBT) i investigar l'associació potencial entre l'activitat oxidativa dels neutròfils i les concentracions d'IFN-γ i els nivells d'anticossos en gossos en diferents estats d'infecció per *L. infantum*. Es van plantejar dues hipòtesis, la primera de les quals era que els gossos infectats seropositius sans o els gossos amb malaltia lleu (estadi I-dermatitis papular) mostraven una major proporció de neutròfils activats en la sang perifèrica que els gossos amb malaltia moderada a greu (estadis II-III), i la segona era que l'IFN-γ estava positivament associat amb els neutròfils activats en la sang perifèrica dels gossos amb infecció per *L. infantum*, així com inversament relacionat amb els nivells d'anticossos.

Els resultats del (NBT) van revelar una activació significativament major dels neutròfils en els gossos malalts en estadi I-dermatitis papular (n=25) en comparació amb el grup sa-seronegatiu (n=25), el grup sa-seropositiu (n=21), el grup en estadi II (n=41) i el grup en estadi III-IV (n=16). Els gossos sans-seropositius també van mostrar valors més alts que tots els altres grups excepte els de l'estadi I.

Pel que fa a l'IFN-γ, els gossos en l'estadi I van exhibir concentracions elevades en

comparació amb els gossos sans-seronegatius, els gossos en l'estadi II i els gossos en els estadis III-IV. No obstant, no es van observar diferències significatives entre les concentracions d'IFN-γ dels gossos sans seropositius i els de l'estadi I. En conclusió, l'activació dels neutròfils va ser més pronunciada en els gossos amb malaltia lleu i els gossos sans-seropositius, i es va observar una correlació entre l'activació dels neutròfils i la producció de IFN-γ.

References

1. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends Parasitol.* **2008**, *24*, 324–330.
2. Moreno, J. Assessment of Vaccine-Induced Immunity against Canine Visceral Leishmaniasis. *Front. Vet. Sci.* **2019**, *6, 168*.
3. Alvar, J.; Vélez, I.D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; de Boer, M. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS One* **2012**, *7*, :e35671.
4. Akhoundi, M.; Downing, T.; Votýpka, J.; Kuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; et al. *Leishmania* Infections: Molecular Targets and Diagnosis. *Mol. Aspects Med.* **2017**, *57*, 1–29.
5. Solano-Gallego, L.; Mirá, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites and Vectors* **2011**, *4*, 1–16.
6. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological Findings and Detection of Toll-like Receptor 2 in Cutaneous Lesions of Canine Leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163.
7. Alvar, J.; Cañavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine Leishmaniasis. *Adv. Parasitol.* **2004**, *57*, 1–88.
8. Toepp, A.J.; Petersen, C.A. The Balancing Act: Immunology of Leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25.
9. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, *64*, 38–43.
10. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, *173*, 6521–6525.
11. Mantovani, A.; Cassatella, M.A.; Costantini, C.; Jaillon, S. Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity. *Nat. Rev. Immunol.* **2011**, *11*, 519–531.
12. Nauseef, W.M.; Borregaard, N. Neutrophils at Work. *Nat. Immunol.* **2014**, *15*, 602–611.
13. Mócsai, A. Diverse Novel Functions of Neutrophils in Immunity, Inflammation, and Beyond. *J. Exp. Med.* **2013**, *210*, 1289–1299.
14. Wardini, A.B.; Pinto-da-Silva, L.H.; Nadaes, N.R.; Nascimento, M.T.; Roatt, B.M.; Reis, A.B.; Viana, K.F.; Giunchetti, R.C.; Saraiva, E.M. Neutrophil Properties in Healthy and *Leishmania Infantum*-Naturally Infected Dogs. *Sci. Rep.* **2019**, *9*, 1–10.

15. Pereira, M.; Valério-Bolas, A.; Santos-Mateus, D.; Alexandre-Pires, G.; Santos, M.; Rodrigues, A.; Rocha, H.; Santos, A.; Martins, C.; Tomas, A.; et al. Canine Neutrophils Activate Effector Mechanisms in Response to *Leishmania Infantum*. *Vet. Parasitol.* **2017**, *248*, 10–20.

16. Panaro MA, Puccini V, Faliero SM, Marzio R, Marangi A, Lisi S, Brandonisio O. *Leishmania donovani* lipophosphoglycan (LPG) inhibits respiratory burst and chemotaxis of dog phagocytes. *New Microbiol.* **1996,** *19,* 107-12.

17. Ponte-Sucre, A.; Gamarro, F.; Dujardin, J.C.; Barrett, M.P.; López-Vélez, R.; García-Hernández, R.; Pountain, A.W.; Mwenechanya, R.; Papadopoulou, B. Drug Resistance and Treatment Failure in Leishmaniasis: A 21st Century Challenge. *PLoS Negl. Trop. Dis.* **2017**, *11*, :e0006052.

18. Croft, S.L.; Sundar, S.; Fairlamb, A.H. Drug Resistance in Leishmaniasis. *Clin. Microbiol. Rev.* **2006**, *19*, 111–126.

19. Santos, D.O.; Coutinho, C.E.R.; Madeira, M.F.; Bottino, C.G.; Vieira, R.T.; Nascimento, S.B.; Bernardino, A.; Bourguignon, S.C.; Corte-Real, S.; Pinho, R.T.; et al. Leishmaniasis Treatment--a Challenge That Remains: A Review. *Parasitol. Res.* **2008**, *103*, 1–10.

20. Yasur-Landau, D.; Jaffe, C.L.; David, L.; Baneth, G. Allopurinol Resistance in *Leishmania Infantum* from Dogs with Disease Relapse. *PLoS Negl. Trop. Dis.* **2016**, *10*, 1–13.

21. Velez, R.; Gállego, M. Commercially Approved Vaccines for Canine Leishmaniosis: A Review of Available Data on Their Safety and Efficacy. *Trop. Med. Int. Health* **2020**, *25*, 540–557.

22. Wylie, C.E.; Carbonell-Antoñanzas, M.; Aiassa, E.; Dhollander, S.; Zagmutt, F.J.; Brodbelt, D.C.; Solano-Gallego, L. A Systematic Review of the Efficacy of Prophylactic Control Measures for Naturally-Occurring Canine Leishmaniosis, Part I: Vaccinations. *Prev. Vet. Med.* **2014**, *117*, 7–18.

Justification

Canine leishmaniosis (CanL) is a zoonotic disease that implies a significant threat to both human and canine populations [1,2]. Among the various clinical presentations of leishmaniosis in dogs, papular dermatitis-stage I mild disease is a common manifestation that lacks clear treatment recommendations [3]. Additionally, understanding the immune response during *Leishmania* infection and the development of an effective vaccine formulation are essential for advancing in the management and prevention of the infection and disease. This PhD thesis is structured in three studies, each aiming to address a crucial aspect of CanL management and contribute to the knowledge base for this complex disease.

Study 1: Testing a new topical treatment for papular dermatitis

Papular dermatitis is a common presentation of CanL [3]. Currently, there is a lack of clear treatment recommendations for this specific clinical presentation, leading to uncertainties in the clinical management of affected dogs. Since papular dermatitis is associated to a Th1 predominant parasite-specific cellular immunity and low humoral immune response, it is characterized by the absence of laboratory abnormalities and others clinical signs. Moreover, skin lesions due to papular dermatitis may undergo spontaneous healing which usually takes between three and six months. For these reasons, dogs are sometimes not treated [4,5]. Until now, when treatment is established, the typical treatment choices have primarily consisted of systemic conventional anti-*Leishmania* drugs, which have been associated with the onset of diverse side effects, making them potentially inappropriate for addressing papular dermatitis [2,6,7]. The use of local treatment for managing cutaneous leishmaniosis in both humans and dogs has gained significant attention as a potential therapeutic approach [8–10]. However, there has been a notable absence of studies evaluating local treatment for papular dermatitis caused by *L. infantum* infection in dogs, despite this being a common manifestation of the disease [2,6,7].

The first study in this thesis aimed to address this gap by conducting a double blinded randomized controlled clinical study to test new topical treatments for papular dermatitis. The study involved a cohort of dogs with papular dermatitis, carefully selected and randomized into treatment and control groups. The treatment group received two different new local formulations, while the control group received a placebo. The study was carried out over an appropriate timeframe (one year follow-up), closely monitoring the progression of the disease, and assessing the efficacy and safety of the new local treatment. The results of this study provided valuable insights into the treatment and management of papular dermatitis and lay the foundation for future clinical recommendations.

Study 2: Characterizing neutrophil oxidative metabolism during different states of *L. infantum* infection and its relationship with parasite specific IFN- γ and antibody response

The immune response during *Leishmania* infection plays a pivotal role in determining the outcome of the disease in dogs. There is increasing evidence demonstrating the significant role of the innate immune response in controlling *Leishmania* infection [11,12]. Among the immune cells involved, neutrophils have been identified as important contributors to both the parasite clearance and immunopathology [11,13–15]. Neutrophils are the first cells to migrate to the site of infection and take up the *Leishmania* promastigote, acting as the first line of defense [16–19]. Once phagocytosed, neutrophils activate their defense mechanisms, including the production of reactive oxygen species (ROS), which is believed to be a crucial antileishmanial mechanism in phagocytes (known as the oxidative burst) [20–24]. It is theorized that if the oxidative burst is successful and limits parasite growth within cells, the infection could be controlled [25,26]. Hence, the ability or predisposition of neutrophils to activate the oxidative burst could significantly impact disease progression or containment. However, their role during different states of canine *L. infantum* infection remains poorly understood.

The second study aimed to bridge this knowledge gap by characterizing neutrophil oxidative metabolism by means of nitroblue tetrazolium reduction test during distinct states of canine *L. infantum* infection. By analyzing blood samples from dogs at different states of infection, the study elucidated the specific role of neutrophils and their oxidative metabolism in infection and disease progression and immune regulation. Furthermore, the article investigated the relationship between neutrophil responses and parasite specific IFN-γ, a critical cytokine involved in the immune response against *Leishmania* infection. While previous research has primarily focused on IFN-γ's influence on adaptive immunity, recent studies have shed light on its ability to modulate the activity of myeloid cells, including neutrophils, in various ways [27,28]. Considering these findings, it is possible that IFN-γ may have an impact on neutrophils' ability to eliminate *Leishmania* amastigotes and promastigotes they have engulfed. This comprehensive characterization provided essential insights into the pathogenesis of CanL and contribute to identifying potential immunomodulatory targets for therapeutic interventions.

Study 3: Pilot study for a *Leishmania infantum* candidate vaccine

Leishmaniosis is a zoonotic disease with a worldwide distribution that is considered endemic in many countries like Brazil, India, Spain or France[29,30]. It is considered a serious public and animal health problem [31]. Available treatments are not always effective and are associated to toxicity, in addition,

an increase in treatment resistance has been reported [6,32–35]. Prevention measures play a crucial role in the epidemiologic management of the infection and disease; however, the currently available tools are deemed insufficient. Therefore, the development of an effective vaccine is of paramount importance to address this issue effectively [36]. Despite significant efforts to develop an effective vaccine against CanL, the currently available vaccines have shown limited efficacy [37].

The third study in this thesis focused on a pilot study for a promising novel vaccine formulation. The primary objectives of the pilot study were to evaluate the immunogenicity and safety of a novel vaccine formulation in a controlled setting. A cohort of dogs with no prior exposure to *Leishmania* were immunized, and their immune responses were closely monitored. Additionally, the study assessed the safety profile of the vaccine by monitoring adverse effects and potential vaccine-related complications. The findings from this pilot study serve as a critical steppingstone for further vaccine development and optimization, potentially leading to a more efficacious and safer vaccine against CanL.

In conclusion, this PhD thesis aimed to significantly advance the management and understanding of CanL. By addressing the lack of clear treatment recommendations for papular dermatitis, characterizing neutrophil oxidative metabolism, and investigating a candidate *Leishmania* vaccine, the thesis contributes to the broader knowledge of leishmaniosis treatment, pathogenesis, immune response, and preventive strategies. Ultimately, the outcomes of this research provide valuable insights that can significantly impact the health and well-being of both dogs and humans affected by this zoonotic disease.

References

1. Alvar, J.; Vélez, I.D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; de Boer, M. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS One* **2012**, *7*, :e35671.
2. Solano-Gallego, L.; Mirá, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites and Vectors* **2011**, *4*, 1–16.
3. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular Dermatitis Due to *Leishmania* Spp. Infection in Dogs with Parasite-Specific Cellular Immune Responses. *Vet. Dermatol.* **2005**, *16*, 187–191.
4. Martínez-Flórez I, Guerrero MJ, Dalmau A, Cabré M, Alcover MM, Berenguer D, Good L, Fisa R, Riera C, Ordeix L, Solano-Gallego L. Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis Due to *Leishmania Infantum* in Dogs. *Pathog.* **2023**, *12*, 821.

5. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular Dermatitis Due to *Leishmania Infantum* Infection in Seventeen Dogs: Diagnostic Features, Extent of the Infection and Treatment Outcome. *Parasites and Vectors* **2014**, *7*, 1–11.
6. Morales-Yuste, M.; Martín-Sánchez, J.; Corpas-Lopez, V. Canine Leishmaniasis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. *Vet. Sci.* **2022**, *9*, :387.
7. Noli, C.; Saridomichelakis, M.N. An Update on the Diagnosis and Treatment of Canine Leishmaniosis Caused by *Leishmania Infantum* (Syn. L. Chagasi). *Vet. J.* **2014**, *202*, 425–435.
8. Piragauta, S.P.; Higuita-Castro, J L; Arbeláez, N; Restrepo, A M; Archbold, R; Quiñones, W; Torres, F; Echeverri, F; Escobar, G; Vélez, I D; et al. Utility of the Combination of Hederagenin Glucoside Saponins and Chromane Hydrazone in the Topical Treatment of Canine Cutaneous Leishmaniasis. An Observational Study. *Parasitol. Res.* **2022**, *121(5), 1419–1428*.
9. Lago, J.; Fraga, D.; Guimarães, L.H.; Lago, T.; Santos, Y.; Lago, E.; Werneck, G.L.; Bacellar, O.; Carvalho, E.M. Efficacy of Intralesional Meglumine Antimoniate in the Treatment of Canine Tegumentary Leishmaniasis: A Randomized Controlled Trial. *PLoS Negl. Trop. Dis.* **2023**, *17*, e0011064.
10. Barbosa Santos, E.G.O.; Marzochi, M.C.A.; Conceição, N.F.; Brito, C.M.M.; Barroso, J.A.; Pacheco, R.S. N-Methylglucamine Antimonate (SbV+): Intralesional Canine Tegumentary Leishmaniasis Therapy. *Parasite* **1998**, *5*, 175–180.
11. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, *64*, 38–43.
12. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, *144*, 95–115.
13. Baneth, G.; Solano-Gallego, L. Leishmaniasis. *Vet. Clin. North Am. - Small Anim. Pract.* **2022**, *52*, 1359–1375.
14. Brandonisio, O.; Panunzio, M.; Faliero, S.M.; Ceci, L.; Fasanella, A.; Puccini, V. Evaluation of Polymorphonuclear Cell and Monocyte Functions in *Leishmania Infantum*-Infected Dogs. *Vet. Immunol. Immunopathol.* **1996**, *53*, 95–103.
15. Panaro MA, Puccini V, Faliero SM, Marzio R, Marangi A, Lisi S, Brandonisio O. *Leishmania donovani* lipophosphoglycan (LPG) inhibits respiratory burst and chemotaxis of dog phagocytes. *New Microbiol.* **1996**, *19,* 107-12.
16. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, *173*, 6521–6525.

17. Mantovani, A.; Cassatella, M.A.; Costantini, C.; Jaillon, S. Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity. *Nat. Rev. Immunol.* **2011**, *11*, 519–531.

18. Nauseef, W.M.; Borregaard, N. Neutrophils at Work. *Nat. Immunol.* **2014**, *15*, 602–611.

19. Mócsai, A. Diverse Novel Functions of Neutrophils in Immunity, Inflammation, and Beyond. *J. Exp. Med.* **2013**, *210*, 1289–1299.

20. Roos, D.; Van Bruggen, R.; Meischl, C. Oxidative Killing of Microbes by Neutrophils. *Microbes Infect.* **2003**, *5*, 1307–1315.

21. Nauseef, W.M. How Human Neutrophils Kill and Degrade Microbes: An Integrated View. *Immunol. Rev.* **2007**, *219*, 88–102.

22. Hampton, M. B., Kettle, A. J., & Winterbourn, C. C. Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. *Blood,* **1998,** *92(9),* 3007–3017.

23. Underhill, D.M.; Ozinsky, A. Phagocytosis of Microbes: Complexity in Action. *Annu. Rev. Immunol.* **2002**, *20*, 825–852.

24. Winterbourn, C.; Kettle, A.; Hampton, M. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **2016**, *85,* 765–792.

25. Kaye, P.; Scott, P. Leishmaniasis: Complexity at the Host-Pathogen Interface. *Nat. Rev. Microbiol.* **2011**, *9*, 604–615.

26. Terrazas, C.; Oghumu, S.; Varikuti, S.; Martinez-Saucedo, D.; Beverley, S.M.; Satoskar, A.R. Uncovering *Leishmania*-Macrophage Interplay Using Imaging Flow Cytometry. *J. Immunol. Methods* **2015**, *423*, 93–98.

27. Ellis, T.N.; Beaman, B.L. Interferon-Gamma Activation of Polymorphonuclear Neutrophil Function. *Immunology* **2004**, *112*, 2–12.

28. Ellison, M.A.; Gearheart, C.M.; Porter, C.C.; Ambruso, D.R. IFN-γ Alters the Expression of Diverse Immunity Related Genes in a Cell Culture Model Designed to Represent Maturing Neutrophils. *PLoS One* **2017**, *12(10),* e0185956.

29. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends Parasitol.* **2008**, *24*, 324–330.

30. Abdellahi, L.; Iraji, F.; Mahmoudabadi, A.; Hejazi, S.H. Vaccination in Leishmaniasis: A Review Article. *Iran. Biomed. J.* **2022**, *26*, 1–35.

31. Sevá, A. da P.; Mao, L.; Galvis-Ovallos, F.; Tucker Lima, J.M.; Valle, D. Risk Analysis and Prediction of Visceral Leishmaniasis Dispersion in São Paulo State, Brazil. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0005353.

32. Yasur-Landau, D.; Jaffe, C.L.; David, L.; Baneth, G. Allopurinol Resistance in *Leishmania Infantum* from Dogs with Disease Relapse. *PLoS Negl. Trop. Dis.* **2016**, *10*, 1–13.

33. Gonçalves, G.; Campos, M.P.; Gonçalves, A.S.; Medeiros, L.C.S.; Figueiredo, F.B. Increased *Leishmania Infantum* Resistance to Miltefosine and Amphotericin B after Treatment of a Dog with Miltefosine and Allopurinol. *Parasites and Vectors* **2021**, *14*, 1–8.

34. Mateo, M.; Maynard, L.; Vischer, C.; Bianciardi, P.; Miró, G. Comparative Study on the Short Term Efficacy and Adverse Effects of Miltefosine and Meglumine Antimoniate in Dogs with Natural Leishmaniosis. *Parasitol. Res.* **2009**, *105*, 155–162.

35. Solano-Gallego, L.; Koutinas, A.; Miró, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. Directions for the Diagnosis, Clinical Staging, Treatment and Prevention of Canine Leishmaniosis. *Vet. Parasitol.* **2009**, *165*, 1–18.

36. Wylie, C.E.; Carbonell-Antoñanzas, M.; Aiassa, E.; Dhollander, S.; Zagmutt, F.J.; Brodbelt, D.C.; Solano-Gallego, L. A Systematic Review of the Efficacy of Prophylactic Control Measures for Naturally-Occurring Canine Leishmaniosis, Part I: Vaccinations. *Prev. Vet. Med.* **2014**, *117*, 7–18.

37. Velez, R.; Gállego, M. Commercially Approved Vaccines for Canine Leishmaniosis: A Review of Available Data on Their Safety and Efficacy. *Trop. Med. Int. Health* **2020**, *25*, 540–557.

Introduction

General Introduction

Leishmania is a genus of parasitic protozoan organisms that cause a spectrum of diseases collectively known as leishmaniosis, ranging from mild cutaneous lesions to severe systemic clinical signs [1,2]. This neglected vector-borne disease has a worldwide distribution and is transmitted through the bites of infected female sandflies from the genus *Phlebotomus* and *Lutzomyia*, which serve as their vector [3,4]. More than 50 distinct species have been identified within *Leishmania* genus, and at least 21 are pathogenic to humans [5]. Although this parasite has been described to infect multiple different mammalian hosts such as humans, dog is considered as the main reservoir host, with a seroprevalence in endemic areas that ranges from 3% to 30% [6,7]. Leishmaniosis has been described to be caused by various *Leishmania* species, with at least 13 reported to infect dogs globally [8,9]. However, among these species, *Leishmania infantum* emerges as the most significant etiological agent [8]. As a result, the focus of this PhD thesis will be directed towards this species.

Additionally, leishmaniosis is considered a zoonotic disease that affects millions of people mainly in developing countries from regions with tropical and subtropical climates, in which it is considered a major public health problem with significant impacts on public health and socioeconomic development [10].

Lifecycle

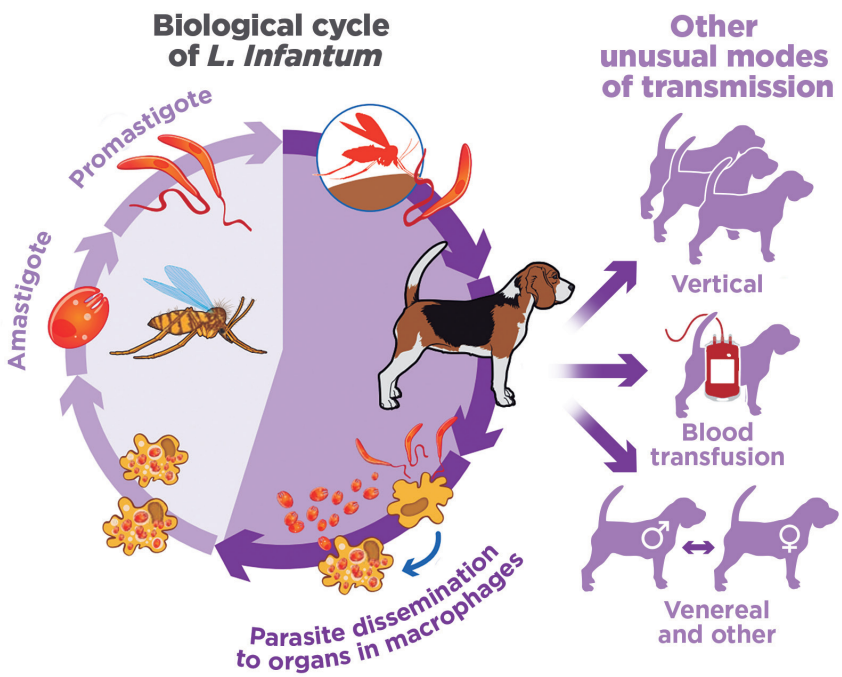


Figure 1. **Biological cycle of *L. infantum* and its transmission routes.**
Adapted from Solano-Gallego et al. 2011 [1].

Leishmania spp. has a digenetic biological cycle that includes a metacyclic promastigote phase and an amastigote phase [11]. The first is an extracellular, flagellated, and motile form that infects the phlebotomine (sand fly) gastrointestinal tract. When the sand fly bites the vertebrate host, it injects the promastigotes under its skin **(Figure 1)**. There, phagocytes such as neutrophils, dendritic cells and monocytes engulf the parasite [12]. Neutrophils act as a "Trojan horse" allowing the promastigote to survive and infect macrophages where it differentiates into the amastigote phase [13]. Through binary division, amastigotes replicate, leading to the enlargement of the host cell and its eventual rupture, facilitating the infection of other mononuclear phagocytic cells by the released amastigotes. Some of these amastigotes are circulating in the blood stream where they can be ingested by a sand fly when it feeds from the host, thus initiating a cycle of repetition [11].

In dogs, other non-vectorial transmission routes have been confirmed, such as venereal transmission [14], transplacental transmission [15], and transmission through blood transfusion [16]. Additionally, a case of transmission by biting has been reported in dogs [17].

As mentioned above, *L. infantum* can infect other mammals, either domestic such as cat [18,19] and equine [20] or wild mammals as wolf [21], fox [22], Mediterranean monk seal [23], among others which could potentially act as reservoirs [24].

Several risk factors associated with *L. infantum* infection have been documented in dogs [25]. For example, the risk of seropositivity increases as dog's age, likely due to repeated exposure to *L. infantum* infection [25–28]. Interestingly, there is a bimodal age distribution, in young dogs (under two years) and another in older dogs (over eight years) [25]. On the other hand, sex might be another risk factor, since male dogs have a higher risk of *L. infantum* exposure compared to females [27,29], though some studies contradict this finding and find no sex differences [30,31]. Moreover, different dog breeds show varied susceptibility to leishmaniosis, with purebred dogs more likely to develop clinical disease [25,32], while mixed-breed dogs experience clinical leishmaniasis less frequently [32,33]. However, certain breeds, like the Ibizan Hound, rarely suffer from clinical leishmaniosis due to their strong parasite specific cellular immune response [33]. Environmental factors also play a critical role considering that dogs living indoors have a lower risk of infection compared to those in kennels or shelters [25]. Moreover, coinfections with other pathogens contribute to the development of clinical disease in dogs finding that dogs with seropositivity to other tick-borne diseases were 1.7 times more likely to be seropositive to *L. infantum* and 11 times more likely to progress to clinical leishmaniosis if infected with three or more tick-borne diseases [34].

Once infection occurs, a wide range of clinical manifestation can be observed from subclinical infection to very severe disease which is highly dependent on the immune response of dogs [1].

Immunity

Leishmania is an obligate intracellular parasite that primarily resides within myeloid lineage cells, including monocytes, macrophages, dendritic cells, and neutrophils [35]. The interaction between this protozoal parasite and the host's immune system is complex and leads to a diverse range of clinical manifestations, with only a small fraction of infected individuals progressing to the disease [1]. This interaction plays a pivotal role not only in the dog's ability to combat the infection and prevent its progression to clinical stages but also in the pathogenicity of the disease [11,35–37]. Thus, the manifestations of leishmaniosis are greatly influenced by the host's immune response, which is highly complex, still largely unknown, and is determined by the dog's genetics and various acquired factors [11,35].

Both the innate and adaptive immune responses, as well as their interaction, have been identified as critical factors in disease development [11,35]. However, innate immune responses in canine leishmaniosis (CanL) have been less extensively studied when compared with adaptive immune responses. The elements of innate immunity consist of a collection of disease-resistant mechanisms that are not tailored to specific pathogens but possess molecular and cellular components capable of recognizing classes of molecules commonly found in pathogens [38]. Cells involved in innate immunity, like dendritic cells and macrophages, directly combat pathogens through processes like phagocytosis. They also trigger the production of cytokines, which aid in the elimination of pathogens. Furthermore, the innate immune response plays a crucial role in guiding the development of durable, pathogen-specific adaptive immune responses [38]. Neutrophils and macrophages play particularly significant roles in the initial ability of dog's immune system to control infection. Following inoculation, *Leishmania* parasites undergo a transformation from the promastigote form, which is engulfed by phagocytic cells like neutrophils and macrophages, to intracellular amastigotes [39,40]. Neutrophils are the first cells to encounter the parasite, internalizing it and acting as a vehicle to evade the immune system and reach their final destination, the macrophages [41–43]. Both neutrophils and macrophages can phagocytize the parasite, which can lead either to its elimination or contribute to its survival, resulting in parasite persistence and dissemination within the host. Thus, disease progression depends on the ability of these cells to eliminate the parasite versus the ability of the amastigotes to evade the cell's killing mechanisms [43–45]. If these cells fail to destroy the parasite, amastigotes can multiply within them until the cell ruptures, releasing

the parasites into the bloodstream or other interstitial spaces to infect other cells. Conversely, if the cells are capable of killing the parasite, its growth can be controlled, leading to a controlled infection [46,47].

Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) play a crucial role in regulating the activation of Th1 or Th2 immunity [38,48]. These receptors recognize lipophosphoglycan molecules present on the surface of the flagellated promastigote form of the protozoan parasite, which act as pathogen-associated molecular patterns (PAMPs) [48]. TLRs are found on the membrane or intracellular compartments of various cell types, including epithelial cells, endothelial cells, fibroblasts, and immune cells such as T and B lymphocytes, macrophages, dendritic cells, and natural killer cells [49]. Upon binding to their specific ligands, TLRs initiate a series of immune responses, including immune cell maturation, phagocytosis, microbicidal activity of phagosomes, and immune activation through various events such as the induction of inflammatory cytokines like tumor necrosis factor-alfa (TNF-α) and IFN-γ [38,50–52]. These processes contribute to the recognition and elimination of pathogens and the subsequent activation of the immune system. Ten TLRs have been described in dogs. It is demonstrated that TLR2, TLR3, TLR4, TLR7 and TLR9 play a role in the infection due to *L. infantum* and in the dissemination of the disease [11]. It seems that TLR2 has a protective role against infection by amplifying the synthesis of the inflammatory cytokines TNF-α and interleukin-6 (IL-6) in sick, "resistant" dogs and non-infected healthy dogs [53]. However, it is associated with the pathogenesis in cutaneous lesions of CanL [48] and it has been demonstrated that the use of the protein aggregate of magnesium ammoniumphospholinoleate palmitoleate anhydride (P-MAPA) which is a TLR agonist (TLRa) in sick dogs improved clinical signs, it was associated with a reduction in the parasite load on the skin, elevated IFN-γ concentration, and decreased interleukin-10 (IL-10) production. [54]

Regarding the TLR3 and TLR7, they also have a protective function of recognition and control of *Leishmania* infection by the production of cytokines. However, TLR7 showed a stronger response against *Leishmania* infection than TLR3 [55–57].

TLR4 plays a role in regulating the growth of *Leishmania* through both innate and adaptive immune responses. Moreover, it acts as a potent regulator of inducible nitric oxide synthase (iNOS), resulting in the death of the parasite [58].

Studies revealed that TLR9 has a crucial function in attracting neutrophils as part of the defensive reaction against *L. infantum* infection, possibly linked to the activation of dendritic cells [59][11].

The adaptive T-cell immunity has been extensively studied and recognized for its critical role in both disease development and resistance. The immune response to *L. infantum* infection in dogs involves a complex interplay of T cell subpopulations and their associated cytokines. In this complex interaction, it has been consistently observed that a predominantly cellular immune response is associated with disease resistance or subclinical forms, whereas a predominant humoral response is linked to clinical illness [3,60]. A Th1-mediated immune response, characterized by the release of cytokines such as IFN-γ, interleukin-2 (IL-2), and TNF-α, has been specifically linked to a protective cell-mediated response [11,61]. IFN-γ, in particular, plays a significant role as it stimulates activated macrophages to produce nitric oxide and reactive oxygen species (ROS), contributing to the elimination of amastigotes and control of *L. infantum* infection [62,63] **(Figure 2)**.

Interleukin-17a (IL-17) could also play an important role, as it has been shown to be implicated in the management of intracellular parasite infections [64]. However, its role in canine leishmaniosis is still a topic of debate. While it has been associated with protection against visceral leishmaniosis and restricting parasite growth in dogs and humans, it may also have an inflammatory function in cutaneous and mucosal forms in humans [65–69].

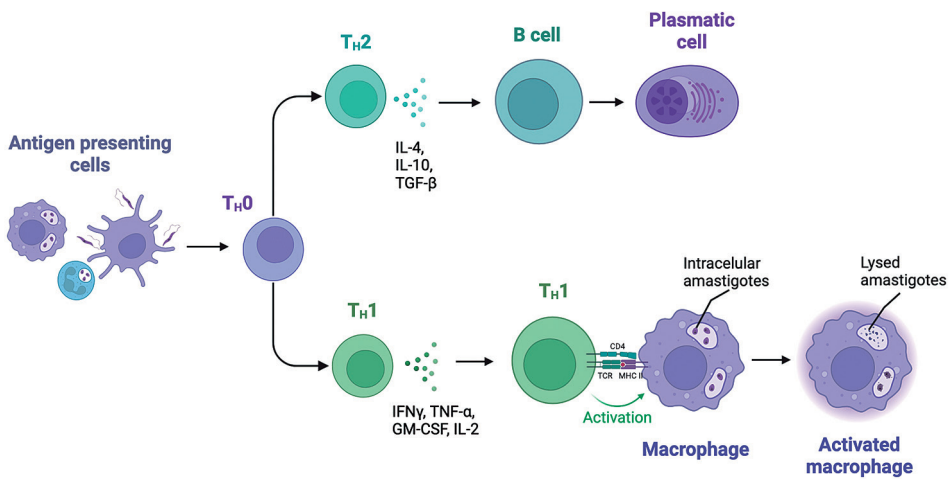


Figure 2. Interaction between the parasite and the host's immune system.
GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor; **IFN-γ**, Interferon-gamma; **IL-4**, Interleukin-4; **IL10**, Interleukin-10; **TGF- β**, transforming growth factor-beta; **TNF- α**, tumor necrosis factor-alfa.

Conversely, a Th2-mediated immune response results in the production of non-protective cytokines, including interleukin-4 (IL-4), (IL-10), and transforming growth factor-beta (TGF-β). These cytokines are associated with a predominantly humoral immune response and disease progression [11,61] **(Figure 2)**. Maintaining a balanced interaction between proinflammatory Th1

CD4+ T cells, which aim to inhibit parasite replication, and immunosuppressive regulatory response mediated by T regulatory 1 cells, which aim to prevent immune overreaction, is crucial for disease control [35].

The immune response requires a balance between inflammatory and regulatory responses to control *L. infantum* infection [35]. Prolonged antigen exposure can lead to immune disbalance resulting in dysfunction [62]. Dogs in a subclinical state and during early stages of infection exhibit a strong immune response characterized by CD4+ T cell proliferation upon exposure to parasite antigen, effectively combating intracellular pathogens through IFN-γ production and macrophage activation [35,70,71]. To mitigate potential adverse effects of these proinflammatory cytokines, a minimal amount of IL-10 is produced, often at levels that are difficult to detect [72,73]. However, as leishmaniosis progresses, the immune system gradually loses its ability to maintain the delicate balance of inflammation without resulting in pathological damage [62,72]. At some point, a switch occurs in the T cell response from a protective Th1 response to a regulatory response. This transition to a regulatory response is accompanied by an increased proliferation of regulatory T cells and the production of regulatory cytokines, particularly IL-10 [74]. This switch is driven by changes in cytokine expression and prolonged antigen exposure. An increased IL-10 production reduces the proliferation of protective Th1 CD4+ T cells, resulting in decreased IFN-γ production and impaired macrophage activation [36,75,76]. This shift in the immune response, combined with increased humoral responses, leads to T cell exhaustion and unrestricted parasite growth [75,77].

T cell exhaustion refers to the dysfunction of T cells resulting from prolonged exposure to antigens, and it is characterized by the heightened expression of inhibitory receptors such as programmed death-1 and cytotoxic T-lymphocyte-associated antigen 4 [77,78]. In the case of CanL, there is evidence supporting the occurrence of T cell exhaustion [62]. CD4+ and CD8+ T cells derived from dogs displaying clinical signs of leishmaniosis exhibit elevated levels of programmed cell death protein-1 (PD-1) expression, reduced proliferation, and impaired functionality, which can include diminished production of IFN-γ [62,75].

B cells also play a key role in the progression of CanL and its pathogenesis. They induce a humoral immune response and act as antigen presenting cells modulating the activation of CD4+ T cells, which in turn activate B cells to produce antibodies [35]. As disease progresses, the production of IgG antibodies increases and bind to *Leishmania* antigen creating immune complexes (type III hypersensitivity reaction) **(Figure 3)**. This process reflects as hypergammaglobulinemia [79]. When deposited in specific tissues, these immune complexes provoke vasculitis and tissue damage in particular organs such as the kidneys, eyes, and skin [80–82] **(Figure 3)**.

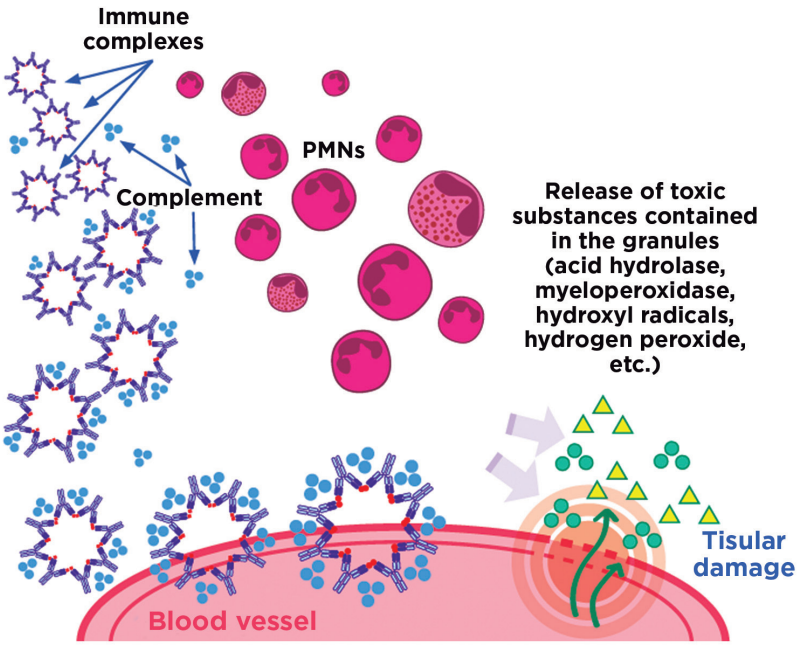


Figure 3. **Deposition of immune complexes in *L. infantum* infection.**
Adapted from Solano-Gallego, 2023 [91].

Glomerulonephritis, vasculitis, uveitis, myositis, or polyarthritis are typical lesions of clinical leishmaniosis in severe stages that can be induced by immunocomplexes formation [81,82]. Immune complexes also induce the production of IL-10 by macrophages, which constitutes a negative feedback loop over the macrophages, reducing its activity and thus, allowing parasite proliferation [83]. On the other hand, a regulatory B cell population also increases IL-10 production and down regulates the Th1 response allowing parasite growth [84,85]. Excessive and continuous stimulation of antibodies produced by B lymphocytes can also trigger type II hypersensitivity reactions associated with complement-mediated antibody-antigen interactions [81,82]. Other inflammatory components, like neutrophils and Th17 cells, also contribute to the inflammatory state, inducing parasite elimination but also leading to pathology [35]. All these immune challenges affect the balance between progression to clinical disease and maintaining a subclinical infection.

Disease and clinical staging

As previously mentioned, when a dog acquires the infection due to *L. infantum*, a wide spectrum of clinical manifestations can be observed. Because of the interaction between the parasite and the host's immune system, the *L. infantum* infection presentation varies from a subclinical infection with no clinical signs nor laboratory abnormalities to a very severe fatal disease [1].

Given the parasite's capability to affect various tissues, organs, or systems, it can give rise to a multitude clinical signs and laboratory abnormalities **(Table 1)**.

Table 1. Clinical signs and laboratory abnormalities found in CanL due to *L. infantum*.
Modified from Solano-Gallego et al., 2011 [1].

Clinical signs

General

- Generalized lymphadenomegaly
- Loss of body weight
- Decreased or increased appetite
- Lethargy
- Mucous membranes pallor
- Splenomegaly, hepatomegaly
- Polyuria and polydipsia
- Fever
- Vomiting
- Diarrhea (small and large bowel inflammation)

Cutaneous

- Non-pruritic exfoliative dermatitis with or without alopecia
- Erosive-ulcerative dermatitis
- Nodular dermatitis
- Papular dermatitis
- Pustular dermatitis
- Onychogryphosis

Ocular

- Blepharitis (exfoliative, ulcerative, or nodular) and conjunctivitis (nodular)
- Keratoconjunctivitis, either common or sicca
- Anterior uveitis/ endophtalmitis

Other

- Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital, and nasal)
- Epistaxis
- Lameness (erosive or non-erosive polyarthritis, osteomyelitis, polymyositis)
- Atrophic masticatory myositis
- Vascular disorders (systemic vasculitis, arterial thromboembolism)
- Neurological disorders
- Respiratory disorders

Laboratory abnormalities

CBC/Hemostasis

- Mild to moderate non-regenerative anemia
- Leukocytosis or leukopenia
- Thrombocytopathy
- Thrombocytopenia
- Impaired secondary hemostasis and fibrinolysis

Biochemical profile
Serum protein electrophoresis
Urinalysis

- Hyperproteinemia
- Hyperglobulinemia (polyclonal beta and/or gammaglobulinemia)
- Hypoalbuminemia
- Decreased A/G ratio
- Renal azotemia
- Elevated liver enzyme activities
- Proteinuria

According to LeishVet guidelines, staging is conducted in canine patients diagnosed with *L. infantum* infection, based on clinical signs and/or clinicopathological abnormalities, in order to facilitate the implementation of appropriate treatment and monitoring strategies for the patient's well-being [82] **(Table 2)**.

Table 2. Clinical staging of CanL based on serological status, clinical signs, laboratory findings, and prognosis. Modified from Solano-Gallego et al., 2017 [82].

Clinical stages	Clinical signs	Laboratory findings*	Quantitative serology**	Prognosis
Stage I (mild disease)	Mild clinical signs (papular dermatitis or localized lymphadenomegaly)	No clinicopathological abnormalities observed. Normal renal profile: creatinine < 1.4 mg/dL, non-proteinuric: UPC< 0.5	Negative to low positive antibody levels	Good
Stage II (moderate disease)	Diffuse or symmetrical cutaneous lesions (exfoliative dermatitis, onychogryphosis, ulcerations in planum nasale, ears, footpads, bony prominences or mucocutaneous junctions, generalized lymphadeno-megaly, loss of appetite and weight loss)	Clinicopathological abnormalities compatible with *L. infantum* infection (mild non-regenerative anemia, ergammaglobulinemia and hypoalbuminemia) **Substage** a) Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 b) Proteinuric: creatinine < 1.4 mg/dL; UPC= 0.5-1	Low to high positive antibody levels	Good to guarded
Stage III (severe disease)	Dogs, which apart from the signs listed in stages I and II, may present signs originating due to immune-complex deposition (e.g., glomerulonephritis, uveitis)	Clinicopathological abnormalities listed in Stage II. CKD IRIS stage 1 with proteinuria UPC > 1 or CKD IRIS stage 2 (creatinine 1.4-2.8 mg/dl)	Medium to high positive antibody levels	Guarded to poor
Stage IV (very severe disease)	Dogs with clinical signs listed in Stage III. Pulmonary thromboembolism, or nephrotic syndrome and end stage renal disease	Clinicopathological abnormalities listed in stages II and III. CKD IRIS stage 3 (creatinine 2.9-5 mg/dl) and CKD IRIS stage 4 (creatinine > 5 mg/dl) or Nephrotic syndrome or marked proteinuria UPC > 5	Medium to high positive antibody levels	Poor

CKD, Chronic Kidney Disease; **IRIS,** International Renal Interest Society; **UPC,** urine protein:creatine ratio.

Papular dermatitis

Papular dermatitis is a common cutaneous sign and is considered a typical presentation in endemic areas. This type of skin manifestation is linked to LeishVet stage I, which corresponds to mild leishmaniosis [1,86]. The presence of an efficient Th1-based parasite-specific cellular immunity and a limited humoral immune response characterizes this cutaneous form. Consequently, laboratory abnormalities are absent, and there is a lack of systemic clinical signs. Although self-healing of the skin lesions is possible, the process generally spans a period of three to six months [87].

Regarding the treatment, papular dermatitis has been scientifically neglected [1]. In dogs classified as LeishVet stage I, there have been instances of short-term treatment involving one or two conventional anti-*Leishmania* drugs (like meglumine antimoniate, miltefosine, and/or allopurinol), alongside immune-potentiating treatments (such as domperidone or nucleotides plus active hexose correlated compound (AHCC)), either individually or combined with the previously mentioned drugs [1,88]. Alternatively, in certain cases, clinical monitoring without treatment might be considered. Limited evidence exists regarding treatment outcomes for dogs in this stage, resulting in the unknown clinical effectiveness of these treatment options [89].

Interestingly, local treatment as administered in cutaneous leishmaniosis in humans [90], has not been explored in the context of LeishVet stage I papular dermatitis caused by canine *L. infantum* infection.

Treatment

Conventional treatment

The recommended dosage, route of administration, adverse effects, and mechanism of action of conventional anti-*Leishmania* drugs in dogs are detailed in **Table 3** [91].

Pentavalent antimonials

Pentavalent antimonials (Sb(V)) are considered first-line drugs for the treatment of canine and human leishmaniosis in many countries [1]. Currently, there are mainly two alternatives: meglumine antimoniate and sodium stibogluconate. Meglumine antimoniate is the most used in dogs and has more studies and reported cases available [92]. It is also recommended as the first-choice drug for the treatment of leishmaniosis in humans by the World Health Organization (WHO). However, its use is associated with certain limitations in both humans and dogs, including the risk of significant adverse effects, the need for daily parenteral administration, and the increasing development of parasite resistance [92]. The effectiveness of Sb(V) has been widely proven, and multiple studies

Table 3. Most commonly used drugs in conventional anti-*Leishmania* treatment (mechanism of action, dosage, side effects, and relapse time). Modified from Solano-Gallego et al., 2023 [77] and Miró et al., 2017 [92].

Active Ingredient	Mechanisms of action	Dosage	Side effects	Relapse time	Refs.
Meglumine antimoniate	Blocking the parasite's energy metabolism through the inhibition of the phosphofructokinase enzyme Prodrug that converts into trivalent antimonial with increased activity and toxicity Promutagenic agent that induces toxicity	100 mg/kg/24 h or 50 mg/kg/12 h S.C. for 4-6 weeks	Gastrointestinal (vomiting, diarrhea, anorexia) * Local reactions at the injection site Musculoskeletal disorders (joint pain and muscle pain) Possible renal and hepatic damage Non-specific: lethargy, weakness, apathy, weight loss, fever, persistent cough	6-12 months	[92, 93, 103, 109, 110]
Miltefosine	Impairment of signaling pathways and synthesis of the cell membrane Immunomodulatory effects (release of interferon-gamma and Th1 response)	2 mg/kg/24 h P.O. for 28 days 1.2 mg/kg/24 h P.O. for 5 consecutive days followed by a dose of 2.5 mg/kg for 25 days P.O.	Gastrointestinal (dysorexia, vomiting, and diarrhea) ** Teratogenic, embryotoxic, and fetotoxic effects ***	4-6 months	[1, 122, 126, 129]
Allopurinol	Interference with the purine pathway, leading to the disruption of protein translation and selective parasite death	10 mg/kg/12 h P.O. for 6-24 months (some cases require longer treatments based on the clinical stage and tolerance)	Urolithiasis, nephrolithiasis, and/or mineralization of the renal pelvis (xanthinuria) * Mild gastrointestinal symptoms (nausea, vomiting, diarrhea) Elevation of liver enzymes (AST, ALT, and ALP)	Insufficient information	[1, 133, 134]

ALT, alanine aminotransferase; **ALP,** alkaline phosphatase; **AST,** aspartate aminotransferase; **P.O.,** per oral; **S.C.,** subcutaneous administration. *Most common side effects; **if administered with food, the risk of these effects is significantly reduced (Solano-Gallego et al., 2011)[1]. ***Its use is contraindicated during pregnancy and lactation (Miró et al., 2009) [129].

suggest the existence of different mechanisms of action **(Table 3)**. There are multiple studies that, collectively, suggest the existence of different mechanisms of action, either as an active principle or by acting as a pro-drug that converts into a trivalent antimonial, which seems to exhibit higher activity and toxicity [93,94].

As an active principle, it has been theorized that Sb(V) has the ability to disrupt the energy metabolism of *Leishmania* amastigotes by forming stable complexes with ribonucleosides, interfering with the parasite's β-oxidation and glycolysis processes, and leading to a depletion of adenosine triphosphate (ATP) levels. Another hypothesis suggests that Sb(V) acts as a pro-drug that transforms into its more toxic trivalent form, Sb (III), which is considered an intermediate (or borderline) metal ion with high affinity for ligands containing nitrogen or sulfhydryl groups [93–95]. Some studies even propose that the *in vivo* transformation to Sb (III) is responsible for both its therapeutic activity and its toxicity [96–98]. One of its anti-*Leishmania* mechanisms is probably related to its interaction with biomolecules containing sulfhydryl groups, including thiols, peptides, proteins, and enzymes [93,99–102]. Additionally, Sb (III) could act as a pro-mutagen *in vivo*, requiring its transformation to exert an effect [103]. One of the described mutagenic mechanisms might be linked to the ability of Sb (III) to modify the redox potential of cells, compromising the activity of antioxidant defense mechanisms like glutathione peroxidase [95,99,103,104]. Thus, antimonial compounds elevate levels of reactive oxygen and nitrogen species, leading to toxicity [105–107]. These same mechanisms could be related to patient harm and the described adverse effects [103]. The toxicity of antimonials is mainly attributed to their binding to sulfhydryl groups, altering the tertiary structure of proteins and disrupting the activity of various enzymes [108].

The described adverse effects can be classified into two major groups: signs of intolerance and signs of intoxication **(Table 3)**. The former usually appear at the beginning of treatment and are not dose-dependent (changes in temperature, arthralgia, vomiting, dry cough, and injection site lesions). The latter can occur even after discontinuing treatment and can affect the heart, liver, kidney, pancreas, and/or the circulatory system. Historically, meglumine antimoniate treatment has been associated with kidney damage. Currently, the most accepted hypothesis is that the main cause of kidney damage during meglumine antimoniate treatment is the deposition of antibody complexes at the glomeruli due to parasite death, although the exact impact of tubular toxicity caused by antimonials is unknown [109–111]. There are multiple studies that evaluate renal biomarkers in dogs treated with meglumine antimoniate. Pardo-Martín et al. (2017) described that there were no changes in symmetric dimethylarginine (SDMA) or tubular damage markers (uGGT/UC) in response

to the administration of meglumine antimoniate combined with allopurinol. However, later, Paltrinieri et al. (2018) did describe a reduction in uGGT/UC [112,113]. On the other hand, Bianciardi et al. (2009) analyzed the effect of meglumine antimoniate treatment on the kidneys of healthy dogs by taking biopsies just before and after 27 days of treatment. They observed significant tubular damage after treatment, although no clinical signs of renal disease appeared in any subjects [114]. Finally, in a study published in 2019 conducted on dogs with *Leishmania*, no alteration in the glomerular filtration rate or urinary density was observed when administering meglumine antimoniate [108]. Although there is still much controversy, currently the most accepted hypothesis is that the main cause of renal damage during leishmaniosis treatment is the deposition of antibody complexes at the glomerular level, although the exact impact of tubular toxicity by antimonials is not known with certainty [115]. Different administration routes have been described, although intramuscular and subcutaneous routes have a longer presence in the body than the intravenous route, maintaining good bioavailability, making them superior [116,117]. Furthermore, Valladares et al., already in 1996, reported that, when comparing the intramuscular route with the subcutaneous route, very similar pharmacokinetic behavior was obtained, making the subcutaneous route preferred due to a lower occurrence of adverse effects (especially related to pain at the injection site) and convenience [117].

Additionally, the main excretion route for meglumine antimoniate is through urine (up to 83% is eliminated via this route within the first 9 hours of administration)[117]. This may be particularly relevant in patients with kidney damage, as drug elimination could be impaired, leading to increased blood concentrations.

Miltefosine and other alkylphospholipids

Miltefosine, along with oleilphosphocholine, belongs to the group of drugs known as alkylphospholipids. These drugs have structural components related to the group of alkyl-lysophospholipids, which are synthetic analogs of lysophosphatidylcholine. Miltefosine is an inhibitor of protein kinase B, which is involved in cell survival [118]. Initially, it was used as a local treatment for cutaneous metastases in breast cancer patients due to its antineoplastic properties. In 1987, Croft et al. first described its anti-*Leishmania* activity *in vitro* and *in vivo* [119].

Miltefosine has been demonstrated to be a safe and effective alternative for the treatment of canine leishmaniosis; however, its mechanism of action has not yet been fully identified [92]. Multiple studies suggest that miltefosine acts through various mechanisms affecting various molecular action points **(Table 3)** [91]. Some studies indicate an association between miltefosine and the

alteration of ether phospholipid metabolism (major components of the parasitic membrane), glycosylphosphatidylinositol synthesis (involved in virulence), and intraparasitic transduction signals [120]. On the other hand, in 2005, it was observed that some strains of *Leishmania* exposed to miltefosine underwent changes in the length and saturation level of fatty acids, indicating that fatty acid metabolism might be a target for the drug [121]. Finally, a study published by Paris et al. suggests that part of the apoptotic machinery is related to protease activity [122]. Furthermore, it has also been reported that miltefosine can exert immunomodulatory effects. It has been observed to induce the release of certain cytokines such as IFN-γ, enhancing macrophage response capability and promoting a Th1 response [123].

Miltefosine is the first effective oral drug described for anti-*Leishmania* treatment. Its use has been described as monotherapy as well as in combination with allopurinol, and some specialists recommend its use in patients with renal disease due to the controversy surrounding meglumine antimoniate and kidney damage. Furthermore, a reduction in proteinuria has been observed in *L. infantum*-infected patients treated with miltefosine and allopurinol [124], similar to what was observed with meglumine antimoniate and allopurinol treatment [125]. In 2020, a study demonstrated that a deviation from the usual miltefosine dosage **(Table 3)** resulted in better tolerance and clinical effectiveness, a reduction in parasite load, and a lower risk of relapse for the patient [126]. Resistance to miltefosine has also been reported in dogs infected with *L. infantum* [124]. Additionally, it has been observed that dogs can continue to infect sandflies weeks after treatment is completed, increasing the transmission of resistant amastigotes [127].

The miltefosine dose and its most commonly described adverse effects are presented in **Table 3**. The most common adverse effects are gastrointestinal, such as anorexia, vomiting, and diarrhea [128]. However, administering miltefosine with food significantly reduces the risk of these effects [1]. Additionally, its use is contraindicated during pregnancy and lactation due to observed teratogenic, embryotoxic, and fetotoxic effects [129].

Allopurinol

Allopurinol is a hypoxanthine analog belonging to the pyrazolopyrimidine family that blocks xanthine oxidase, interrupting purine metabolism [124]. Its anti-*Leishmania* activity was first described in 1974 by Pfaller and Marr [130] and is attributed to the inhibition of the parasite's hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme. HGPRT is involved in the parasite's purine salvage pathway, converting dephosphorylated purines into nucleoside monophosphates. The most accepted theory is that phosphorylated allopurinol is incorporated into nucleic acids, leading to the disruption of

protein translation and the selective death of the parasite [131–133]. Allopurinol is widely used due to its activity against *L. infantum* and its low toxicity, making it ideal for dogs with kidney damage. It is typically used in combination with other drugs such as antimonials and miltefosine for prolonged treatment. However, it can also be used as monotherapy in dogs with milder clinical stages (I or IIa) or in seropositive healthy dogs with high antibody levels [124]. It is widely available worldwide, cost-effective, with infrequent and generally mild adverse effects, and is used for the treatment of human leishmaniosis [133].

The recommended initial dose is shown in **Table 3**, along with the adverse effects of this drug [91]. The duration of allopurinol treatment depends on the severity of the disease (clinical stages), response to treatment, and tolerance to the drug. In some particularly susceptible patients, indefinite treatment may be necessary [115]. The decision of when to discontinue allopurinol treatment should be based on a comprehensive clinical examination (physical examination, complete blood count, biochemistry, and urinalysis) that should show complete physical and clinical-pathological recovery, as well as a quantitative serological test indicating a marked decrease in antibody levels (seroreversion or borderline). Treatment may also be stopped in cases of xanthinuria that does not improve with low-purine diets or by reducing the total daily dose [1]. In patients with xanthinuria, if discontinuing treatment is contraindicated, the administration of a diet based on specific proteins (casein) and increased water consumption can be considered to reduce the risk of urolithiasis [1]. The development of resistance to *L. infantum* treatment is one of the factors that can lead to relapse in patients with leishmaniosis [133]. Yasur-Landau et al. in 2016 demonstrated, for the first time in a clinical study, the emergence of allopurinol resistance in dogs with leishmaniosis and linked it to an increased risk of relapse [133].

Other potential anti-*Leishmania* drugs

Antibiotics

Aminoglycosides: paromomycin

Paromomycin is a broad-spectrum aminoglycoside that inhibits the 30S ribosomal subunit of the parasite [124]. When used as monotherapy, relapses have been reported in 73.8% of cases during the first few months after treatment discontinuation, as well as dose-dependent significant adverse effects such as kidney damage or hearing loss [124]. However, in a study where it was combined with allopurinol, among the 8 dogs that completed the study, no relapse was observed [135]. Regarding the recommended dosage, different clinical studies have described a high percentage of patients with a positive clinical response (86.6-100%) using a subcutaneous dose of 5 mg/kg every 12 hours [124]. On the other hand, using higher doses than the one described did not result in

increased efficacy but did lead to increased adverse effects [136]. However, a more recent study concluded that a dose of 15 mg/kg once daily for 21 days could be beneficial without causing renal damage [137]. Another study compared the combined treatment of paromomycin (15 mg/kg subcutaneously once daily for 28 days) and allopurinol with conventional treatment (meglumine antimoniate and allopurinol) and concluded that although the first treatment was safe and effective, conventional treatment showed a higher percentage of cases in complete remission [135]. Therefore, the use of paromomycin and allopurinol can be considered as a second-line treatment, especially important in countries where meglumine antimoniate is not available or when its use needs to be reduced [135].

It is important to highlight that, similar to what is observed with other drugs, repeated exposure of the parasite to the drug results in the emergence of resistance, leading to a decrease in effectiveness and the potential transmission of the resistant parasite through sandflies [138].

Imidazole derivatives: ketoconazole, metronidazole, and combinations

Imidazoles are antifungal compounds that can have antibacterial and antiprotozoal activity depending on the specific drug. It has been observed that they can induce parasite death by decreasing glycogen reserves through the activation of glycogenolysis after phosphorylase activation. They have also been shown to inhibit nucleic acid synthesis [136].

There are some clinical studies reporting the use of metronidazole and its combination with other antibiotics (metronidazole/espiramycin and metronidazole/enrofloxacin and metronidazole/marbofloxacin) in patients with leishmaniosis, with varying responses, although none of the combinations have proven to be as effective as conventional treatments [124,136]. There is not enough evidence to recommend the use of imidazoles in the treatment of canine leishmaniosis.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are components of the innate immune system in multicellular organisms whose function is to protect against external pathogens. The mechanism of action against *Leishmania* involves disruption of the plasma membrane by altering the composition of phospholipids, leading to increased molecular permeability, binding to intracellular targets, and apoptosis [139].

There is also evidence of modulation of the innate immune response and modification of the adaptive immune response, for example, by modulating gene expression, inhibiting the production of proinflammatory cytokines,

stimulating chemokine expression, promoting wound healing, or inducing changes in dendritic cells [139].

Artemisinin and derivatives

Artemisinin and its derivatives (ART) have been shown to be effective in the treatment of certain protozoan parasites such as *Plasmodium spp.* and *Perkinsus spp.* [140]. Currently, there is very little information regarding their effectiveness in the treatment of leishmaniosis. Some *in vitro* studies have reported anti-*Leishmania* activity of certain artemisinin derivatives against *L. infantum* [141]. Furthermore, their effectiveness has been documented in the treatment of experimentally induced visceral leishmaniosis in mice [142]. In a randomized field study comparing conventional treatment (meglumine antimoniate and allopurinol) with artesunate in dogs with leishmaniosis, it was observed that the artesunate-treated group had a lower clinical grade, lower parasitemia, and a more significant reduction in antibody levels 6 months after starting treatment. Additionally, all dogs tolerated artesunate well, and no related adverse effects were observed [143].

Immunomodulation

The dosage and adverse effects of nucleotides along with domperidone and active hexose compounds, cytokines and toll-like receptors and autovaccines are detailed in **Table 4**.

Table 4. Commonly used immunomodulatory drugs.
Modified from Solano-Gallego et al., 2017 [82].

Active Ingredient	Posology	Side Effects	References
Domperidone	0,5 mg/kg/24 h P.O. for 1 month*	Galactorrhea Gastrointestinal Behavioral	[144,145]
Nucleotides + AHCC	Follow manufacturer's instructions. P.O. for 6-12 months	Gastrointestinal Teratogenic, embryotoxic, and fetotoxic effects	[144,147]
Cytokines and Toll-like receptors	Follow manufacturer's instructions. Parenteral administration	No side effects reported Minor local swelling	[54,144,152]
Autovaccines	Two booster vaccinations 1 ml S.C. followed at 2-week intervals	No side effects reported	[151]

AHCC, Active hexose correlated compound; **P.O.,** per oral; **S.C.,** subcutaneous administration. *Only registered in some European countries for the treatment of mild disease (stage I); however, published clinical studies are very limited and uncontrolled.

Domperidone

Domperidone is a dopamine D2 receptor antagonist. This drug modulates the immune response by increasing prolactin levels, which is related to serotonin release by the hypothalamus. Prolactin induces an increase in CD4+ T lymphocytes, resulting in the release of cytokines such as IL-2, IFN-γ, and TNF-α, characteristic of the Th1 immune response, which is vital in the control of leishmaniosis [144]. It is used for both the treatment of mild disease (clinical stage I) and the prevention of disease in seronegative and seropositive healthy dogs [124,145,146].

Nucleotides and active hexose compounds

Oral administration of nucleotides has been observed to modulate the immune response. They are low molecular weight compounds that positively influence lipid metabolism and tissue growth and repair [144]. AHCC is a dietary supplement extracted from basidiomycete mushrooms that has shown the ability to stimulate the immune system by increasing Th1-mediated response [124,147]. There is limited evidence for its use in combination with meglumine antimoniate for the treatment of sick dogs or for the prevention of disease in healthy dogs infected with *Leishmania* [147].

Cytokines and Toll-like receptors

TLRs are type I transmembrane proteins considered part of the first line of defense against pathogens. They are distributed in various locations, such as the inner membranes of inflammatory cells like macrophages, dendritic cells, natural killer cells, and T and B lymphocytes. Their function is to recognize PAMPs and trigger maturation, phagocytosis, and induction of various inflammatory cytokines (IL-12, TNF-α, and IFN-γ) [11,144].

Some TLRa such as imiquimod and resiquimod have shown efficacy in the treatment of cutaneous leishmaniosis in humans when combined with antimonials [148]. However, due to their high costs, their use is currently not feasible in veterinary medicine.

Regarding cytokines, their protective role in dogs that develop a Th1-type cellular response has been well-documented. One of the cytokines released in this pathway is IFN-γ, which stimulates macrophage activation to promote parasite death [11]. In some studies, conducted in humans with leishmaniosis, the combination of recombinant human IFN-γ with pentavalent antimonials was found to increase treatment effectiveness [149,150].

Autovaccines

In 2012, an autovaccine called Leishtop® (Diomune SL.) was introduced in Spain as a treatment for CanL.

It is prepared from an aspirate of lymph nodes or bone marrow from the infected animal to collect *Leishmania amastigotes,* which are then cultured and obtained as promastigotes. These promastigotes are inactivated and subjected to a stabilization process.

After the administration of the autovaccine (an initial immunization followed by a second one 15 days later), the Th1 cellular response is activated, leading to increased production of cytokines such as IL-12, TNF-α, and IFN-γ.

In a study conducted with 44 dogs naturally infected with *L. infantum*, 85% of the animals showed clinical improvement after 2 months of treatment [151].

Therapeutic vaccines

Although commercialized vaccines are not approved for the treatment of infected healthy or sick dogs, some veterinarians use them off-label for modulating immunity in patients with recurrent or refractory relapses.

Some of the commercialized vaccines for prevention, as well as other vaccines in development, have been tested as potential immunotherapeutic vaccines [153–160]. The findings from all these studies were compared in a recent article which reported that they consistently demonstrate positive outcomes, including clinical improvement in treated dogs particularly notable in cases of mild or moderate disease, although the impact on severe disease was more limited [144]. Furthermore, some studies also observed a reduction in the parasitic load [144].

Nanomedicine

Over the past few years, there has been a notable advancement in the management and therapy of leishmaniosis, facilitated by the integration of nanotechnological approaches that enhance conventional diagnostic methods and introduce novel therapeutic agents and strategies [161,162]. Nanomedicine has emerged as a pivotal player in the enhancement of drug formulations, primarily due to its emphasis on targeted delivery and controlled release, achieved through the application of nanodevices and nanomaterials [162,163].

Nanoparticles (NPs) find application in the development of new pharmaceutical formulations due to their ability to facilitate significant interaction with the biological environment, achieve sustained and controlled release of the desired compound, shield the compound from potential enzymatic and hydrolytic degradation, and decreased toxic effects [164]. Nanoparticles can be produced through a variety of techniques and using different materials, fitting into categories like metallic, lipid, or polymeric nanoparticles, as well as including liposomes, nanoemulsions, and others [165,166]. They can be administered through a range of routes, including oral, intravenous, pulmonary, topical, and subcutaneous. Depending on how they are made and what materials are used,

nanoparticles can display unique physical, chemical, mechanical, and magnetic characteristics, resulting in various reactions when introduced to a biological environment [167].

A strategy in this context is the utilization of drug delivery systems that enhance pharmacokinetic properties, surmounting common drug limitations such as poor solubility, limited permeability, and enzymatic and hydrolytic degradation. Furthermore, this enhanced drug efficiency translates to reduced toxicity by minimizing the required dosage. These drug delivery systems are employed for precise targeting, augmenting drug concentrations within the intended cells or tissues [162].

Local or topical treatment

Although there is scarce information regarding local treatments in patients with *Leishmania* in veterinary medicine [168], the WHO and the Pan American Health Organization (PAHO) recommend it as an alternative treatment for cutaneous leishmaniosis in humans when there are at least four lesions with a diameter smaller than 4 cm [169]. Local treatments offer simpler application, lower cost, and lower risk of adverse effects compared to conventional treatments [169]. Intralesional injections of antimonials (such as sodium stibogluconate or meglumine antimoniate), cryotherapy with liquid nitrogen, thermotherapy (localized current field radiofrequency heat), and topical formulations (creams with paromomycin) have been described [169–171].

Despite cutaneous manifestations are the most commonly findings observed during clinical examination and might be the sole presentation of the disease [91], local treatment has barely studied.

Following the LeishVet guidelines, canine cutaneous leishmaniosis (CCL) has no specific treatment and will be manage according to the stage. New local treatments have been successfully tested in dogs as the use of an ointment containing 2% hederagenin glucoside saponins (SS) and 2% chromane-derived hydrazone (TC2) [172]. Papular dermatitis is considered as stage I-mild leishmaniosis and its treatment has been scientifically neglected [1]. However, a short-term use of one or two conventional anti-*Leishmania* drugs (such as meglumine antimoniate, miltefosine, and/or allopurinol), has been documented [91].

Additionally, immune-potentiating treatments involving domperidone or nucleotides, often combined with AHCC, have been explored alone or in conjunction with the aforementioned drugs [1,88]. Alternatively, a monitoring approach without active treatment could be considered in select cases. However, the efficacy of these treatment options in this stage remains uncertain due to limited available evidence [89].

Treatment and prognosis according to clinical stage

The main objectives of treating a patient with leishmaniosis are:

- Achieving remission of clinical and clinicopathological signs by reducing the parasite load using anti-*Leishmania drugs*.
- Reducing the risk of relapse by using drugs that interfere with parasite proliferation or stimulate the immune system to induce the development of effective cellular immunity.
- Minimizing the occurrence of adverse effects, as far as possible.

In order to accomplish these objectives, therapeutic decisions will depend on the patient's clinical condition and the severity of the disease [92].

Clinical stages established by the LeishVet group [82] allow organizing patients according to the severity of the disease and help determine the need for treatment and type of treatment, as well as establish the prognosis **(Table 5)**.

Table 5. Clinical stages, treatment, and prognosis.
Modified from Solano-Gallego et al., 2017 [82].

Clinical stages	Treatment	Prognosis
Stage I (mild disease)	Two posible approaches: Close monitoring without treatment Short-term treatment with: a) Anti-*Leishmania* drug alone (meglumine antimoniate, miltefosine, or allopurinol) b) Immunomodulators alone c) Combination of both	Good
Stage II (moderate disease)	Meglumine antimoniate + allopurinol Miltefosine + allopurinol Substage b: follow IRIS guidelines for CKD.	Good to guarded
Stage III (severe disease)	Meglumine antimoniate + allopurinol Miltefosine + allopurinol Follow IRIS guidelines for CKD	Guarded to poor
Stage IV (very severe disease)	Assess the appropriate treatment for *Leishmania* on a case-by-case basis for each patient Follow IRIS guidelines for CKD	Poor

CKD, Chronic Kidney Disease; **IRIS,** International Renal Interest Society.

Currently, there is limited evidence to establish specific treatment recommendations for dogs in stage I.

Managing patients with severe stages (stages III and IV) is more complex due to the presence of advanced kidney disease. In these cases, specific therapeutic options for leishmaniosis should be individualized on a case-by-case basis.

Prevention

When it comes to preventing *Leishmania* infection, there are two main mechanisms. The first involves preventing transmission by targeting the vector responsible for spreading the disease and the second approach focuses on enhancing the host's ability to resist the disease by stimulating a robust immune response [91,92].

To date, the most effective strategy for preventing *Leishmania* infection is centered around vector management [124,173]. Since transmission primarily occurs through the bite of phlebotomine sand flies, measures aimed at reducing their population or preventing their bites have proven to be effective [124,173]. This can be achieved through environmental management practices or the use of repellents [115].

Environmental control

The strategies for environmental control primarily aim to eliminate ideal breeding sites for sandflies and directly reduce the sandfly population using insecticides. Sandflies tend to breed in damp and sheltered locations where organic debris like leaf litter or firewood is abundant [92]. They have successfully adapted to urban environments and can be found in shaded areas of gardens, containers, or drains. To effectively control sandflies, it is recommended to thoroughly clean these areas, removing any organic debris, and apply effective insecticides specifically targeted at sandflies [92]. Additionally, avoiding outdoor activities during periods when the vector is most active, typically at dawn and dusk can also be considered to reduce the risk of *Phlebotomus* biting, as well as installing fine-mesh mosquito nets (0.3-0.4 mm). Lastly, implementing indoor insecticide treatments can further contribute to the prevention of sand fly bites [115].

Vector control

Regarding the use of repellents, there are various formulations available, such as spot-on treatments, sprays, and collars, which have shown effectiveness in repelling sand flies, reducing the risk of new infections, and providing protection against sand fly bites for both healthy and infected dogs [20,174–181]. These repellents typically contain synthetic pyrethroids, permethrin, or deltamethrin as active ingredients [92,177–181]. They offer a dual effect, acting as a deterrent to prevent vector bites and as an insecticide that can eliminate the sand fly upon contact with the dog, effectively interrupting transmission between individuals [92].

However, it is important to note that while repellents are helpful, they may not be completely sufficient in preventing the disease on their own. It is recommended to combine various preventive measures to further reduce the risk as much as possible.

L. infantum infection control

Immunomodulators

Numerous strategies have been investigated to develop immunoprophylaxis against *Leishmania*; however, their effectiveness remains limited. These approaches can be broadly classified into two categories: vaccines and immunomodulators [91]. Currently, the availability of these products in Europe is limited. Domperidone stands as the sole immunomodulator specifically designed for the prevention of *L. infantum* infection [145,146].

Prophylactic vaccines

Vaccines play a crucial role in preventing infectious diseases by providing effective and protective immunity, and this could include CanL. However, achieving this level of immunity through vaccination remains challenging [182,183]. When evaluating potential vaccines against *Leishmania*, it is essential to understand the characteristics of protective immunity. Since *Leishmania* is an intracellular pathogen, the primary goal of a vaccine against it is to elicit a predominantly Th1-type cellular immune response [182–184]. This response is characterized by an efficient antigen presentation with a subsequent IFN-γ production by T cells [183,184]. PRRs, such as Toll-like receptors and nucleotide oligomerization domain (NOD)-like receptors, are vital for recognizing the parasite's surface molecules, known as PAMPs, and for initiating an appropriate immune response [12]. Many existing *Leishmania* vaccines incorporate PAMPs as antigens, adjuvants, or both.

The innate immune system also plays a crucial role in clearing *Leishmania* infections by enabling intracellular killing through the production of oxygen radicals within infected macrophage phagolysosomes [35,92]. If PRRs correctly recognize antigens and activate an appropriate Th1 response, IFN-γ will be released, inducing macrophages and other immune cells to produce oxygen radicals, increasing the chances of killing the parasite and controlling the infection [35,92].

Currently, there are two licensed vaccines available for the prevention of leishmaniosis in dogs. These include Leish-Tec®, which contains a recombinant single-protein antigen, and Letifend®, which consists of a recombinant polypeptide antigen [92,185]. Leish-Tec® is authorized for use in Brazil, while Letifend® is approved for use in Europe [124,185]. Additionally, two other vaccines, namely Leishmune® and Canileish®, were previously marketed. Leishmune® was available in Brazil, while Canileish® was available in Europe. However, both vaccines have been withdrawn from the market [185]. Additionally, a new vaccine, Neoleish, has recently been presented at the WorldLeish congress 2022. Field trials performed on commercially approved CanL vaccines are summarized in **Table 6**.

Table 6. Summary of field trials performed on commercially approved CanL vaccines. Modified from Velez and Gállego, 2020 [185].

Vaccine	Vaccinal Formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	Refs.
Leishmune®	FML antigen (1.5 mg) plus R adjuvant (500 µg)	Mixed population of native privately owned dogs (n = 117)	Protection against clinical CanL	**Serological:** FML-ELISA. *L. infantum* IFAT **Cellular:** DTH test **Molecular:** Parasite detection on blood and BM samples **Parasitological:** Giemsa-stained tissue smears	2 years	76% (92% protection against disease)	N.D.	[235]
	FML antigen (1.5 mg) plus saponin adjuvant (1 mg)	Mixed population of native privately owned dogs (n = 85)	*Leishmania* infection Protection against clinical CanL	**Serological:** FML-ELISA **Cellular:** DTH test **Molecular:** Parasite detection on blood and BM samples **Parasitological:** Giemsa-stained tissue smears	3.5 years	80% (95% protection against disease)	N.D.	[236]
	FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg)	Mixed population of native privately owned dogs (n = 600)	Vaccine safety and toxicity	N.D. (only physical examination was performed)	14 days after each vaccine dose	N.D.	Mostly mild and transient reactions were observed	[237]
	FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg)	Mixed population of native privately owned dogs (n = 550)*	Protection against clinical CanL. Survival rate changes in the incidence of CanL	**Serological:** FML-ELISA (only vaccine group). Seroconversion to *L. infantum* (only control group) **Cellular:** DTH test (only vaccine group). PBMC Immunophenotype analysis by flow cytometry **Molecular:** Parasite detection on blood and LN samples (only vaccine group) **Parasitological:** Giemsa-stained LN smears (only vaccine group)	2 years	N.D.	Mostly mild and transient reactions were observed	[193]

Table 6 (*cont.*). Summary of field trials performed on commercially approved CanL vaccines. Modified from Velez and Gállego, 2020 [185].

Vaccine	Vaccinal Formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	Refs.
Leish-Tec®	rA2 antigen (100 µg) plus saponin adjuvant (250 µg)	Mixed population of native privately owned dogs (n = 140)	Seroconversion in vaccinated dogs: specificity, levels, and duration of antibody responses	**Serological:** LPA-IFAT. DPP-CVL test. rA2-ELISA. Immunoblot analysis	14 months	N.D.	No significant adverse reactions were observed	[159]
	rA2 antigen (100 µg) plus saponin adjuvant (500 µg)	Mixed population of native privately owned dogs (n = 847)	CanL cases (defined as serology (+) and parasite detection by parasitological methods) Dog infectiousness to sandflies	**Serological:** cELISA. IFAT test. KD test **Parasitological:** Giemsa-stained tissue smears. Culture on BM samples **Xenodiagnosis**	18 months	71.4%† 58.1%‡ 80.8%§	N.D.	[224]
	rA2 antigen (100 µg) plus saponin adjuvant (500 µg)	Vaccine group: mixed population of native privately owned dogs (n = N.S.) Control group: introduced sentinel beagle dogs and newly recruited healthy dogs (n = N.S.)	Cases of *L. infantum* infection Cases of clinical CanL	**Serological:** A2-ELISA. LPA-ELISA **Histopathology:** Liver, spleen, LN and ear skin	2 years	No significant differences were observed between vaccine and control groups	No significant adverse reactions were observed	[225]

Table 6 (*cont.*). Summary of field trials performed on commercially approved CanL vaccines. Modified from Velez and Gállego, 2020 [185].

Vaccine	Vaccinal Formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	Refs.
Leishmune® and Leish-Tec®	Leishmune®: FML antigen (1.5 mg) plus saponin adjuvant (0.5 mg) Leish-Tec®: rA2 antigen (100 µg) plus saponin adjuvant (500 µg)	Mixed population of native privately owned dogs (n = 180). No seronegative control group	Vaccine comparison regarding vaccine reactogenicity, seroconversion, parasitism, CanL clinical signs, and dog infectiousness to sandflies	**Serological:** LPA-ELISA **Parasitological:** Culture on spleen samples **Xenodiagnosis** **Molecular:** Parasite detection on spleen samples and on phlebotomine sandflies after xenodiagnosis	11 months	No significant differences were observed between vaccine groups with respect to CanL clinical signs, parasitism, seropositivity, or dog infectiousness	Observed adverse reactions in the Leish-Tec® group were more frequent and severe	[201]
CaniLeish®	LiESAP antigen (100 µg) plus MDP adjuvant (200 µg)	Mixed population of native privately owned dogs (n = 414)	Protection against *L. infantum* infection Prevention of disease development	**Serological:** *Leishmania* IFAT. LiESAP-ELISA **Cellular:** Macrophage killing ability. NO production. IFN-γ and IL-4 production on stimulated PBMC **Molecular:** Parasite detection on BM aspirate **Parasitological:** Culture on BM aspirates	2 years	92%	No significant adverse reactions were observed	[238]
	LiESP antigen (100 µg) plus QA-21 adjuvant (60 µg)	Naïve beagle dogs, aged 5– 7.5 months and kept in kennels (n = 90)	Protection against *L. infantum* infection Prevention of disease development	**Serological:** *Leishmania* IFAT. ESP-ELISA. PSA-ELISA **Molecular:** Parasite detection on BM aspirates **Parasitological:** Culture on BM and LN aspirates **Others:** Haematological and biochemical parameters	2 years	68.4% (92.7% protection level, i.e., % of non-symptomatic vaccinated dogs)	No significant adverse reactions were observed	[215]

Table 6 (*cont.*). Summary of field trials performed on commercially approved CanL vaccines. Modified from Velez and Gállego, 2020 [185].

Vaccine	Vaccinal Formulation	Canine population	Measured outcome	Laboratory techniques	Trial duration	Vaccine efficacy	Vaccine safety	Refs.
CaniLeish®	LiESP antigen (100 µg) plus QA-21 adjuvant (60 µg)	Mixed population of shelter dogs, aged < 18 months (n = 224)	Active symptomatic *Leishmania* infection Detection of *Leishmania* parasites in BM samples	**Serological:** *Leishmania* IFAT **Molecular:** Parasite detection on BM and skin samples **Parasitological:** BM smears stained with MGG Quick Stain	1 year	No significant differences were observed between vaccine and control groups	N.D.	[220]
	LiESP antigen (100 µg) plus QA-21 adjuvant (60 µg)	Mixed population of native privately owned dogs (n = 177)	Active *Leishmania* infection Evaluation of vaccine-induced CMI	**Serological:** CTLA-ELISA **Molecular:** Parasite detection on LN aspirates **Cellular:** IFN-γ production in stimulated PBMC **Others:** Hematological and biochemical parameters	1 year	No significant differences were observed between vaccine and control groups	No significant adverse reactions were observed	[239]
LetiFend®	Recombinant protein Q from *L. infantum* MON-1 (≥36.7 ELISA units)	Mixed population of native privately owned dogs (n = 549)	Confirmed CanL cases No. of dogs with positive *Leishmania* serology. No. of dogs with presence of parasites on BM or LN at the last time point No. of dogs presenting clinical signs at the last time point	**Serological:** PQ-ELISA. SLA-ELISA. *Leishmania* IFAT **Molecular:** Parasite detection on LN and BM aspirates **Parasitological:** LN and BM smears **Others:** Hematological and biochemical parameters	2 years	72%	No local or systemic side effects were observed	[231]

BM, bone marrow; **cELISA,** crude antigen ELISA; **CMI,** cellular-mediated immunity; **CTLA,** crude total *L. infantum* antigen; **DPP-CVL,** Dual-Path Platform® Canine Visceral Leishmaniasis Rapid Test (BioManguinhos); **DTH,** delayed-type hypersensitivity; **FML,** fucose-mannose ligand; **FML-ELISA,** fucose-mannose ligand enzyme-linked immunosorbent assay; **IFAT,** indirect fluorescent antibody test; **IFN-γ,** interferon gamma; **IL-4,** interleukin 4; **KD,** Kalazar DetectTM Rapid Test for Visceral Leishmaniasis (InBios); **LiESAP,** excreted/secreted antigens of *L. infantum* promastigotes; **LiESP,** *Leishmania infantum* excreted/secreted proteins; **LN,** lymph node; **LPA,** *Leishmania* promastigote antigen; **MGG,** May-Grünwald Giemsa; **MDP,** muramyl dipeptide; **N.D.,** not determined; **NO,** nitric oxide; **N.S.,** not stated; **PBMC,** peripheral blood mononuclear cells; **PQ,** protein Q; **PSA,** parasite surface antigen; **QA-21,** Purified extract of *Quillaja saponaria*; **rA2,** A2 recombinant protein; **R,** Riedel de Haën saponin; **SLA,** soluble *L. infantum* antigen. *Control group (n = 588) belonged to a different CanL area in Brazil, with a similar CanL incidence; different criteria were used to detect *Leishmania* infection in dogs from vaccine and control groups. †According to parasitological examinations, imprinting, culture and/or histopathology of dog tissues (skin, lymph nodes, spleen, and bone marrow). ‡According to parasitological examinations plus xenodiagnosis. §According to anti-A2 serology.

Leishmune® (Fort Dodge Wyeth, later Zoetis, Brazil)

Leishmune®, introduced as the first licensed vaccine for CanL in 2004, is a second-generation vaccine composed of the fucose-mannose ligand (FML) of *L. donovani*, combined with a saponin adjuvant [186].

Immunogenicity studies conducted on FML-vaccine in mice demonstrated its ability to induce specific seroconversion, enhanced lymphoproliferative response, and a positive delayed-type hypersensitivity (DTH) reaction to *L. donovani* antigens [187–190]. The vaccine also showed promise in reducing parasite burden in the liver of previously immunized animals during experimental infection [188–190], and it exhibited cross-protective efficacy against *L. infantum* infection [191].

Phase III field trials conducted in endemic areas in Brazil evaluated the efficacy of the FML-vaccine. The first trial used FML antigen with Riedel de Haën saponin as the adjuvant and reported a vaccine efficacy of 76% and a protection rate against CanL of 92% [192]. However, the study design had some limitations, such as a lack of randomization and unclear criteria to classify dogs as infected or diseased.

The second trial utilized FML antigen with QuilA saponin as the adjuvant and reported a vaccine efficacy of 80% and 95% vaccine protection against CanL [186]. Although it employed a similar case detection methodology to the previous trial [192].

A large-scale field study involving 600 owned dogs in CanL endemic areas provided further evaluation of Leishmune® [193,194]. The vaccine was found to be safe and well-tolerated, with no severe adverse reactions observed [193]. Vaccinated dogs showed sustained specific seroconversion and positive DTH reaction [194]. Compared to the control group, vaccinated dogs exhibited higher rates of asymptomatic and healthy survivors [194]. However, due to potential variations in infection rates across different locations regarding control and infected groups, and the use of different criteria to diagnose infections in dogs, statistical comparisons between the vaccine and control groups may not be entirely reliable [194].

Subsequent studies confirmed a T-cell-dependent profile promoted by Leishmune®, including the up-regulation of CD8+ lymphocytes, enhanced levels of IFN-γ, NO, anti-*L. infantum* IgG2, IL-17a, TNF-α, as well as the stimulation of neutrophils and monocytes [195–199].

Leishmune® was considered a transmission-blocking vaccine and showed potential in inhibiting parasite binding to sandfly midguts [200,201]. However, the methodology of comparative studies assessing the infectiousness rate in Leishmune®-vaccinated dogs compared to controls was also not reliable [202].

However, vaccination with Leishmune® was reported to reduce the incidence of CanL and human leishmaniosis (HL) in Brazilian endemic areas, with correlations observed between the number of vaccinated dogs and a decrease in CanL and HL cases [203].

Regarding canine serological screening, variations in seropositivity detection using different diagnostic tests were observed, making it challenging to distinguish vaccinated dogs from naturally infected dogs in some cases [204–207].

Finally, the production and marketing license of Leishmune® were withdrawn in 2014 by the Brazilian Ministry of Agriculture due to insufficient evidence of effectiveness in phase III trials [208].

Canileish® (Virbac, France)

Canileish® was a licensed vaccine for CanL introduced in Europe in 2011 [185,209]. It contains purified excreted-secreted proteins of *L. infantum* (LiESP) and is adjuvanted with a purified fraction of the *Quilaja saponaria* saponin (QA-21) [185,209].

Before the release of Canileish, studies evaluated LiESAp with the adjuvant muramyl dipeptide (MDP) [210–213]. These studies demonstrated that vaccination with LiESAp induced a specific humoral response characterized by IgG2 antibodies against LiESAp, along with a predominantly Th1-type cellular immune response. The vaccine conferred high protection against experimental *L. infantum* infection in both laboratory and field studies, with efficacy rates of 100% and 99.4%, respectively [210–212]. Notably, no adverse effects, apart from mild local reactions, were reported [210–212]. However, this formulation was never authorized, instead a new adjuvant was added in substitution of MDP to create Canileish®, which was licensed in Europe.

In an initial phase I experimental study conducted on Beagle dogs, Canileish® vaccination induced specific antibodies to LiESP and parasite surface antigen (PSA), with a bias towards an IgG2 profile. The vaccine also elicited a cellular immune response, as vaccinated animals showed a specific T-cell response and increased production of IFN-γ. Furthermore, monocyte-derived macrophages from vaccinated dogs exhibited enhanced capacity for parasite killing, expression of inducible nitric oxide synthase (iNOS), and production of nitrogen dioxide (NO) [209]. These markers were evaluated for up to one year after vaccination, showing persistent changes [214].

However, efficacy field trials of Canileish® in endemic areas reported varying results. The first field trial conducted on naturally exposed dogs showed a significant difference in the number of dogs with active infection (33.3% in the control group vs. 12.2% in the vaccinated group) and symptomatic cases (23.1%

in the control group vs. 7.3% in the vaccinated group) between the two groups. However, no significant difference was observed in the proportion of dogs with a positive PCR result, suggesting that the vaccine does not prevent the entry and migration of the parasite to deeper tissues. Vaccinated dogs also exhibited a reduced progression to fatal stages compared to the control group [215].

However, in additional studies performed later, no significant difference was found in the development of active symptomatic infections or the number of PCR-positive dogs between vaccinated dogs and the control group [26,216]. The vaccine was also assessed for its impact on the infectiousness potential of *Leishmania*-infected dogs through a xenodiagnosis study, which showed no difference in the rate of sandfly infection between the vaccinated and control groups [217]. However, CaniLeish® reduces the intensity of infection in *Phlebotomus perniciosus* fed on *L. infantum* infected dog

An additional limitation of using this vaccine is its interference with the serologic diagnosis of leishmaniosis, as the assay cannot distinguish between vaccine-induced antibodies and those produced in response to natural infection [215,218–221]. Canileish® was withdrawn from the market in 2021.

Leish-Tec® (Hertape Calier Saude Animal, later Ceva, Brazil)

Leish-Tec® is an anti-*Leishmania* vaccine formulated with a recombinant A2 protein derived from *L. donovani* amastigotes, along with saponin as an adjuvant which was authorized in Brazil in 2007 [185]. Studies conducted in murine models showed that immunization with the recombinant A2 protein resulted in significant protection against experimental *L. donovani* infection, as evidenced by lower parasite burden in vaccinated animals compared to controls [222]. The vaccine induced a specific humoral immune response and triggered a mixed Th1-Th2 cell-mediated immune response, characterized by increased production of IFN-γ [222].

In Beagle dogs subjected to a high-dose challenge with *L. infantum*, vaccinated dogs exhibited elevated levels of anti-A2 IgG2 antibodies and higher production of IFN-γ. These dogs also experienced a delayed onset of clinical signs compared to the control group, although the parasite was still detectable in the bone marrow samples of 57% of the vaccinated dogs [223]. Thus, while the vaccine demonstrated the induction of protective immunity, it provided only partial protection against the parasite [223]. However, in a xenodiagnosis study, Leish-Tec® was found to significantly reduce the infectivity of dogs to sandflies [202].

Safety analysis indicated that the vaccine only caused mild, site-specific adverse reactions in 3.09% of vaccinated dogs [159]. Furthermore, it did not induce non-specific seroconversion or cross-reactivity with diagnostic tests in the majority of vaccinated animals [224].

Two field trials have been conducted to assess the efficacy of Leish-Tec®. The first trial showed a significant reduction in the number of CanL cases based on various criteria, including parasitological tests, xenodiagnosis, and seroconversion to A2 antigen. However, there was no statistically significant difference in the prevalence of sandfly pools feeding on vaccinated dogs, indicating no reduction in infectiveness [225]. The second efficacy trial reported a lower incidence of infection in the vaccine group (27%) compared to the control group (42%). However, among seropositive animals, a higher proportion of vaccinated dogs developed the disease, suggesting limited effectiveness under field conditions [226]. Additionally, a systematic review had previously raised questions about the efficacy of the Leish-Tec® vaccine, further adding to the controversy [227].

Finally, a comparative study between Leishmune® and Leish-Tec® found no significant differences in humoral response or infection and transmission rates to the sandfly vector, except for a higher rate of adverse reactions observed in the Leish-Tec® group [25].

Letifend® (Laboratorios LETI, Spain)

Letifend®, developed by Laboratorios LETI in Spain, is a licensed recombinant vaccine for CanL in Europe available since 2016 [185]. It consists of a chimerical protein known as protein Q, which is composed of five antigenic fragments derived from different *L. infantum* proteins (ribosomal proteins LiP2a, LiP2b, LiP0, and histone H2A). Notably, Letifend® does not contain any adjuvant [185].

The potential of protein Q to induce immunization against *L. infantum* was initially demonstrated in murine models [228,229]. In early experiments, protein Q was combined with live bacillus Calmette-Guérin (BCG) as an adjuvant, and when vaccinated dogs and mice were challenged with *L. infantum* experimental infection, the vaccine exhibited the ability to prevent parasite establishment [228]. However, due to safety concerns associated with BCG, alternative adjuvant combinations were tested, but no significant differences were observed comparing these to a control group, suggesting that the previously observed protective effect may have been attributed to live BCG [230,231].

Nevertheless, in a separate study where protein Q was administered without adjuvants, as in the commercial LetiFend® formulation, a protective effect was observed in vaccinated dogs [232]. Additionally, in a phase III clinical trial involving 549 dogs exposed to natural infection, the vaccinated group showed a significantly lower proportion of infected dogs (4.7%) compared to the control group (10.2%) after a two-year study period [233]. The vaccine demonstrated a vaccine efficacy of 72% in preventing clinical signs of CanL and significantly reduced the likelihood of confirmed CanL cases or the development of clinical signs in vaccinated dogs [233].

Importantly, no adverse effects were observed in laboratory or field studies, and LetiFend® did not produce false-positive results in *L. infantum* serological diagnostic tests [232–234]. These findings indicate a good safety profile and compatibility with existing diagnostic methods.

Overall, although its limited effectiveness, LetiFend® has shown promising results and has demonstrated a favorable safety profile. However, further studies are needed to better understand its long-term effectiveness.

New vaccines: Neoleish® (in development by VZ Vaccines, Spain)

A new vaccine against *Leishmania infantum* in dogs has been recently reported in the 7th WorldLeish congress [240], the following information has been obtained from this congress, but it has to be taken in account that these studies have not yet been published in indexed journals. This vaccine has been developed utilizing a bacterial plasmid (DNA) that encodes the LACK gene of *L. infantum*, and without any adjuvants or preservatives. The vaccine is administered intranasally in two doses over a two-week period. The LACK protein, is an analog of the activated C-Kinase receptor and is expressed in both amastigotes and promastigotes. This protein is highly conserved, and it is known to elicit an immunological response [241].

Clinical studies have been conducted to evaluate the efficacy and safety of this vaccine. In phase I studies involving healthy dogs, the vaccine demonstrated no local or systemic reactions, as well as no hematological or biochemical alterations. Additionally, the vaccine did not interfere with serology results.

Phase II studies conducted with experimentally infected dogs showed promising results. Dogs vaccinated with the DNA vaccine exhibited a lower parasite burden in bone marrow and tissues, along with a reduction in clinical signs. The vaccine also stimulated a Th1 cellular immune response both post-vaccination and post-infection, indicating its ability to induce a protective immune response. Notably, the dogs received an infectious dose 10^6 times higher than the natural dose.

In phase III field studies involving a larger sample size of 180 vaccinated dogs compared to 180 control dogs, no observed adverse reactions were reported. Additionally, vaccinated dogs showed a reduction in parasite burden, a lower number of infections, and increased resistance to the disease. Additionally, the severity of clinical signs was reduced in vaccinated dogs.

These findings suggest that the vaccine is apparently safe and can stimulate a Th1 immune response, resulting in a decrease in parasite burden and a lower incidence of active infections. It is important to remind once again that all the mentioned studies are currently unpublished. Further research and publication of the studies are necessary to provide additional evidence and establish its potential as an effective preventive measure against canine leishmaniosis.

Other non-commercialized vaccines

Numerous other *Leishmania* antigens have been identified as potential vaccine candidates; nonetheless, only a few have undergone rigorous evaluation in field trials [227]. The quality of evidence from many existing studies is compromised by methodological limitations and insufficient statistical power, which hampers the comprehensive assessment of the effectiveness of this vaccines [227]. An overview of *Leishmania* antigens that have been evaluated as potential vaccine candidates (either in murine or canine) are summarized in **Table 7**.

Table 7. ***Leishmania*** **antigens evaluated as potential vaccines candidates.**
Modified from Keerti and N.K., 2015 [242].

First generation vaccine candidates			
Antigen	**Description of antigen**	**Adjuvant**	**Refs.**
Life attenuated *Leishmania* parasite			
L. donovani	Radio-attenuated *L. donovani*	No	[243]
L. infantum	SIR2 (silent information regulatory 2) deficient *L. infantum* amastigotes	No	[244]
L. donovani, L. infantum	Long term culture of LiESP promastigotes	Gentamycin	[245]
L. donovani	BT1 (biopterin transporter) knock out promastigotes	No	[246]
L. tarentolae	Live attenuated nonpathogenic *L. tarentolae* promastigotes	No	[247]
L. amazonensis	Photodynamic vaccination with suicidal mutants of *L. amazonensis*	No	[248]
Life attenuated *Leishmania* parasite			
L. major	Alum precipitated autoclaved *L. major*	BCG (Bacillus Clamette Guerin)	[249]
L. donovani	Autoclaved or heat killed *L. donovani*	No	[250]
L. major	Autoclaved *L. major*	BCG (Bacillus Clamette Guerin)	[251]
Second generation or purified antigen			
Antigen	**Description of antigen**	**Adjuvant**	**Refs.**
P0	Acidic ribosomal protein P0 of *L. infantum*	No	[252]
CPI and CPII	*L. infantum* cysteine proteinases A and B	Cationic solid lipid nanoparticles	[253]
GP63	Surface expressed glycoprotein GP63, or leishmanolysin Fucose	*Salmonella typhimurium* or BCG or cationic liposomes or monophosphoryl lipid A-trehalose dicorynomycolate (MPL-TDM)	[254]

Table 7 (*cont.*). *Leishmania* antigens evaluated as potential vaccines candidates.
Modified from Keerti and N.K., 2015 [242].

Second generation or purified antigen			
Antigen	**Description of antigen**	**Adjuvant**	**Refs.**
FML	Fucose mannose ligand Purified	Saponins (Riedel De Haen, QuilA, QS21), IL12, BCG	[255]
GP36	Purified antigen from FML complex	Saponin	[190]
LiESAp	Naturally excreted/secreted antigens purified from culture supernatant of *L. infantum* promastigotes	MDP (Muramyl dipeptide)	[256]
rLdccys1	Recombinant cysteine proteinase	BCG/ Propionibacterium acnes	[257]
SLA	Soluble leishmanial antigen	Monophosphoryl lipid-trehalose dicorynomycolate (MPL-TDM)	[258]
rLdp45	Recombinant *L. donovani* p45 (rLdp45). Ldp45 is a member of the methionine aminopeptidase family	No	[259]
rLelF-2	*L. donovani* elongation factor-2 protein (LelF-2)	No	[260]
rLdPDI	Protein disulfide isomerase (PDI) is a chaperone involved in virulence and survival of *Leishmania* parasite. (rLdPDI: recombinant *L. donovani* protein disulfide isomerase)	No	[261]
rA2	Recombinant protein specific to *L. donovani* amastigotes	*P. acnes*	[262]
KMP-11	Kinetoplastid membrane protein-11 (KMP-11)	CpG-ODN	[263]
rLeish-111f (Polyprotein vaccine)	Leish-111f is a polyprotein derived by fusion of three antigens namely *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), *L. major stress* inducible protein 1 (LmSTI1) & *L. braziliensis* elongation and initiation factor (LelF)	MPL-SE (monophosphoryl lipid A plus squalene) and/or Glucantime	[158]
Ldp27	*L. donovani* amastigote-specific protein p27 (Ldp27), which is a component of an active cytochrome C oxidase complex	No	[264]
pSP	Secretory *L. donovani* serine protease	IL-12	[265]
rSMT	Sterol 24-c-methyltransferase (SMT) is one of the identified Ags. SMT is an enzyme involved in biosynthesis of ergosterol, which is a target molecule of leishmanicidal and fungicidal drug amphotericin B	MPL-SE (monophosphoryl lipid A plus squalene) and/or Glucantime	[266]

Table 7 (*cont.*). *Leishmania* antigens evaluated as potential vaccines candidates.
Modified from Keerti and N.K., 2015 [242].

Second generation or purified antigen			
Antigen	**Description of antigen**	**Adjuvant**	**Refs.**
Histone H1	Bone marrow-derived dendritic cells (BM-DCs) pulsed with the *L. infantum* histone H1	CpG ODNs	[267]
HSP70 and HSP83	Heat shock protein	MPLA, ALD	[268]
LBSap	*L. braziliensis* antigens plus saponin (LBSap) vaccine	Saponins	[152,269]
GP63 and HSP70	Cocktail vaccine (GP63 + HSP70 + MPL-A and GP63 + HSP70 + ALD)	MPL-A, ALD	[270]
rLiHyp1	Hypothetical *Leishmania* amastigote-specific protein (LiHyp1)	Saponin	[27]
Aldolase	Central glycolytic enzyme in carbohydrate metabolism	Freund's adjuvant	[272]
***Lir*CyP1**	Found in several intracellular compartments and extracellularly. Possess peptidylprolylcis–trans isomerase activity	Mouse recombinant IL-12	[273]
LdSir2HP	Characterized by a conserved catalytic domain that exerts unique NAD-dependent deacetylase activity. Potent immunestimulatory protein	Freund's adjuvant	[274
L3 and L5	*Leishmania major* ribosomal proteins	Phosphorothioate-modified CpG-ODN	[275]
NS protein (LEISH-F3)	Chimeric protein by the combination of nucleoside hydrolase and sterol 24-c-methyltransferase	Glucopyranosyl lipid A	[276
8E/p21/SMT	Chimeric fusion protein by the contiguous expression of the 8E, p21 and SMT genes	GLA-SE	[277]
Third generation vaccines			
Antigen	**Description of antigen**	**Expression vector**	**Refs.**
Antigen-encoding DNA plasmid vaccines			
ORFF	Open reading frame fragment	pcDNA3.1	[278]
A2	Antigen specific to *L. donovani* amastigotes	A2-expressing *Lactococcus lactis*	[279]
LACK	*Leishmania* homologue of receptors for activated C kinase	pCI-neo	[280]
KMP-11	Kinetoplastid membrane protein-11	pCMV-LIC	[281]
NH36	Nucleoside hydrolase (NH36) is a vital enzyme of *L. donovani* used in the synthesis of parasite DNA	VR1012	[282]

Table 7 (*cont.*). *Leishmania* antigens evaluated as potential vaccines candidates. Modified from Keerti and N.K., 2015 [242].

Third generation vaccines			
Antigen	**Description of antigen**	**Expression vector**	**Refs.**
Heterologous prime-boost vaccines			
LACK	*Leishmania* homologue of receptors for activated C kinase prime boost vaccine	Recombinant vaccinia virus (rVV)/ modified vaccinia virus Ankara (MVA)	[283]
CPI + CPII	*L. infantum* cysteine proteinase type I (CPI) and II (CPII) prime boost vaccine	PCB6	[284]
CPIII	*L. infantum* cysteine proteinase type III (CPIII) prime boost vaccine	pcDNA	[285]
KMP-11	Kinetoplastid membrane protein-11	rVV	[286]
A2 + CPI + CPII	A2 antigen and cysteine proteinases (CPI and CPII) of *L. donovani*	pcDNA	[287]
rLdP1	Recombinant *L. donovani* ribosomal P1 gene (rLdP1)	pVAX	[288]
TRYP	Tryparedoxin peroxidase (TRYP)	Modified vaccinia virus Ankara (MVA)	[257]
Ldccys1	Cysteine proteinase of 30 kDa from *L. infantum*	pcDNA3	[289]

References

1. Solano-Gallego, L.; Mirá, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites and Vectors* **2011**, *4*, 1–16.
2. Colmenares, M.; Kar, S.; Goldsmith-Pestana, K.; McMahon-Pratt, D. Mechanisms of Pathogenesis: Differences amongst *Leishmania* Species. *Trans. R. Soc. Trop. Med. Hyg.* **2002**, *96 Suppl 1*, 3-7.
3. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends Parasitol.* **2008**, *24*, 324–330.
4. Moreno, J. Assessment of Vaccine-Induced Immunity against Canine Visceral Leishmaniasis. *Front. Vet. Sci.* **2019**, *6*, 168.
5. Akhoundi, M.; Downing, T.; Votýpka, J.; Kuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; et al. *Leishmania* Infections: Molecular Targets and Diagnosis. *Mol. Aspects Med.* **2017**, *57*, 1–29.
6. Gradoni, L. Epidemiological Surveillance of Leishmaniasis in the European Union: Operational and Research Challenges. *Euro Surveill.* **2013**, *18*, :20539.

7. Belo, V.S.; Werneck, G.L.; Barbosa, D.S.; Simões, T.C.; Nascimento, B.W.L.; da Silva, E.S.; Struchiner, C.J. Factors Associated with Visceral Leishmaniasis in the Americas: A Systematic Review and Meta-Analysis. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2182.

8. Dantas-Torres, F.; Solano-Gallego, L.; Baneth, G.; Ribeiro, V.M.; de Paiva-Cavalcanti, M.; Otranto, D. Canine Leishmaniosis in the Old and New Worlds: Unveiled Similarities and Differences. *Trends Parasitol.* **2012**, *28*, 531–538.

9. Iatta, R.; Mendoza-Roldan, J.A.; Latrofa, M.S.; Cascio, A.; Brianti, E.; Pombi, M.; Gabrielli, S.; Otranto, D. *Leishmania Tarentolae* and *Leishmania Infantum* in Humans, Dogs and Cats in the Pelagie Archipelago, Southern Italy. *PLoS Negl. Trop. Dis.* **2021**, *15*, e0009817.

10. Alvar, J.; Vélez, I.D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; de Boer, M. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS One* **2012**, *7*, e35671.

11. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, *144*, 95–115.

12. Kaye, P.; Scott, P. Leishmaniasis: Complexity at the Host–Pathogen Interface. *Nat. Rev. Microbiol.* **2011**, *9*, 604–615.

13. Faria, M.S.; Reis, F.C.G.; Lima, A.P.C.A. Toll-like Receptors in *Leishmania* Infections: Guardians or Promoters? *J. Parasitol. Res.* **2012**, :930257.

14. Naucke, T.J.; Lorentz, S. First Report of Venereal and Vertical Transmission of Canine Leishmaniosis from Naturally Infected Dogs in Germany. *Parasites and Vectors* **2012**, *5*, 1–5.

15. Toepp, A.J.; Bennett, C.; Scott, B.; Senesac, R.; Oleson, J.J.; Petersen, C.A. Maternal *Leishmania Infantum* Infection Status Has Significant Impact on Leishmaniasis in Offspring. *PLoS Negl. Trop. Dis.* **2019**, *13*, e0007058.

16. De Freitas, E.; Melo, M.N.; Da Costa-Val, A.P.; Michalick, M.S.M. Transmission of *Leishmania Infantum* via Blood Transfusion in Dogs: Potential for Infection and Importance of Clinical Factors. *Vet. Parasitol.* **2006**, *137*, 159–167.

17. Naucke, T.J.; Amelung, S.; Lorentz, S. First Report of Transmission of Canine Leishmaniosis through Bite Wounds from a Naturally Infected Dog in Germany. *Parasites and Vectors* **2016**, *9*, 1–4.

18. Alcover, M.M.; Basurco, A.; Fernandez, A.; Riera, C.; Fisa, R.; Gonzalez, A.; Verde, M.; Garrido, A.M.; Ruíz, H.; Yzuel, A.; et al. A Cross-Sectional Study of *Leishmania Infantum* Infection in Stray Cats in the City of Zaragoza (Spain) Using Serology and PCR. *Parasites and Vectors* **2021**, *14*, 1–14.

19. Pennisi, M.G.; Cardoso, L.; Baneth, G.; Bourdeau, P.; Koutinas, A.; Miró, G.; Oliva, G.; Solano-Gallego, L. LeishVet Update and Recommendations on Feline Leishmaniosis. *Parasit. Vectors* **2015**, *8*, 302.

20. Gramiccia, M.; Gradoni, L. The Current Status of Zoonotic Leishmaniases and Approaches to Disease Control. *Int. J. Parasitol.* **2005**, *35*, 1169–1180.

21. Sastre, N.; Francino, O.; Ramírez, O.; Enseñat, C.; Sánchez, A.; Altet, L. Detection of *Leishmania Infantum* in Captive Wolves from Southwestern Europe. *Vet. Parasitol.* **2008**, *158*, 117–120.

22. Dipineto, L.; Manna, L.; Baiano, A.; Gala, M.; Fioretti, A.; Gravino, A.E.; Menna, L.F. Presence of *Leishmania Infantum* in Red Foxes (Vulpes Vulpes) in Southern Italy. *J. Wildl. Dis.* **2007**, *43*, 518–520.

23. Toplu, N.; Aydoğan, A.; Oguzoglu, T.C. Visceral Leishmaniosis and Parapoxvirus Infection in a Mediterranean Monk Seal (Monachus Monachus). *J. Comp. Pathol.* **2007**, *136*, 283–287.

24. Azami-conesa, I.; Gómez-muñoz, M.T.; Martínez-díaz, R.A. A Systematic Review (1990-2021) of Wild Animals Infected with Zoonotic Leishmania. *Microorganisms* **2021**, *9*, 1101.

25. Rombolà, P.; Barlozzari, G.; Carvelli, A.; Scarpulla, M.; Iacoponi, F.; Macrì, G. Seroprevalence and Risk Factors Associated with Exposure to *Leishmania Infantum* in Dogs, in an Endemic Mediterranean Region. *PLoS One* **2021**, *16*, e0244923.

26. Velez, R.; Ballart, C.; Domenech, E.; Abras, A.; Fernández-Arévalo, A.; Gómez, S.A.; Tebar, S.; Muñoz, C.; Cairó, J.; Gállego, M. Seroprevalence of Canine *Leishmania Infantum* Infection in the Mediterranean Region and Identification of Risk Factors: The Example of North-Eastern and Pyrenean Areas of Spain. *Prev. Vet. Med.* **2019**, *162*, 67–75.

27. Tamponi, C.; Scarpa, F.; Carta, S.; Knoll, S.; Sanna, D.; Gai, C.; Pipia, A.P.; Dessì, G.; Casu, M.; Varcasia, A.; et al. Seroprevalence and Risk Factors Associated with *Leishmania Infantum* in Dogs in Sardinia (Italy), an Endemic Island for Leishmaniasis. *Parasitol. Res.* **2020**, *120*, 289.

28. Almeida, M.; Maia, C.; Cristóvão, J.M.; Morgado, C.; Barbosa, I.; Ibars, R.F.; Campino, L.; Gonçalves, L.; Cortes, S. Seroprevalence and Risk Factors Associated with *Leishmania* Infection in Dogs from Portugal. *Microorganisms* **2022**, *10*, 2262.

29. Gálvez, R.; Miró, G.; Descalzo, M.A.; Nieto, J.; Dado, D.; Martín, O.; Cubero, E.; Molina, R. Emerging Trends in the Seroprevalence of Canine Leishmaniasis in the Madrid Region (Central Spain). *Vet. Parasitol.* **2010**, *169*, 327–334.

30. 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.

31. Travi, B.L.; Cordeiro-da-Silva, A.; Dantas-Torres, F.; Miró, G. Canine Visceral Leishmaniasis: Diagnosis and Management of the Reservoir Living among Us. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006082.

32. Cabré, M.; Planellas, M.; Ordeix, L.; Solano-Gallego, L. Is Signalment

Associated with Clinicopathological Findings in Dogs with Leishmaniosis? *Vet. Rec.* **2021**, *189*, e451.

33. Solano-Gallego, L.; Llull, J.; Ramos, G.; Riera, C.; Arboix, M.; Alberola, J.; Ferrer, L. The Ibizian Hound Presents a Predominantly Cellular Immune Response against Natural *Leishmania* Infection. *Vet. Parasitol.* **2000**, *90*, 37–45.
34. Toepp, A.J.; Monteiro, G.R.G.; Coutinho, J.F.V.; Lima, A.L.; Larson, M.; Wilson, G.; Grinnage-Pulley, T.; Bennett, C.; Mahachi, K.; Anderson, B.; et al. Comorbid Infections Induce Progression of Visceral Leishmaniasis. *Parasit. Vectors* **2019**, *12*, 54.
35. Toepp, A.J.; Petersen, C.A. The Balancing Act: Immunology of Leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25.
36. Solano-Gallego, L.; Montserrrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania Infantum*-Specific Production of IFN-γ and IL-10 in Stimulated Blood from Dogs with Clinical Leishmaniosis. *Parasites and Vectors* **2016**, *9*, 317.
37. Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P.; Solano-Gallego, L. Parasite Specific Antibody Levels, Interferon-γ and TLR2 and TLR4 Transcripts in Blood from Dogs with Different Clinical Stages of Leishmaniosis. *Vet. Sci.* **2018**, *5*, 5–31.
38. Kumar, H.; Kawai, T.; Akira, S. Pathogen Recognition by the Innate Immune System. *Int. Rev. Immunol.* **2011**, *30*, 16–34.
39. Handman, E.; Bullen, D.V.R. Interaction of *Leishmania* with the Host Macrophage. *Trends Parasitol.* **2002**, *18*, 332–334.
40. Rittig, M.G.; Bogdan, C. *Leishmania*-Host-Cell Interaction: Complexities and Alternative Views. *Parasitol. Today* **2000**, *16*, 292–297.
41. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, *173*, 6521–6525.
42. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, *64*, 38–43.
43. Gueirard, P.; Laplante, A.; Rondeau, C.; Milon, G.; Desjardins, M. Trafficking of *Leishmania Donovani* Promastigotes in Non-Lytic Compartments in Neutrophils Enables the Subsequent Transfer of Parasites to Macrophages. *Cell. Microbiol.* **2008**, *10*, 100–111.
44. Brandonisio, O.; Panaro, M.A.; Marzio, R.; Marangi, A.; Faliero, S.M.; Jirillo, E. Impairment of the Human Phagocyte Oxidative Responses Caused by *Leishmania* Lipophosphoglycan (LPG): In Vitro Studies. *FEMS Immunol. Med. Microbiol.* **1994**, *8*, 57–62.
45. Panaro MA, Puccini V, Faliero SM, Marzio R, Marangi A, Lisi S,

Brandonisio O. *Leishmania donovani* lipophosphoglycan (LPG) inhibits respiratory burst and chemotaxis of dog phagocytes. *New Microbiol.* **1996**, *19,* 107-12.

46. Kaye, P.; Scott, P. Leishmaniasis: Complexity at the Host-Pathogen Interface. *Nat. Rev. Microbiol.* **2011**, *9*, 604–615.

47. Terrazas, C.; Oghumu, S.; Varikuti, S.; Martinez-Saucedo, D.; Beverley, S.M.; Satoskar, A.R. Uncovering *Leishmania*-Macrophage Interplay Using Imaging Flow Cytometry. *J. Immunol. Methods* **2015**, *423*, 93–98.

48. Pereira-Fonseca, D.C.M.; Oliveira-Rovai, F.M.; Rodas, L.A.C.; Beloti, C.A.C.; Torrecilha, R.B.P.; Ito, P.K.R.K.; Avanço, S. V.; Cipriano, R.S.; Utsunomiya, Y.T.; Hiramoto, R.M.; et al. Dog Skin Parasite Load, TLR-2, IL-10 and TNF-α Expression and Infectiousness. *Parasite Immunol.* **2017**, *39*, 1–7.

49. Carpenter, S.; O'Neill, L.A.J. How Important Are Toll-like Receptors for Antimicrobial Responses? *Cell. Microbiol.* **2007**, *9*, 1891–1901.

50. Kawai, T.; Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **2011**, *34*, 637–650.

51. Ordeix, L.; Montserrat-Sangrà, S.; Martínez-Orellana, P.; Baxarias, M.; Solano-Gallego, L. Toll-like Receptors 2, 4 and 7, Interferon-Gamma and Interleukin 10, and Programmed Death Ligand 1 Transcripts in Skin from Dogs of Different Clinical Stages of Leishmaniosis. *Parasit. Vectors* **2019**, *12*, 575.

52. Martínez-Orellana, P.; Baxarias, M.; Good, L.; Solano-Gallego, L. The Effects of Polyhexamethylene Biguanide (PHMB) and TLR Agonists Alone or as Polyplex Nanoparticles against *Leishmania Infantum* Promastigotes and Amastigotes. *Vet. Sci.* **2020**, *7*, 1–14.

53. Martínez-Orellana, P.; Quirola-Amores, P.; Montserrat-Sangrà, S.; Ordeix, L.; Llull, J.; Álvarez-Fernández, A.; Solano-Gallego, L. The Inflammatory Cytokine Effect of Pam3CSK4 TLR2 Agonist Alone or in Combination with *Leishmania Infantum* Antigen on Ex-Vivo Whole Blood from Sick and Resistant Dogs. *Parasit. Vectors* **2017**, *10*, 123.

54. Santiago, M.E.B.; Neto, L.S.; Alexandre, E.C.; Munari, D.P.; Andrade, M.M.C.; Somenzari, M.A.; Ciarlini, P.C.; De Lima, V.M.F. Improvement in Clinical Signs and Cellular Immunity of Dogs with Visceral Leishmaniasis Using the Immunomodulator P-MAPA. *Acta Trop.* **2013**, *127*, 174–180.

55. Hosein, S.; Rodríguez-Cortés, A.; Blake, D.P.; Allenspach, K.; Alberola, J.; Solano-Gallego, L. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 Cytokines in a Susceptible Experimental Model of Canine *Leishmania Infantum* Infection. *PLoS One* **2015**, *10*, e0140325.

56. Flandin, J.F.; Chano, F.; Descoteaux, A. RNA Interference Reveals a

Role for TLR2 and TLR3 in the Recognition of *Leishmania Donovani* Promastigotes by Interferon–γ-Primed Macrophages. *Eur. J. Immunol.* **2006**, *36*, 411–420.

57. Martínez-Orellana, P.; Montserrat-Sangrà, S.; Quirola-Amores, P.; González, N.; Solano-Gallego, L. Cytokine Effect of TLR3, TLR4, and TLR7 Agonists Alone or Associated with *Leishmania Infantum* Antigen on Blood from Dogs. *Biomed Res. Int.* **2018**, 5693736.
58. Kropf, P.; Freudenberg, M.A.; Modolell, M.; Price, H.P.; Herath, S.; Antoniazi, S.; Galanos, C.; Smith, D.F.; Müller, I. Toll-like Receptor 4 Contributes to Efficient Control of Infection with the Protozoan Parasite *Leishmania Major. Infect. Immun.* **2004**, *72*, 1920–1928.
59. Sacramento, L.; Trevelin, S.C.; Nascimento, M.S.; Lima-Jùnior, D.S.; Costa, D.L.; Almeida, R.P.; Cunha, F.Q.; Silva, J.S.; Carregaro, V. Toll-Like Receptor 9 Signaling in Dendritic Cells Regulates Neutrophil Recruitment to Inflammatory Foci Following *Leishmania Infantum* Infection. *Infect. Immun.* **2015**, *83*, 4604.
60. Alvar, J.; Cañavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine Leishmaniasis. *Adv. Parasitol.* **2004**, *57*, 1–88.
61. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous Immune Mechanisms in Canine Leishmaniosis Due to *Leishmania Infantum*. *Vet. Immunol. Immunopathol.* **2015**, *163*, 94–102.
62. Boggiatto, P.M.; Ramer-tait, A.E.; Metz, K.; Kramer, E.E.; Gibson-corley, K.; Mullin, K.; Hostetter, J.M.; Gallup, J.M.; Jones, D.E.; Petersen, C.A. Immunologic Indicators of Clinical Progression during Canine *Leishmania Infantum* Infection. **2010**, *17*, 267–273.
63. Ambruso, D.R.; Briones, N.J.; Baroffio, A.F.; Murphy, J.R.; Tran, A.D.; Gowan, K.; Sanford, B.; Ellison, M.; Jones, K.L. In Vivo Interferon-Gamma Induced Changes in Gene Expression Dramatically Alter Neutrophil Phenotype. *PLoS One* **2022**, *17*, 1–22.
64. Banerjee, A.; Bhattacharya, P.; Joshi, A.B.; Ismail, N.; Dey, R.; Nakhasi, H.L. Role of Pro-Inflammatory Cytokine IL-17 in *Leishmania* Pathogenesis and in Protective Immunity by *Leishmania* Vaccines. *Cell. Immunol.* **2016**, *309*, 37–41.
65. Nascimento, M.S.L.; Albuquerque, T.D.R.; Nascimento, A.F.S.; Caldas, I.S.; Do-Valle-Matta, M.A.; Souto, J.T.; Talvani, A.; Bahia, M.T.; Galvão, L.M.C.; Câmara, A.C.J.; et al. Impairment of Interleukin-17A Expression in Canine Visceral Leishmaniosis Is Correlated with Reduced Interferon-γ and Inducible Nitric Oxide Synthase Expression. *J. Comp. Pathol.* **2015**, *153*, 197–205.
66. Pitta, M.G.R.; Romano, A.; Cabantous, S.; Henri, S.; Hammad, A.; Kouriba, B.; Argiro, L.; El Kheir, M.; Bucheton, B.; Mary, C.; et al. IL-17 and IL-22 Are Associated with Protection against Human Kala Azar Caused by *Leishmania Donovani*. *J. Clin. Invest.* **2009**, *119*, 2379–2387.
67. Wu, W.; Huang, L.; Mendez, S. A Live *Leishmania* Major Vaccine

Containing CpG Motifs Induces the de Novo Generation of Th17 Cells in C57BL/6 Mice. *Eur. J. Immunol.* **2010**, *40*, 2517–2527.

68. Bacallar, O.; Faria, D.; Nascimento, M.; Cardoso, T.M.; Gollob, K.J.; Dutra, W.O.; Scott, P.; Carvalho, E.M. Interleukin 17 Production among Patients with American Cutaneous Leishmaniasis. *J. Infect. Dis.* **2009**, *200*, 75–78.

69. Boaventura, V.S.; Santos, C.S.; Cardoso, C.R.; De Andrade, J.; Dos Santos, W.L.C.; Clarêncio, J.; Silva, J.S.; Borges, V.M.; Barral-Netto, M.; Brodskyn, C.I.; et al. Human Mucosal Leishmaniasis: Neutrophils Infiltrate Areas of Tissue Damage That Express High Levels of Th17-Related Cytokines. *Eur. J. Immunol.* **2010**, *40*, 2830–2836.

70. Sacks, D.; Noben-Trauth, N. The Immunology of Susceptibility and Resistance to *Leishmania Major* in Mice. *Nat. Rev. Immunol.* **2002**, *2*, 845–858.

71. Vida, B.; Toepp, A.; Schaut, R.G.; Esch, K.J.; Juelsgaard, R.; Shimak, R.M.; Petersen, C.A. Immunologic Progression of Canine Leishmaniosis Following Vertical Transmission in United States Dogs. *Vet. Immunol. Immunopathol.* **2016**, *169*, 34–38.

72. Carrillo, E.; Moreno, J. Cytokine Profiles in Canine Visceral Leishmaniasis. *Vet. Immunol. Immunopathol.* **2009**, *128*, 67–70.

73. Chamizo, C.; Moreno, J.; Alvar, J. Semi-Quantitative Analysis of Cytokine Expression in Asymptomatic Canine Leishmaniasis. *Vet. Immunol. Immunopathol.* **2005**, *103*, 67–75.

74. Liu, D.; Uzonna, J.E. The Early Interaction of *Leishmania* with Macrophages and Dendritic Cells and Its Influence on the Host Immune Response. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 83.

75. Esch, K.J.; Juelsgaard, R.; Martinez, P.A.; Jones, D.E.; Petersen, C.A. PD-1-Mediated T Cell Exhaustion during Visceral Leishmaniasis Impairs Phagocyte Function. *J. Immunol.* **2013**, *191*, 5542.

76. Nylén, S.; Maurya, R.; Eidsmo, L.; Das Manandhar, K.; Sundar, S.; Sacks, D. Splenic Accumulation of IL-10 MRNA in T Cells Distinct from CD4+CD25+ (Foxp3) Regulatory T Cells in Human Visceral Leishmaniasis. *J. Exp. Med.* **2007**, *204*, 805–817.

77. Wherry, E.J. T Cell Exhaustion. *Nat. Immunol.* **2011**, *12*, 492–499.

78. Sharpe, A.H.; Wherry, E.J.; Ahmed, R.; Freeman, G.J. The Function of Programmed Cell Death 1 and Its Ligands in Regulating Autoimmunity and Infection. *Nat. Immunol.* **2007**, *8*, 239–245.

79. Gibson-Corley, K. N., Hostetter, J. M., Hostetter, S. J., Mullin, K., Ramer-Tait, A. E., Boggiatto, P. M., & Petersen, C. A. Disseminated *Leishmania infantum* infection in two sibling foxhounds due to possible vertical transmission. *Can. vet. journal* **2008**, *49*(10), 1005–1008.

80. Esch, K.J.; Schaut, R.G.; Lamb, I.M.; Clay, G.; Lima, L.M.; Nascimento,

R.P.; Whitley, E.M.; Jeronimo, S.M.B.; Sutterwala, F.S.; Haynes, J.S.; et al. Activation of Autophagy and Nucleotide-Binding Domain Leucine-Rich Repeat-Containing-Like Receptor Family, Pyrin Domain-Containing 3 Inflammasome during *Leishmania Infantum*-Associated Glomerulonephritis. *Am. J. Pathol.* **2015**, *185*, 2105–2117.

81. Cacheiro-llaguno, C.; Parody, N.; Escutia, M.R.; Carnés, J. Role of Circulating Immune Complexes in the Pathogenesis of Canine Leishmaniasis: New Players in Vaccine Development. *Microorganisms* **2021**, *9*, 712.

82. Solano-Gallego, L.; Cardoso, L.; Pennisi, M.G.; Petersen, C.; Bourdeau, P.; Oliva, G.; Miró, G.; Ferrer, L.; Baneth, G. Diagnostic Challenges in the Era of Canine *Leishmania Infantum* Vaccines. *Trends parasitol.* **2017**, *33*(9), 706–717.

83. Gibson-corley, K.N.; Boggiatto, P.M.; Mukbel, R.M.; Petersen, C.A.; Jones, D.E. A Deficiency in the B Cell Response of C57BL / 6 Mice Correlates with Loss of Macrophage-Mediated Killing of *Leishmania Amazonensis*. *Int. J. Parasitol.* **2010**, *40*, 157–161.

84. Vlugt, L.E.P.M. Van Der; Mlejnek, E.; Bonas, M.J.; Dijksman, T.R.; Labuda, L.A.; Schot, R. CD24 Hi CD27 + B Cells from Patients with Allergic Asthma Have Impaired Regulatory Activity in Response to Lipopolysaccharide Experimental Allergy. *Clin. Exp. Allergy* **2013**, *44*, 517–528.

85. Wang, R.; Yu, C.; Dambuza, I.M.; Mahdi, R.M.; Dolinska, M.B.; Sergeev, Y. V; Wingfield, P.T.; Kim, S.; Egwuagu, C.E. Interleukin-35 Induces Regulatory B Cells That Suppress Autoimmune Disease. *Nat. Med.* **2014**, *20*, 633–641.

86. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological Findings and Detection of Toll-like Receptor 2 in Cutaneous Lesions of Canine Leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163.

87. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular Dermatitis Due to *Leishmania Infantum* Infection in Seventeen Dogs: Diagnostic Features, Extent of the Infection and Treatment Outcome. *Parasites and Vectors* **2014**, *7*, 1–11.

88. Dea-Ayuela, M.A.; Segarra, S.; Serrano, D.R.; Bolás-Fernández, F. Nucleotides and AHCC Enhance Th1 Responses in Vitro in *Leishmania*-Stimulated/Infected Murine Cells. *Molecules* **2020**, *25*, 3918.

89. Ordeix, L.; Rodríguez, A.; Martínez-Orellana, Pamela Montserrat Sangrà, S.; Solano-Gallego, L. Clinical Follow up of a Series of Dogs with Papular Dermatitis Due to *Leishmania Infantum*. *In: 29th Annual Congress of the ESVD-ECVD,* **2017**.

90. Garza-Tovar, T.F.; Sacriste-Hernández, M.I.; Juárez-Durán, E.R.; Arenas, R. An Overview of the Treatment of Cutaneous Leishmaniasis. *Fac. Rev.* **2020**, *9*, 28.

91. Solano-Gallego, L. *La Leishmaniosis*. Ed: SERVET. 2023.

92. Miró, G.; Petersen, C.; Cardoso, L.; Bourdeau, P.; Baneth, G.; Solano-Gallego, L.; Pennisi, M.G.; Ferrer, L.; Oliva, G. Novel Areas for Prevention and Control of Canine Leishmaniosis. *Trends Parasitol.* **2017**, *33*, 718–730.

93. Frézard, F.; Demicheli, C.; Ribeiro, R.R. Pentavalent Antimonials: New Perspectives for Old Drugs. *Molecules* **2009**, *14*, 2317–2336.

94. Shaked-Mishant, P.; Ulrich, N.; Ephros, M.; Zilberstein, D. Novel Intracellular SbV Reducing Activity Correlates with Antimony Susceptibility in *Leishmania Donovani*. *J. Biol. Chem.* **2001**, *276*, 3971–3976.

95. Dos Santos Ferreira, C.; Silveira Martins, P.; Demicheli, C.; Brochu, C.; Ouellette, M.; Frézard, F. Thiol-Induced Reduction of Antimony(V) into Antimony(III): A Comparative Study with Trypanothione, Cysteinyl-Glycine, Cysteine and Glutathione. *Biometals* **2003**, *16*, 441–446.

96. Frézard, F.; Schettini, D.A.; Rocha, O.G.F.; Demicheli, C. Lipossomas: Propriedades Físico-Químicas e Farmacológicas, Aplicações Na Quimioterapia à Base de Antimônio. *Quim. Nova* **2005**, *28*, 511–518.

97. De Lima, E.B.; Da Motta, J.O.C.; Porto, C.; Sampaio, R.N.R. Tratamento Da Leishmaniose Tegumentar Americana. *An. Bras. Dermatol.* **2007**, *82*, 111–124.

98. Friedrich, K.; Vieira, F.A.; Porrozzi, R.; Marchevsky, R.S.; Miekeley, N.; Grimaldi, G.; Paumgartten, F.J.R. Disposition of Antimony in Rhesus Monkeys Infected with *Leishmania Braziliensis* and Treated with Meglumine Antimoniate. *J. Toxicol. Environ. Health. A* **2012**, *75*, 63–75.

99. Frézard, F.; Demicheli, C.; Ferreira, C.S.; Costa, M.A.P. Glutathione-Induced Conversion of Pentavalent Antimony to Trivalent Antimony in Meglumine Antimoniate. *Antimicrob. Agents Chemother.* **2001**, *45*, 913–916.

100. Roatt, B.M.; de Oliveira Cardoso, J.M.; De Brito, R.C.F.; Coura-Vital, W.; de Oliveira Aguiar-Soares, R.D.; Reis, A.B. Recent Advances and New Strategies on Leishmaniasis Treatment. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 8965–8977.

101. Yan, S.; Li, F.; Ding, K.; Sun, H. Reduction of Pentavalent Antimony by Trypanothione and Formation of a Binary and Ternary Complex of Antimony(III) and Trypanothione. *J. Biol. Inorg. Chem.* **2003**, *8*, 689–697.

102. Sun, H.; Yan, S.C.; Cheng, W.S. Interaction of Antimony Tartrate with the Tripeptide Glutathione Implication for Its Mode of Action. *Eur. J. Biochem.* **2000**, *267*, 5450–5457.

103. Moreira, V.R.; De Jesus, L.C.L.; Soares, R.E.P.; Silva, L.D.M.; Pinto, B.A.S.; Melo, M.N.; De AndradePaes, A.M.; Pereira, S.R.F. Meglumine Antimoniate (Glucantime) Causes Oxidative Stress-Derived DNA Damage in BALB/c Mice Infected by *Leishmania* (*Leishmania*) *Infantum*. *Antimicrob. Agents Chemother.* **2017**, *61*, e02360-16.

104. Wyllie, S.; Cunningham, M.L.; Fairlamb, A.H. Dual Action of Antimonial Drugs on Thiol Redox Metabolism in the Human Pathogen *Leishmania Donovani*. *J. Biol. Chem.* **2004**, *279*, 39925–39932.

105. Rais, S.; Perianin, A.; Lenoir, M.; Sadak, A.; Rivollet, D.; Paul, M.; Deniau, M. Sodium Stibogluconate (Pentostam) Potentiates Oxidant Production in Murine Visceral Leishmaniasis and in Human Blood. *Antimicrob. Agents Chemother.* **2000**, *44*, 2406–2410.

106. Basu, J.M.; Mookerjee, A.; Sen, P.; Bhaumik, S.; Sen, P.; Banerjee, S.; Naskar, K.; Choudhuri, S.K.; Saha, B.; Raha, S.; et al. Sodium Antimony Gluconate Induces Generation of Reactive Oxygen Species and Nitric Oxide via Phosphoinositide 3-Kinase and Mitogen-Activated Protein Kinase Activation in *Leishmania Donovani*-Infected Macrophages. *Antimicrob. Agents Chemother.* **2006**, *50*, 1788–1797.

107. Bento, D.B.; de Souza, B.; Steckert, A. V.; Dias, R.O.; Leffa, D.D.; Moreno, S.E.; Petronilho, F.; de Andrade, V.M.; Dal-Pizzol, F.; Romão, P.R. Oxidative Stress in Mice Treated with Antileishmanial Meglumine Antimoniate. *Res. Vet. Sci.* **2013**, *95*, 1134–1141.

108. Daza González, M.A.; Miró, G.; Fermín Rodríguez, M.; Rupérez Noguer, C.; Fragío Arnold, C. Short Term Impacts of Meglumine Antimoniate Treatment on Kidney Function in Dogs with Clinical Leishmaniosis. *Res. Vet. Sci.* **2019**, *126*, 131–138.

109. Ikeda-Garcia, F.A.; Lopes, R.S.; Ciarlini, P.C.; Marques, F.J.; Lima, V.M.F.; Perri, S.H.V.; Feitosa, M.M. Evaluation of Renal and Hepatic Functions in Dogs Naturally Infected by Visceral Leishmaniasis Submitted to Treatment with Meglumine Antimoniate. *Res. Vet. Sci.* **2007**, *83*, 105–108.

110. Mateo, M.; Maynard, L.; Vischer, C.; Bianciardi, P.; Miró, G. Comparative Study on the Short Term Efficacy and Adverse Effects of Miltefosine and Meglumine Antimoniate in Dogs with Natural Leishmaniosis. *Parasitol. Res.* **2009**, *105*, 155–162.

111. Zatelli, A.; Borgarelli, M.; Santilli, R.; Bonfanti, U.; Nigrisoli, E.; Zanatta, R.; Tarducci, A.; Guarraci, A. Glomerular Lesions in Dogs Infected with *Leishmania* Organisms. *Am. J. Vet. Res.* **2003**, *64*, 558–561.

112. Pardo-Marín, L.; Martínez-Subiela, S.; Pastor, J.; Tvarijonaviciute, A.; Garcia-Martinez, J.D.; Segarra, S.; Cerón, J.J. Evaluation of Various Biomarkers for Kidney Monitoring during Canine Leishmaniosis Treatment. *BMC Vet. Res.* **2017**, *13*, 31.

113. Paltrinieri, S.; Mangiagalli, G.; Ibba, F. Use of Urinary γ-Glutamyl Transferase (GGT) to Monitor the Pattern of Proteinuria in Dogs with Leishmaniasis Treated with N-Methylglucamine Antimoniate. *Res. Vet. Sci.* **2018**, *119*, 52–55.

114. Bianciardi, P.; Brovida, C.; Valente, M.; Aresu, L.; Cavicchioli, L.; Vischer, C.; Giroud, L.; Castagnaro, M. Administration of Miltefosine and Meglumine Antimoniate in Healthy Dogs: Clinicopathological Evaluation of the Impact on the Kidneys. *Toxicol. Pathol.* **2009**, *37*, 770–775.

115. Solano-Gallego, L.; Koutinas, A.; Miró, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. Directions for the Diagnosis, Clinical Staging, Treatment and Prevention of Canine Leishmaniosis. *Vet. Parasitol.* **2009**, *165*, 1–18.

116. Valladares, J.E.; Riera, C.; Alberola, J.; Gállego, M.; Portús, M.; Cristòfol, C.; Franquelo, C.; Arboix, M. Pharmacokinetics of Meglumine Antimoniate after Administration of a Multiple Dose in Dogs Experimentally Infected with *Leishmania Infantum*. *Vet. Parasitol.* **1998**, *75*, 33–40.

117. Valladares, J.E.; Alberola, J.; Esteban, M.; Arboix, M. Disposition of Antimony after the Administration of N-Methylglucamine Antimoniate to Dogs. *Vet. Rec.* **1996**, *138*, 181–183.

118. Dorlo, T.P.C.; Balasegaram, M.; Beijnen, J.H.; Vries, P.J. De Miltefosine: A Review of Its Pharmacology and Therapeutic Efficacy in the Treatment of Leishmaniasis. **2012**, 2576–2597.

119. Croft, S.L.; Neal, R.A.; Pendergast, W.; Chan, J.H. The Activity of Alkyl Phosphorylcholines and Related Derivatives against *Leishmania Donovani*. *Biochem. Pharmacol.* **1987**, *36*, 2633–2636.

120. Lux, H.; Hart, D.T.; Parker, P.J.; Klenner, T. Ether Lipid Metabolism, GPI Anchor Biosynthesis, and Signal Transduction Are Putative Targets for Anti-Leishmanial Alkyl Phospholipid Analogues. *Adv. Exp. Med. Biol.* **1996**, *416*, 201–211.

121. Rakotomanga, M.; Saint-Pierre-Chazalet, M.; Loiseau, P.M. Alteration of Fatty Acid and Sterol Metabolism in Miltefosine-Resistant *Leishmania Donovani* Promastigotes and Consequences for Drug-Membrane Interactions. *Antimicrob. Agents Chemother.* **2005**, *49*, 2677–2686.

122. Paris, C.; Loiseau, P.M.; Bories, C.; Bréard, J. Miltefosine Induces Apoptosis-like Death in *Leishmania Donovani* Promastigotes. *Antimicrob. Agents Chemother.* **2004**, *48*, 852–859.

123. Wadhone, P.; Maiti, M.; Agarwal, R.; Kamat, V.; Martin, S.; Saha, B. Miltefosine Promotes IFN-Gamma-Dominated Anti-Leishmanial Immune Response. *J. Immunol.* **2009**, *182*, 7146–7154.

124. Morales-Yuste, M.; Martín-Sánchez, J.; Corpas-Lopez, V. Canine Leishmaniasis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. *Vet. Sci.* **2022**, *9*, 387.

125. Pierantozzi, M.; Roura, X.; Paltrinieri, S.; Poggi, M.; Zatelli, A. Variation of Proteinuria in Dogs with Leishmaniasis Treated with Meglumine Antimoniate and Allopurinol: A Retrospective Study. *J. Am. Anim. Hosp. Assoc.* **2013**, *49*(4), 231–236.

126. Iarussi, F.; Paradies, P.; Foglia Manzillo, V.; Gizzarelli, M.; Caratozzolo, M.F.; Navarro, C.; Greco, B.; Rubino, G.T.R.; Oliva, G.; Sasanelli, M. Comparison of Two Dosing Regimens of Miltefosine, Both in Combination With Allopurinol, on Clinical and Parasitological Findings of Dogs With Leishmaniosis: A Pilot Study. *Front. Vet. Sci.* **2020**, *7*, 1068.

127. Gonçalves, G.; Campos, M.P.; Gonçalves, A.S.; Medeiros, L.C.S.; Figueiredo, F.B. Increased *Leishmania Infantum* Resistance to Miltefosine and Amphotericin B after Treatment of a Dog with Miltefosine and Allopurinol. *Parasites and Vectors* **2021**, *14*, 1–8.

128. Soto, J.A.; Berman, J.D. Miltefosine Treatment of Cutaneous Leishmaniasis. **2021**, *73*, 2463–2464.

129. Miró, G.; Oliva, G.; Cruz, I.; Cañavate, C.; Mortarino, M.; Vischer, C.; Bianciardi, P. Multicentric, Controlled Clinical Study to Evaluate Effectiveness and Safety of Miltefosine and Allopurinol for Canine Leishmaniosis. *Vet. Dermatol.* **2009**, *20*, 397–404.

130. Pfaller, M.A.; Marr, J.J. Antileishmanial Effect of Allopurinol. *Antimicrob. Agents Chemother.* **1974**, *5*, 469.

131. Baneth, G.; Shaw, S.E. Chemotherapy of Canine Leishmaniosis. **2002**, *106*, 315–324.

132. Croft, S.L.; Sundar, S.; Fairlamb, A.H. Drug Resistance in Leishmaniasis. *Clin. Microbiol. Rev.* **2006**, *19*, 111–126.

133. Yasur-Landau, D.; Jaffe, C.L.; David, L.; Baneth, G. Allopurinol Resistance in *Leishmania Infantum* from Dogs with Disease Relapse. *PLoS Negl. Trop. Dis.* **2016**, *10*, 1–13.

134. Jesus, L.; Arenas, C.; Domínguez-Ruiz, M.; Silvestrini, P.; Englar, R.E.; Roura, X.; Oliveira Leal, R. Xanthinuria Secondary to Allopurinol Treatment in Dogs with Leishmaniosis: Current Perspectives of the Iberian Veterinary Community. *Comp. Immunol. Microbiol. Infect. Dis.* **2022**, *83*, 101783.

135. Kasabalis, D.; Chatzis, M.K.; Apostolidis, K.; Petanides, T.; Athanasiou, L. V.; Xenoulis, P.G.; Mataragka, A.; Ikonomopoulos, J.; Leontides, L.S.; Saridomichelakis, M.N. A Randomized, Blinded, Controlled Clinical Trial Comparing the Efficacy of Aminosidine (Paromomycin)-Allopurinol Combination with the Efficacy of Meglumine Antimoniate-Allopurinol Combination for the Treatment of Canine Leishmaniosis Due to *Leishmania Inf. Exp. Parasitol.* **2020**, *214*, 107903.

136. Noli, C.; Auxilia, S.T. Treatment of Canine Old World Visceral Leishmaniasis: A Systematic Review. *Vet. Dermatol.* **2005**, *16*, 213–232.

137. Athanasiou, L. V.; Batzias, G.C.; Saridomichelakis, M.N.; Delis, G.; Soubasis, N.; Kontos, V.I.; Rallis, T.S. Pharmacokinetics and Tolerability of Aminosidine after Repeated Administrations Using an Optimal Dose Regimen in Healthy Dogs and in Dogs with Leishmaniosis. *Vet. Parasitol.* **2014**, *205*, 365–370.

138. Hendrickx, S.; Van Bockstal, L.; Aslan, H.; Sadlova, J.; Maes, L.; Volf, P.; Caljon, G. Transmission Potential of Paromomycin-Resistant *Leishmania Infantum* and *Leishmania Donovani*. **2019**, *75*(4), 951–957.

139. Marr, A.K.; McGwire, B.S.; McMaster, W.R. Modes of Action of Leishmanicidal Antimicrobial Peptides. *Future Microbiol.* **2012**, *7*, 1047–1059.

140. Loo, C.S.N.; Lam, N.S.K.; Yu, D.; Su, X. zhuan; Lu, F. Artemisinin and Its Derivatives in Treating Protozoan Infections beyond Malaria. *Pharmacol. Res.* **2017**, *117*, 192–217.

141. Cortes, S.; Albuquerque, A.; Cabral, L.I.L.; Lopes, L.; Campino, L.; Cristiano, M.L.S. In Vitro Susceptibility of *Leishmania Infantum* to Artemisinin Derivatives and Selected Trioxolanes. *Antimicrob. Agents Chemother.* **2015**, *59*, 5032–5035.

142. Want, M.Y.; Islammudin, M.; Chouhan, G.; Ozbak, H.A.; Hemeg, H.A.; Chattopadhyay, A.P.; Afrin, F. Nanoliposomal Artemisinin for the Treatment of Murine Visceral Leishmaniasis. *Int. J. Nanomedicine* **2017**, *12*, 2189–2204.

143. Medkour, H.; Bitam, I.; Laidoudi, Y.; Lafri, I.; Lounas, A.; Hamidat, H.K.; Mekroud, A.; Varloud, M.; Davoust, B.; Mediannikov, O. Potential of Artesunate in the Treatment of Visceral Leishmaniasis in Dogs Naturally Infected by *Leishmania Infantum*: Efficacy Evidence from a Randomized Field Trial. *PLoS Negl. Trop. Dis.* **2020**, *14*, e0008947.

144. Baxarias, M.; Martínez-Orellana, P.; Baneth, G.; Solano-Gallego, L. Immunotherapy in Clinical Canine Leishmaniosis: A Comparative Update. *Res. Vet. Sci.* **2019**, *125*, 218–226.

145. Baxarias, M.; Homedes, J.; Mateu, C.; Attipa, C.; Solano-Gallego, L. Use of Preventive Measures and Serological Screening Tools for *Leishmania Infantum* Infection in Dogs from Europe. *Parasit. Vectors* **2022**, *15*, 134.

146. Sabaté, D.; Llinás, J.; Homedes, J.; Sust, M.; Ferrer, L. A Single-Centre, Open-Label, Controlled, Randomized Clinical Trial to Assess the Preventive Efficacy of a Domperidone-Based Treatment Programme against Clinical Canine Leishmaniasis in a High Prevalence Area. *Prev. Vet. Med.* **2014**, *115*, 56–63.

147. Segarra, S. Nutritional Modulation of the Immune Response Mediated by Nucleotides in Canine Leishmaniosis. *Microorganisms* **2021**, *9*, 2601.

148. Pinart, M.; Rueda, J.R.; Romero, G.A.S.; Pinzón-Flórez, C.E.; Osorio-Arango, K.; Silveira Maia-Elkhoury, A.N.; Reveiz, L.; Elias, V.M.; Tweed, J.A. Interventions for American Cutaneous and Mucocutaneous Leishmaniasis. *Cochrane Database Syst. Rev.* **2020**, *8*(8), CD004834.

149. Badaro, R.; Falcoff, E.; Badaro, F.S.; Carvalho, E.M.; Pedral-Sampaio, D.; Barral, A.; Carvalho, J.S.; Barral-Netto, M.; Brandely, M.; Silva, L.; et al. Treatment of Visceral Leishmaniasis with Pentavalent Antimony and Interferon Gamma. *N. Engl. J. Med.* **1990**, *322*, 16–21.

150. Sundar, S.; Murray, H.W. Effect of Treatment with Interferon-Gamma Alone in Visceral Leishmaniasis. *J. Infect. Dis.* **1995**, *172*, 1627–1629.

151. Hernández Martínez, L. Estudio de La Infección Por *Leishmania Infantum* En El Perro: Utilidad de Las Técnicas Diagnósticas No Invasivas y Nuevas Alternativas Terapéuticas. *In: Tesis doctoral Universidad Complutense de Madrid* **2016**.

152. Vitoriano-Souza, J.; Moreira, N. das D.; Menezes-Souza, D.; Roatt, B.M.; De Oliveira Aguiar-Soares, R.D.; Siqueira-Mathias, F.A.; De Oliveira Cardoso, J.M.; Giunchetti, R.C.; De Sá, R.G.; Corrêa-Oliveira, R.; et al. Dogs Immunized with LBSap Vaccine Displayed High Levels of IL-12 and IL-10 Cytokines and CCL4, CCL5 and CXCL8 Chemokines in the Dermis. *Mol. Immunol.* **2013**, *56*, 540–548.

153. Borja-Cabrera, G.P.; Mendes, A.C.; Paraguai De Souza, E.; Okada, L.Y.H.; Trivellato, F.A.D.A.; Kawasaki, J.K.A.; Costa, A.C.; Reis, A.B.; Genaro, O.; Batista, L.M.M.; et al. Effective Immunotherapy against Canine Visceral Leishmaniasis with the FML-Vaccine. *Vaccine* **2004**, *22*, 2234–2243.

154. Borja-Cabrera, G.P.; Santos, F.N.; Santos, F.B.; Trivellato, F.A. de A.; Kawasaki, J.K.A.; Costa, A.C.; Castro, T.; Nogueira, F.S.; Moreira, M.A.B.; Luvizotto, M.C.R.; et al. Immunotherapy with the Saponin Enriched-Leishmune® Vaccine versus Immunochemotherapy in Dogs with Natural Canine Visceral Leishmaniasis. *Vaccine* **2010**, *28*, 597–603.

155. Santos, F.N.; Borja-Cabrera, G.P.; Miyashiro, L.M.; Grechi, J.; Reis, A.B.; Moreira, M.A.B.; Martins Filho, O.A.; Luvizotto, M.C.R.; Menz, I.; Pessôa, L.M.; et al. Immunotherapy against Experimental Canine Visceral Leishmaniasis with the Saponin Enriched-Leishmune® Vaccine. *Vaccine* **2007**, *25*, 6176–6190.

156. Viana, K.F.; Lacerda, G.; Teixeira, N.S.; Rodrigues Cangussu, A.S.; Sousa Aguiar, R.W.; Giunchetti, R.C. Therapeutic Vaccine of Killed *Leishmania Amazonensis* plus Saponin Reduced Parasite Burden in Dogs Naturally Infected with *Leishmania Infantum*. *Vet. Parasitol.* **2018**, *254*, 98–104.

157. Roatt, B.M.; Aguiar-Soares, R.D. de O.; Reis, L.E.S.; Cardoso, J.M. de O.; Mathias, F.A.S.; de Brito, R.C.F.; da Silva, S.M.; Gontijo, N.D.F.; Ferreira, S. de A.; Valenzuela, J.G.; et al. A Vaccine Therapy for Canine Visceral Leishmaniasis Promoted Significant Improvement of Clinical and Immune Status with Reduction in Parasite Burden. *Front. Immunol.* **2017**, *8*, 232017.

158. Trigo, J.; Abbehusen, M.; Netto, E.M.; Nakatani, M.; Pedral-Sampaio, G.; de Jesus, R.S.; Goto, Y.; Guderian, J.; Howard, R.F.; Reed, S.G. Treatment of Canine Visceral Leishmaniasis by the Vaccine Leish-111f + MPL-SE. *Vaccine* **2010**, *28*, 3333–3340.

159. Toepp, A.; Larson, M.; Tara, G.P.; Bennett, C.; Anderson, M.; Parrish, M.; Fowler, H.; Wilson, G.; Gibson-Corely, K.; Gharpure, R.; et al. Safety Analysis of *Leishmania* Vaccine Used in a Randomized Canine Vaccine/Immunotherapy Trial. *Am. J. Trop. Med. Hyg.* **2018**, *98*, 1332–1338.

160. Ferreira, J.H.L.; Silva, L. dos S.; Longo-Maugéri, I.M.; Katz, S.; Barbiéri, C.L. Use of a Recombinant Cysteine Proteinase from *Leishmania (Leishmania) Infantum Chagasi* for the Immunotherapy of Canine Visceral Leishmaniasis. *PLoS Negl. Trop. Dis.* **2014**, *8*, e2729.

161. Nafari, A.; Cheraghipour, K.; Sepahvand, M.; Shahrokhi, G.; Gabal, E.; Mahmoudvand, H. Nanoparticles: New Agents toward Treatment of Leishmaniasis. *Parasite Epidemiol. Control* **2020**, *10*, e00156.

162. Assolini, J.P.; Carloto, A.C.M.; Bortoleti, B.T. da S.; Gonçalves, M.D.; Tomiotto Pellissier, F.; Feuser, P.E.; Cordeiro, A.P.; Hermes de Araújo, P.H.; Sayer, C.; Miranda Sapla, M.M.; et al. Nanomedicine in Leishmaniasis: A Promising Tool for Diagnosis, Treatment and Prevention of Disease - An Update Overview. *Eur. J. Pharmacol.* **2022**, *923*, 174934.

163. Zahin, N.; Anwar, R.; Tewari, D.; Kabir, M.T.; Sajid, A.; Mathew, B.; Uddin, M.S.; Aleya, L.; Abdel-Daim, M.M. Nanoparticles and Its Biomedical Applications in Health and Diseases: Special Focus on Drug Delivery. *Environ. Sci. Pollut. Res. Int.* **2020**, *27*, 19151–19168.

164. dos Santos Ramos, M.A.; dos Santos, K.C.; da Silva, P.B.; de Toledo, L.G.; Marena, G.D.; Rodero, C.F.; de Camargo, B.A.F.; Fortunato, G.C.; Bauab, T.M.; Chorilli, M. Nanotechnological Strategies for Systemic Microbial Infections Treatment: A Review. *Int. J. Pharm.* **2020**, *589*, 119780.

165. Assolini, J.P.; Concato, V.M.; Gonçalves, M.D.; Carloto, A.C.M.; Conchon-Costa, I.; Pavanelli, W.R.; Melanda, F.N.; Costa, I.N. Nanomedicine Advances in Toxoplasmosis: Diagnostic, Treatment, and Vaccine Applications. *Parasitol. Res.* **2017**, *116*, 1603–1615.

166. Gutiérrez, V.; Seabra, A.B.; Reguera, R.M.; Khandare, J.; Calderón, M. New Approaches from Nanomedicine for Treating Leishmaniasis. *Chem. Soc. Rev.* **2016**, *45*, 152–168.

167. Wang, E.C.; Wang, A.Z. Nanoparticles and Their Applications in Cell and Molecular Biology. *Integr. Biol. (Camb).* **2014**, *6*, 9–26.

168. Martínez-Flórez I, Guerrero MJ, Dalmau A, Cabré M, Alcover MM, Berenguer D, Good L, Fisa R, Riera C, Ordeix L, Solano-Gallego L. Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis Due to *Leishmania Infantum* in Dogs. *Pathog.* **2023**, *12*, 821.

169. Garza-tovar, T.F.; Sacriste-hernández, M.I. An Overview of the Treatment of Cutaneous Leishmaniasis. *Faculty reviews* **2020,** *9*, 28.

170. Uribe-Restrepo, A.F.; Prieto, M.D.; Cossio, A.; Desai, M.M.; Del Mar Castro, M. Eligibility for Local Therapies in Adolescents and Adults with Cutaneous Leishmaniasis from Southwestern Colombia: A Cross-Sectional Study. *Am. J. Trop. Med. Hyg.* **2019**, *100*, 306–310.

171. Nassif, P.W.; De Mello, T.F.P.; Navasconi, T.R.; Mota, C.A.; Demarchi, I.G.; Aristides, S.M.A.; Lonardoni, M.V.C.; Teixeira, J.J.V.; Silveira, T.G.V. Safety and Efficacy of Current Alternatives in the Topical Treatment of Cutaneous Leishmaniasis: A Systematic Review. *Parasitology* **2017**, *144*, 995–1004.

172. Piragauta, S.P.; Higuita-Castro, · J L; Arbeláez, · N; Restrepo, · A M; Archbold, · R; Quiñones, · W; Torres, · F; Echeverri, · F; Escobar, · G; Vélez, · I D; et al. Utility of the Combination of Hederagenin Glucoside Saponins and Chromane Hydrazone in the Topical Treatment of Canine Cutaneous Leishmaniasis. An Observational Study. *Parasitol. Res.* **2022**, *121*, 1419–1428.

173. Apostolopoulos, N.; Mitropoulou, A.; Thom, N.; Moritz, A. Update on Therapy and Prevention of Canine Leishmaniasis. *Tierarztl. Prax. Ausg. K. Kleintiere. Heimtiere.* **2018**, *46*, 1–7.

174. Killick-Kendrick, R.; Killick-Kendrick, M.; Focheux, C.; Dereure, J.; Puech, M.P.; Cadiergues, M.C. Protection of Dogs from Bites of Phlebotomine Sandflies by Deltamethrin Collars for Control of Canine Leishmaniasis. *Med. Vet. Entomol.* **1997**, *11*, 105–111.

175. Miró, G.; Gálvez, R.; Mateo, M.; Montoya, A.; Descalzo, M.A.; Molina, R. Evaluation of the Efficacy of a Topically Administered Combination of Imidacloprid and Permethrin against Phlebotomus Perniciosus in Dog. *Vet. Parasitol.* **2007**, *143*, 375–379.

176. Mencke, N.; Volf, P.; Volfova, V.; Stanneck, D. Repellent Efficacy of a Combination Containing Imidacloprid and Permethrin against Sand Flies (Phlebotomus Papatasi) in Dogs. *Parasitol. Res.* **2003**, *90*, 108–111.

177. Otranto, D.; Paradies, P.; Lia, R.P.; Latrofa, M.S.; Testini, G.; Cantacessi, C.; Mencke, N.; Galli, G.; Capelli, G.; Stanneck, D. Efficacy of a Combination of 10% Imidacloprid/50% Permethrin for the Prevention of Leishmaniasis in Kennelled Dogs in an Endemic Area. *Vet. Parasitol.* **2007**, *144*, 270–278.

178. Maroli, M.; Mizzoni, V.; Siragusa, C.; D'Orazi, A.; Gradoni, L. Evidence for an Impact on the Incidence of Canine Leishmaniasis by the Mass Use of Deltamethrin-Impregnated Dog Collars in Southern Italy. *Med. Vet. Entomol.* **2001**, *15*, 358–363.

179. Foglia Manzillo, V.; Oliva, G.; Pagano, A.; Manna, L.; Maroli, M.; Gradoni, L. Deltamethrin-Impregnated Collars for the Control of Canine Leishmaniasis: Evaluation of the Protective Effect and Influence on the Clinical Outcome of *Leishmania* Infection in Kennelled Stray Dogs. *Vet. Parasitol.* **2006**, *142*, 142–145.

180. Otranto, D.; de Caprariis, D.; Lia, R.P.; Tarallo, V.; Lorusso, V.; Testini, G.; Dantas-Torres, F.; Latrofa, S.; Diniz, P.P.V.P.; Mencke, N.; et al. Prevention of Endemic Canine Vector-Borne Diseases Using Imidacloprid 10% and Permethrin 50% in Young Dogs: A Longitudinal Field Study. *Vet. Parasitol.* **2010**, *172*, 323–332.

181. Mazloumi Gavgani, A.S.; Hodjati, M.H.; Mohite, H.; Davies, C.R. Effect of Insecticide-Impregnated Dog Collars on Incidence of Zoonotic Visceral Leishmaniasis in Iranian Children: A Matched-Cluster Randomised Trial. *Lancet* **2002**, *360*, 374–379.

182. Gradoni, L. Canine *Leishmania* Vaccines: Still a Long Way to Go. *Vet. Parasitol.* **2015**, *208*, 94–100.

183. Engwerda, C.R.; Matlashewski, G. Development of *Leishmania* Vaccines in the Era of Visceral Leishmaniasis Elimination. *Trans. R. Soc. Trop. Med. Hyg.* **2015**, *109*, 423–424.

184. Kaye, P.M.; Aebischer, T. Visceral Leishmaniasis: Immunology and Prospects for a Vaccine. *Clin. Microbiol. Infect.* **2011**, *17*, 1462–1470.

185. Velez, R.; Gállego, M. Commercially Approved Vaccines for Canine Leishmaniosis: A Review of Available Data on Their Safety and Efficacy. *Trop. Med. Int. Health* **2020**, *25*, 540–557.

186. Borja-Cabrera, G.P.; Correia Pontes, N.N.; Da Silva, V.O.; Paraguai De Souza, E.; Santos, W.R.; Gomes, E.M.; Luz, K.G.; Palatnik, M.; Palatnik De Sousa, C.B. Long Lasting Protection against Canine Kala-Azar Using the FML-QuilA Saponin Vaccine in an Endemic Area of Brazil (São Gonçalo Do Amarante, RN). *Vaccine* **2002**, *20*, 3277–3284.

187. Santos, W.R.; Paraguai de Souza, E.; Palatnik, M.; Palatnik de Sousa, C.B. Vaccination of Swiss Albino Mice against Experimental Visceral Leishmaniasis with the FML Antigen of *Leishmania Donovani*. *Vaccine* **1999**, *17*, 2554–2561.

188. Palatnik-de-Sousa, C.; Paraguai-de-Souza, E.; Gomes, E.; Borojevic, R. Experimental Murine *Leishmania Donovani* Infection: Immunoprotection by the Fucose-Mannose Ligand (FML). *Braz J Med Biol Res.* **1994**, *27*, 547–551.

189. Palatnik de Sousa, C.B.; Gomes, E.M.; Paraguai De Souza, E.; Dos Santos, W.R.; De Macedo, S.R.; De Medeiros, L. V.; Luz, K. The FML (Fucose Mannose Ligand) of *Leishmania Donovani*. a New Tool in Diagnosis, Prognosis, Transfusional Control and Vaccination against Human Kala-Azar. *Rev. Soc. Bras. Med. Trop.* **1996**, *29*, 153–163.

190. Paraguai de Souza, E.; Bernardo, R.R.; Palatnik, M.; Palatnik de Sousa, C.B. Vaccination of Balb/c Mice against Experimental Visceral Leishmaniasis with the GP36 Glycoprotein Antigen of *Leishmania Donovani*. *Vaccine* **2001**, *19*, 3104–3115.

191. Aguilar-Be, I.; Da Silva Zardo, R.; De Souza, E.P.; Borja-Cabrera, G.P.; Rosado-Vallado, M.; Mut-Martin, M.; García-Miss, M.D.R.; Palatnik De Sousa, C.B.; Dumonteil, E. Cross-Protective Efficacy of a Prophylactic *Leishmania Donovani* DNA Vaccine against Visceral and Cutaneous Murine Leishmaniasis. *Infect. Immun.* **2005**, *73*, 812–819.

192. Da Silva, V.O.; Borja-Cabrera, G.P.; Correia Pontes, N.N.; De Souza, E.P.; Luz, K.G.; Palatnik, M.; Palatnik De Sousa, C.B. A Phase III Trial of Efficacy of the FML-Vaccine against Canine Kala-Azar in an Endemic Area of Brazil (São Gonçalo Do Amaranto, RN). *Vaccine* **2000**, *19*, 1082–1092.

193. Parra, L.E.; Borja-Cabrera, G.P.; Santos, F.N.; Souza, L.O.P.; Palatnik-de-Sousa, C.B.; Menz, I. Safety Trial Using the Leishmune Vaccine against Canine Visceral Leishmaniasis in Brazil. *Vaccine* **2007**, *25*, 2180–2186.

194. Borja-Cabrera, G.P.; Santos, F.N.; Bauer, F.S.; Parra, L.E.; Menz, I.; Morgado, A.A.; Soares, I.S.; Batista, L.M.M.; Palatnik-de-Sousa, C.B. Immunogenicity Assay of the Leishmune Vaccine against Canine Visceral Leishmaniasis in Brazil. *Vaccine* **2008**, *26*, 4991–4997.

195. Araújo, M.S.S.; de Andrade, R.A.; Sathler-Avelar, R.; Teixeira-Carvalho, A.; Andrade, M.C.; Vianna, L.R.; Mayrink, W.; Reis, A.B.; Malaquias, L.C.C.; Mello, M.N.; et al. T-Cell-Derived Cytokines, Nitric Oxide Production by Peripheral Blood Monocytes and Seric Anti-*Leishmania* (*Leishmania*) *Chagasi* IgG Subclass Patterns Following Immunization against Canine Visceral Leishmaniasis Using Leishvaccine and Leishmune. *Vaccine* **2009**, *27*, 1008–1017.

196. Araújo, M.S.S.; de Andrade, R.A.; Sathler-Avelar, R.; Magalhães, C.P.; Carvalho, A.T.; Andrade, M.C.; Campolina, S.S.; Mello, M.N.; Vianna, L.R.; Mayrink, W.; et al. Immunological Changes in Canine Peripheral Blood Leukocytes Triggered by Immunization with First or Second Generation Vaccines against Canine Visceral Leishmaniasis. *Vet. Immunol. Immunopathol.* **2011**, *141*, 64–75.

197. de Lima, V.M.F.; Ikeda, F.A.; Rossi, C.N.; Feitosa, M.M.; de Oliveira Vasconcelos, R.; Nunes, C.M.; Goto, H. Diminished CD4+/CD25+ T Cell and Increased IFN-Gamma Levels Occur in Dogs Vaccinated with Leishmune in an Endemic Area for Visceral Leishmaniasis. *Vet. Immunol. Immunopathol.* **2010**, *135*, 296–302.

198. Moreira, M.L.; Costa-Pereira, C.; Alves, M.L.R.; Marteleto, B.H.; Ribeiro, V.M.; Peruhype-Magalhães, V.; Giunchetti, R.C.; Martins-Filho, O.A.; Araújo, M.S.S. Vaccination against Canine Leishmaniosis Increases the Phagocytic Activity, Nitric Oxide Production and Expression of Cell Activation/Migration Molecules in Neutrophils and Monocytes. *Vet. Parasitol.* **2016**, *220*, 33–45.

199. Costa-Pereira, C.; Moreira, M.L.; Soares, R.P.; Marteleto, B.H.; Ribeiro, V.M.; França-Dias, M.H.; Cardoso, L.M.; Viana, K.F.; Giunchetti, R.C.; Martins-Filho, O.A.; et al. One-Year Timeline Kinetics of Cytokine-Mediated Cellular Immunity in Dogs Vaccinated against Visceral Leishmaniasis. *BMC Vet. Res.* **2015**, *11*, 92.

200. Nogueira, F.S.; Moreira, M.A.B.; Borja-Cabrera, G.P.; Santos, F.N.; Menz, I.; Parra, L.E.; Xu, Z.; Chu, H.J.; Palatnik-de-Sousa, C.B.; Luvizotto, M.C.R. Leishmune Vaccine Blocks the Transmission of Canine Visceral Leishmaniasis: Absence of *Leishmania* Parasites in Blood, Skin and Lymph Nodes of Vaccinated Exposed Dogs. *Vaccine* **2005**, *23*, 4805–4810.

201. Saraiva, E.M.; Barbosa, A.D.F.; Santos, F.N.; Borja-Cabrera, G.P.; Nico, D.; Souza, L.O.P.; Mendes-Aguiar, C.D.O.; De Souza, E.P.; Fampa, P.; Parra, L.E.; et al. The FML-Vaccine (Leishmune) against Canine Visceral Leishmaniasis: A Transmission Blocking Vaccine. *Vaccine* **2006**, *24*, 2423–2431.

202. Fernandes, C.B.; Junior, J.T.M.; De Jesus, C.; Da Silva Souza, B.M.P.; Larangeira, D.F.; Fraga, D.B.M.; Tavares Veras, P.S.; Barrouin-Melo, S.M. Comparison of Two Commercial Vaccines against Visceral Leishmaniasis in Dogs from Endemic Areas: IgG, and Subclasses, Parasitism, and Parasite Transmission by Xenodiagnosis. *Vaccine* **2014**, *32*, 1287–1295.

203. Palatnik-de-Sousa, C.B.; Silva-Antunes, I.; Morgado, A. de A.; Menz, I.; Palatnik, M.; Lavor, C. Decrease of the Incidence of Human and Canine Visceral Leishmaniasis after Dog Vaccination with Leishmune in Brazilian Endemic Areas. *Vaccine* **2009**, *27*, 3505–3512.

204. Marcondes, M.; Ikeda, F.A.; Vieira, R.F.C.; Day, M.J.; Lima, V.M.F.; Rossi, C.N.; Perri, S.H.V.; Biondo, A.W. Temporal IgG Subclasses Response in Dogs Following Vaccination against *Leishmania* with Leishmune®. *Vet. Parasitol.* **2011**, *181*, 153–159.

205. Marcondes, M.; de Lima, V.M.F.; de Araújo, M. de F.L.; Hiramoto, R.M.; Tolezano, J.E.; Vieira, R.F.C.; Biondo, A.W. Longitudinal Analysis of Serological Tests Officially Adopted by the Brazilian Ministry of Health for the Diagnosis of Canine Visceral Leishmaniasis in Dogs Vaccinated with Leishmune®. *Vet. Parasitol.* **2013**, *197*, 649–652.

206. Ribeiro, R.A.N.; Teixeira-Neto, R.G.; Belo, V.S.; Ferreira, E.C.; Schallig, H.D.F.H.; Silva, E.S. Ability of Immunodiagnostic Tests to Differentiate between Dogs Naturally Infected with *Leishmania Infantum* and Leishmune(®)-Vaccinated Dogs. *Vet. Res. Commun.* **2015**, *39*, 87–95.

207. De Amorim, I.F.G.; Freitas, E.; Alves, C.F.; Tafuri, W.L.; Melo, M.N.; Michalick, M.S.M.; da Costa-Val, A.P. Humoral Immunological Profile and Parasitological Statuses of Leishmune Vaccinated and Visceral Leishmaniasis Infected Dogs from an Endemic Area. *Vet. Parasitol.* **2010**, *173*, 55–63.

208. Ministério da Agricultura Pecuária e Abastecimento. Suspensão Da Licença de Fabricação e Comercialização Do Produto Leishmune® **2014**.

209. Moreno, J.; Vouldoukis, I.; Martin, V.; McGahie, D.; Cuisinier, A.M.; Gueguen, S. Use of a LiESP/QA-21 Vaccine (CaniLeish) Stimulates an Appropriate Th1-Dominated Cell-Mediated Immune Response in Dogs. *PLoS Negl. Trop. Dis.* **2012**, *6*, :e1683.

210. Bourdoiseau, G.; Hugnet, C.; Gonçalves, R.B.; Vézilier, F.; Petit-Didier, E.; Papierok, G.; Lemesre, J.L. Effective Humoral and Cellular Immunoprotective Responses in Li ESAp-MDP Vaccinated Protected Dogs. *Vet. Immunol. Immunopathol.* **2009**, *128*, 71–78.

211. Lemesre, J.L.; Holzmuller, P.; Gonçalves, R.B.; Bourdoiseau, G.; Hugnet, C.; Cavaleyra, M.; Papierok, G. Long-Lasting Protection against Canine Visceral Leishmaniasis Using the LiESAp-MDP Vaccine in Endemic Areas of France: Double-Blind Randomised Efficacy Field Trial. *Vaccine* **2007**, *25*, 4223–4234.

212. Lemesre, J.L.; Holzmuller, P.; Cavaleyra, M.; Gonçalves, R.B.; Hottin, G.; Papierok, G. Protection against Experimental Visceral Leishmaniasis Infection in Dogs Immunized with Purified Excreted Secreted Antigens of *Leishmania Infantum* Promastigotes. *Vaccine* **2005**, *23*, 2825–2840.

213. Holzmuller, P.; Cavaleyra, M.; Moreaux, J.; Kovacic, R.; Vincendeau, P.; Papierok, G.; Lemesre, J.L. Lymphocytes of Dogs Immunised with

Purified Excreted-Secreted Antigens of *Leishmania Infantum* Co-Incubated with *Leishmania* Infected Macrophages Produce IFN Gamma Resulting in Nitric Oxide-Mediated Amastigote Apoptosis. *Vet. Immunol. Immunopathol.* **2005**, *106*, 247–257.

214. Moreno, J.; Vouldoukis, I.; Schreiber, P.; Martin, V.; Mcgahie, D.; Gueguen, S.; Cuisinier, A.M. Primary Vaccination with the LiESP/QA-21 Vaccine (CaniLeish) Produces a Cell-Mediated Immune Response Which Is Still Present 1 Year Later. *Vet. Immunol. Immunopathol.* **2014**, *158*, 199–207.

215. Martin, V.; Vouldoukis, I.; Moreno, J.; McGahie, D.; Gueguen, S.; Cuisinier, A.M. The Protective Immune Response Produced in Dogs after Primary Vaccination with the LiESP/QA-21 Vaccine (CaniLeish®) Remains Effective against an Experimental Challenge One Year Later. *Vet. Res.* **2014**, *45*, 69.

216. Brianti, E.; Napoli, E.; Gaglio, G.; Falsone, L.; Giannetto, S.; Solari Basano, F.; Nazzari, R.; Latrofa, M.S.; Annoscia, G.; Tarallo, V.D.; et al. Field Evaluation of Two Different Treatment Approaches and Their Ability to Control Fleas and Prevent Canine Leishmaniosis in a Highly Endemic Area. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004987.

217. Bongiorno, G.; Paparcone, R.; Manzillo, V.F.; Oliva, G.; Cuisinier, A.M.; Gradoni, L. Vaccination with LiESP/QA-21 (CaniLeish®) Reduces the Intensity of Infection in *Phlebotomus Perniciosus* Fed on *Leishmania Infantum* Infected Dogs--a Preliminary Xenodiagnosis Study. *Vet. Parasitol.* **2013**, *197*, 691–695.

218. Oliva, G.; Nieto, J.; Foglia Manzillo, V.; Cappiello, S.; Fiorentino, E.; Di Muccio, T.; Scalone, A.; Moreno, J.; Chicharro, C.; Carrillo, E.; et al. A Randomised, Double-Blind, Controlled Efficacy Trial of the LiESP/QA-21 Vaccine in Naïve Dogs Exposed to Two *Leishmania Infantum* Transmission Seasons. *PLoS Negl. Trop. Dis.* **2014**, *8*, :e3213.

219. Montoya, A.; Checa, R.; Marino, V.; Galvez, R.; Ruperez, C.; Marie, K. Antibodies Elicited by Primary Vaccination and Annual Booster with CaniLeish® in Dogs from Spain: A Clinical Field Study. In Proceedings of the Proceedings of Worldleish 6 International Congress; **2017**; 1460-1125.

220. Sagols, E.; Ferraz, F.; Claret, E.; McGahie, D. Evaluation of the Humoral Immune Response after the First Annual CaniLeish® Booster Vaccination. In Proceedings of the 79th SCIVAC National Congress; **2013**; 155– 156.

221. Velez, R.; Domenech, E.; Cairó, J.; Gállego, M. The Impact of Canine Leishmaniosis Vaccination with Canileish® in *Leishmania Infantum* Infection Seroprevalence Studies. *Acta Trop.* **2020**, *202*, 105259.

222. Ghosh, A.; Zhang, W.W.; Matlashewski, G. Immunization with A2 Protein Results in a Mixed Th1/Th2 and a Humoral Response Which Protects Mice against *Leishmania Donovani* Infections. *Vaccine* **2001**, *20*, 59–66.

223. Fernandes, A.P.; Costa, M.M.S.; Coelho, E.A.F.; Michalick, M.S.M.; de Freitas, E.; Melo, M.N.; Luiz Tafuri, W.; Resende, D. de M.; Hermont,

V.; Abrantes, C. de F.; et al. Protective Immunity against Challenge with *Leishmania* (*Leishmania*) *Chagasi* in Beagle Dogs Vaccinated with Recombinant A2 Protein. *Vaccine* **2008**, *26*, 5888–5895.

224. Testasicca, M.C. d. S.; dos Santos, M.S.; Machado, L.M.; Serufo, A.V.; Doro, D.; Avelar, D.; Tibúrcio, A.M.L.; Abrantes, C. de F.; Machado-Coelho, G.L.L.; Grimaldi, G.; et al. Antibody Responses Induced by Leish-Tec®, an A2-Based Vaccine for Visceral Leishmaniasis, in a Heterogeneous Canine Population. *Vet. Parasitol.* **2014**, *204*, 169–176.

225. Regina-Silva, S.; Feres, A.M.L.T.; França-Silva, J.C.; Dias, E.S.; Michalsky, É.M.; de Andrade, H.M.; Coelho, E.A.F.; Ribeiro, G.M.; Fernandes, A.P.; Machado-Coelho, G.L.L. Field Randomized Trial to Evaluate the Efficacy of the Leish-Tec® Vaccine against Canine Visceral Leishmaniasis in an Endemic Area of Brazil. *Vaccine* **2016**, *34*, 2233–2239.

226. Grimaldi, G.; Teva, A.; Dos-Santos, C.B.; Santos, F.N.; Pinto, I.D.S.; Fux, B.; Leite, G.R.; Falqueto, A. Field Trial of Efficacy of the Leish-Tec® Vaccine against Canine Leishmaniasis Caused by *Leishmania Infantum* in an Endemic Area with High Transmission Rates. *PLoS One* **2017**, *12*, e0185438.

227. Wylie, C.E.; Carbonell-Antoñanzas, M.; Aiassa, E.; Dhollander, S.; Zagmutt, F.J.; Brodbelt, D.C.; Solano-Gallego, L. A Systematic Review of the Efficacy of Prophylactic Control Measures for Naturally-Occurring Canine Leishmaniosis, Part I: Vaccinations. *Prev. Vet. Med.* **2014**, *117*, 7–18.

228. Molano, I.; García Alonso, M.; Mirón, C.; Redondo, E.; Requena, J.M.; Soto, M.; Gómez Nieto, C.; Alonso, C. A *Leishmania Infantum* Multi-Component Antigenic Protein Mixed with Live BCG Confers Protection to Dogs Experimentally Infected with L. Infantum. *Vet. Immunol. Immunopathol.* **2003**, *92*, 1–13.

229. Parody, N.; Soto, M.; Requena, J.M.; Álonso, C. Adjuvant Guided Polarization of the Immune Humoral Response against a Protective Multicomponent Antigenic Protein (Q) from *Leishmania Infantum*. A CpG + Q Mix Protects Balb/c Mice from Infection. *Parasite Immunol.* **2004**, *26*, 283–293.

230. Poot, J.; Janssen, L.H.M.; van Kasteren-Westerneng, T.J.; van der Heijden-Liefkens, K.H.A.; Schijns, V.E.J.C.; Heckeroth, A. Vaccination of Dogs with Six Different Candidate Leishmaniasis Vaccines Composed of a Chimerical Recombinant Protein Containing Ribosomal and Histone Protein Epitopes in Combination with Different Adjuvants. *Vaccine* **2009**, *27*, 4439–4446.

231. Reguera, R.M.; Morán, M.; Pérez-Pertejo, Y.; García-Estrada, C.; Balaña-Fouce, R. Current Status on Prevention and Treatment of Canine Leishmaniasis. *Vet. Parasitol.* **2016**, *227*, 98–114.

232. Carcelén, J.; Iniesta, V.; Fernández-Cotrina, J.; Serrano, F.; Parejo, J.C.; Corraliza, I.; Gallardo-Soler, A.; Marañón, F.; Soto, M.; Alonso, C.; et al.

The Chimerical Multi-Component Q Protein from *Leishmania* in the Absence of Adjuvant Protects Dogs against an Experimental *Leishmania Infantum* Infection. *Vaccine* **2009**, *27*, 5964–5973.

233. Fernández Cotrina, J.; Iniesta, V.; Monroy, I.; Baz, V.; Hugnet, C.; Marañon, F.; Fabra, M.; Gómez-Nieto, L.C.; Alonso, C. A Large-Scale Field Randomized Trial Demonstrates Safety and Efficacy of the Vaccine LetiFend® against Canine Leishmaniosis. *Vaccine* **2018**, *36*, 1972–1982.

234. Iniesta, V.; Fernández-Cotrina, J.; Solano-Gallego, L.; Monroy, I.; Gomez-Luque, A.; Muñoz-Madrid, R. Vaccination with LetiFend®, a Novel Canine Leishmaniosis Vaccine, Does Not Interfere with Serological Diagnostic Tests. In Proceedings of the X Southern European Veterinary Conference; **2016**; 20–22.

235. Palatnik-de-Sousa, C.B.; Barbosa, A.D.F.; Oliveira, S.M.; Nico, D.; Bernardo, R.R.; Santos, W.R.; Rodrigues, M.M.; Soares, I.; Borja-Cabrera, G.P. FML Vaccine against Canine Visceral Leishmaniasis: From Second-Generation to Synthetic Vaccine. *Expert Rev. Vaccines* **2008**, *7*, 833–851.

236. Principles of Validation and Quality Control of Polymerase Chain Reaction Methods Used for the Diagnosis of Infectious Diseases. In *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*; **2004**; Chapter I. 1-4.

237. Dantas-Torres, F. Leishmune® Vaccine: The Newest Tool for Prevention and Control of Canine Visceral Leishmaniosis and Its Potential as a Transmission-Blocking Vaccine. *Vet. Parasitol.* **2006**, *141*, 1–8.

238. Bourdoiseau, G.; Hugnet, C.; Gonçalves, R.B.; Vézilier, F.; Petit-Didier, E.; Papierok, G.; Lemesre, J.L. Effective Humoral and Cellular Immunoprotective Responses in Li ESAp-MDP Vaccinated Protected Dogs. *Vet. Immunol. Immunopathol.* **2009**, *128*, 71–78.

239. Velez, R.; Domenech, E.; Rodríguez-Cortés, A.; Barrios, D.; Tebar, S.; Fernández-Arévalo, A.; Aguilar, R.; Dobaño, C.; Alberola, J.; Cairó, J.; et al. Evaluation of Canine Leishmaniosis Vaccine CaniLeish® under Field Conditions in Native Dog Populations from an Endemic Mediterranean Area–A Randomized Controlled Trial. *Acta Trop.* **2020**, *205*, 105387.

240. Sotelo-Girón, E. First Naked Plasmid Vaccine against *Leishmania Infantum* in Dogs (Oral Presentation). In Proceedings of the Proceedings of Worldleish 7 International Congress; **2022**.

241. Rodríguez-Cortés, A.; Ojeda, A.; López-Fuertes, L.; Timón, M.; Altet, L.; Solano-Gallego, L.; Sánchez-Robert, E.; Francino, O.; Alberola, J. Vaccination with Plasmid DNA Encoding KMPII , TRYP , LACK and GP63 Does Not Protect Dogs against *Leishmania Infantum* Experimental Challenge. *Vaccine* **2007**, *25*, 7962–7971.

242. Jain, K.; Jain, N.K. Vaccines for Visceral Leishmaniasis: A Review. *J. Immunol. Methods* **2015**, *422*, 1–12.

243. Datta, S.; Roy, S.; Manna, M. Therapy with Radio-Attenuated Vaccine in Experimental Murine Visceral Leishmaniasis Showed Enhanced T Cell and Inducible Nitric Oxide Synthase Levels, Suppressed Tumor Growth Factor-Beta Production with Higher Expression of Some Signaling Molecules. *Braz. J. Infect. Dis.* **2015**, *19*, 36–42.

244. Silvestre, R.; Cordeiro-Da-Silva, A.; Santarém, N.; Vergnes, B.; Sereno, D.; Ouaissi, A. SIR2-Deficient *Leishmania Infantum* Induces a Defined IFN-Gamma/IL-10 Pattern That Correlates with Protection. *J. Immunol.* **2007**, *179*, 3161–3170.

245. Daneshvar, H.; Coombs, G.H.; Hagan, P.; Phillips, R.S. *Leishmania Mexicana* and *Leishmania Major*: Attenuation of Wild-Type Parasites and Vaccination with the Attenuated Lines. *J. Infect. Dis.* **2003**, *187*, 1662–1668.

246. Papadopoulou, B.; Roy, G.; Breton, M.; Kündig, C.; Dumas, C.; Fillion, I.; Singh, A.K.; Olivier, M.; Ouellette, M. Reduced Infectivity of a *Leishmania Donovani* Biopterin Transporter Genetic Mutant and Its Use as an Attenuated Strain for Vaccination. *Infect. Immun.* **2002**, *70*, 62–68.

247. Breton, M.; Tremblay, M.J.; Ouellette, M.; Papadopoulou, B. Live Nonpathogenic Parasitic Vector as a Candidate Vaccine against Visceral Leishmaniasis. *Infect. Immun.* **2005**, *73*, 6372–6382.

248. Kumari, S.; Samani, M.; Khare, P.; Misra, P.; Dutta, S.; Kolli, B.K.; Sharma, S.; Chang, K.P.; Dube, A. Photodynamic Vaccination of Hamsters with Inducible Suicidal Mutants of *Leishmania Amazonensis* Elicits Immunity against Visceral Leishmaniasis. *Eur. J. Immunol.* **2009**, *39*, 178–191.

249. Khalil, E.A.G.; Musa, A.M.; Modabber, F.; El-Hassan, A.M. Safety and Immunogenicity of a Candidate Vaccine for Visceral Leishmaniasis (Alum-Precipitated Autoclaved *Leishmania Major* + BCG) in Children: An Extended Phase II Study. *Ann. Trop. Paediatr.* **2006**, *26*, 357–361.

250. Nagill, R.; Mahajan, R.; Sharma, M.; Kaur, S. Induction of Cellular and Humoral Responses by Autoclaved and Heat-Killed Antigen of *Leishmania Donovani* in Experimental Visceral Leishmaniasis. *Parasitol. Int.* **2009**, *58*, 359–366.

251. Satti, I.N.; Y. Osman, H.; Daifalla, N.S.; Younis, S.A.; Khalil, E.A.G.; Zijlstra, E.E.; El Hassan, A.M.; Ghalib, H.W. Immunogenicity and Safety of Autoclaved *Leishmania Major* plus BCG Vaccine in Healthy Sudanese Volunteers. *Vaccine* **2001**, *19*, 2100–2106.

252. Pereira, L.; Abbehusen, M.; Teixeira, C.; Cunha, J.; Nascimento, I.P.; Fukutani, K.; dos-Santos, W.; Barral, A.; de Oliveira, C.I.; Barral-Netto, M.; et al. Vaccination with *Leishmania Infantum* Acidic Ribosomal P0 but Not with Nucleosomal Histones Proteins Controls *Leishmania Infantum* Infection in Hamsters. *PLoS Negl. Trop. Dis.* **2015**, *9*, e0003490.

253. Saljoughian, N.; Zahedifard, F.; Doroud, D.; Doustdari, F.; Vasei, M.; Papadopoulou, B.; Rafati, S. Cationic Solid-Lipid Nanoparticles Are

as Efficient as Electroporation in DNA Vaccination against Visceral Leishmaniasis in Mice. *Parasite Immunol.* **2013**, *35*, 397–408.

254. Elfaki, M.E.E.; Khalil, E.A.G.; De Groot, A.S.; Musa, A.M.; Gutierrez, A.; Younis, B.M.; Salih, K.A.M.; El-Hassan, A.M. Immunogenicity and Immune Modulatory Effects of in Silico Predicted *L. Donovani* Candidate Peptide Vaccines. *Hum. Vaccin. Immunother.* **2012**, *8*, 1769–1774.

255. Oliveira-Freitas, E.; Casas, C.P.; Borja-Cabrera, G.P.; Santos, F.N.; Nico, D.; Souza, L.O.P.; Tinoco, L.W.; da Silva, B.P.; Palatnik, M.; Parente, J.P.; et al. Acylated and Deacylated Saponins of Quillaja Saponaria Mixture as Adjuvants for the FML-Vaccine against Visceral Leishmaniasis. *Vaccine* **2006**, *24*, 3909–3920.

256. Lemesre, J.L.; Holzmuller, P.; Gonçalves, R.B.; Bourdoiseau, G.; Hugnet, C.; Cavaleyra, M.; Papierok, G. Long-Lasting Protection against Canine Visceral Leishmaniasis Using the LiESAp-MDP Vaccine in Endemic Areas of France: Double-Blind Randomised Efficacy Field Trial. *Vaccine* **2007**, *25*, 4223–4234.

257. Ferreira, J.H.L.; Gentil, L.G.; Dias, S.S.; Fedeli, C.E.C.; Katz, S.; Barbiéri, C.L. Immunization with the Cysteine Proteinase Ldccys1 Gene from *Leishmania* (*Leishmania*) *Chagasi* and the Recombinant Ldccys1 Protein Elicits Protective Immune Responses in a Murine Model of Visceral Leishmaniasis. *Vaccine* **2008**, *26*, 677–685.

258. Ravindran, R.; Maji, M.; Ali, N. Vaccination with Liposomal Leishmanial Antigens Adjuvanted with Monophosphoryl Lipid-Trehalose Dicorynomycolate (MPL-TDM) Confers Long-Term Protection against Visceral Leishmaniasis through a Human Administrable Route. *Mol. Pharm.* **2012**, *9*, 59–70.

259. Gupta, R.; Kushawaha, P.K.; Tripathi, C.D.P.; Sundar, S.; Dube, A. A Novel Recombinant *Leishmania Donovani* P45, a Partial Coding Region of Methionine Aminopeptidase, Generates Protective Immunity by Inducing a Th1 Stimulatory Response against Experimental Visceral Leishmaniasis. *Int. J. Parasitol.* **2012**, *42*, 429–435.

260. Kushawaha, P.K.; Gupta, R.; Sundar, S.; Sahasrabuddhe, A.A.; Dube, A. Elongation Factor-2, a Th1 Stimulatory Protein of *Leishmania Donovani*, Generates Strong IFN-γ and IL-12 Response in Cured *Leishmania*-Infected Patients/Hamsters and Protects Hamsters against *Leishmania* Challenge. *J. Immunol.* **2011**, *187*, 6417–6427.

261. Kushawaha, P.K.; Gupta, R.; Tripathi, C.D.P.; Sundar, S.; Dube, A. Evaluation of *Leishmania Donovani* Protein Disulfide Isomerase as a Potential Immunogenic Protein/Vaccine Candidate against Visceral Leishmaniasis. *PLoS One* **2012**, *7*, e35670.

262. Fernandes, A.P.; Coelho, E.A.F.; Machado-Coelho, G.L.L.; Grimaldi, G.; Gazzinelli, R.T. Making an Anti-Amastigote Vaccine for Visceral Leishmaniasis: Rational, Update and Perspectives. *Curr. Opin. Microbiol.* **2012**, *15*, 476–485.

263. Agallou, M.; Margaroni, M.; Karagouni, E. Cellular Vaccination with Bone Marrow-Derived Dendritic Cells Pulsed with a Peptide of *Leishmania Infantum* KMP-11 and CpG Oligonucleotides Induces Protection in a Murine Model of Visceral Leishmaniasis. *Vaccine* **2011**, *29*, 5053–5064.

264. Dey, R.; Dagur, P.K.; Selvapandiyan, A.; McCoy, J.P.; Salotra, P.; Duncan, R.; Nakhasi, H.L. Live Attenuated *Leishmania Donovani* P27 Gene Knockout Parasites Are Nonpathogenic and Elicit Long-Term Protective Immunity in BALB/c Mice. *J. Immunol.* **2013**, *190*, 2138–2149.

265. Choudhury, R.; Das, P.; De, T.; Chakraborti, T. 115 KDa Serine Protease Confers Sustained Protection to Visceral Leishmaniasis Caused by *Leishmania Donovani* via IFN-γ Induced down-Regulation of TNF-α Mediated MMP-9 Activity. *Immunobiology* **2013**, *218*, 114–126.

266. Goto, Y.; Bogatzki, L.Y.; Bertholet, S.; Coler, R.N.; Reed, S.G. Protective Immunization against Visceral Leishmaniasis Using *Leishmania* Sterol 24-c-Methyltransferase Formulated in Adjuvant. *Vaccine* **2007**, *25*, 7450–7458.

267. Agallou, M.; Smirlis, D.; Soteriadou, K.P.; Karagouni, E. Vaccination with *Leishmania* Histone H1-Pulsed Dendritic Cells Confers Protection in Murine Visceral Leishmaniasis. *Vaccine* **2012**, *30*, 5086–5093.

268. Chakravarty, J.; Kumar, S.; Trivedi, S.; Rai, V.K.; Singh, A.; Ashman, J.A.; Laughlin, E.M.; Coler, R.N.; Kahn, S.J.; Beckmann, A.M.; et al. A Clinical Trial to Evaluate the Safety and Immunogenicity of the LEISH-F1+MPL-SE Vaccine for Use in the Prevention of Visceral Leishmaniasis. *Vaccine* **2011**, *29*, 3531–3537.

269. Resende, L.A.; Roatt, B.M.; Aguiar-Soares, R.D. de O.; Viana, K.F.; Mendonça, L.Z.; Lanna, M.F.; Silveira-Lemos, D.; Corrêa-Oliveira, R.; Martins-Filho, O.A.; Fujiwara, R.T.; et al. Cytokine and Nitric Oxide Patterns in Dogs Immunized with LBSap Vaccine, before and after Experimental Challenge with *Leishmania Chagasi* plus Saliva of *Lutzomyia Longipalpis*. *Vet. Parasitol.* **2013**, *198*, 371–381.

270. Kaur, T.; Thakur, A.; Kaur, S. Protective Immunity Using MPL-A and Autoclaved *Leishmania Donovani* as Adjuvants along with a Cocktail Vaccine in Murine Model of Visceral Leishmaniasis. *J. Parasit. Dis.* **2013**, *37*, 231–239.

271. Martins, V.T.; Chávez-Fumagalli, M.A.; Costa, L.E.; Canavaci, A.M.C.; Martins, A.M.C.C.; Lage, P.S.; Lage, D.P.; Duarte, M.C.; Valadares, D.G.; Magalhães, R.D.M.; et al. Antigenicity and Protective Efficacy of a *Leishmania* Amastigote-Specific Protein, Member of the Super-Oxygenase Family, against Visceral Leishmaniasis. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2148.

272. Gupta, R.; Kumar, V.; Kushawaha, P.K.; Tripathi, C.P.; Joshi, S.; Sahasrabuddhe, A.A.; Mitra, K.; Sundar, S.; Siddiqi, M.I.; Dube, A. Characterization of Glycolytic Enzymes - RAldolase and REnolase

of *Leishmania Donovani*, Identified as Th1 Stimulatory Proteins, for Their Immunogenicity and Immunoprophylactic Efficacies against Experimental Visceral Leishmaniasis. *PLoS One* **2014**, *9*, e86073.

273. Santos-Gomes, G.M.; Rodrigues, A.; Teixeira, F.; Carreira, J.; Alexandre-Pires, G.; Carvalho, S.; Santos-Mateus, D.; Martins, C.; Vale-Gato, I.; Marques, C.; et al. Immunization with the *Leishmania Infantum* Recombinant Cyclophilin Protein 1 Confers Partial Protection to Subsequent Parasite Infection and Generates Specific Memory T Cells. *Vaccine* **2014**, *32*, 1247–1253.

274. Baharia, R.K.; Tandon, R.; Sharma, T.; Suthar, M.K.; Das, S.; Siddiqi, M.I.; Saxena, J.K.; Sunder, S.; Dube, A. Recombinant NAD-Dependent SIR-2 Protein of *Leishmania Donovani*: Immunobiochemical Characterization as a Potential Vaccine against Visceral Leishmaniasis. *PLoS Negl. Trop. Dis.* **2015**, *9*, 1–27.

275. Ramirez, L.; Corvo, L.; Duarte, M.C.; Chávez-Fumagalli, M.A.; Valadares, D.G.; Santos, D.M.; De Oliveira, C.I.; Escutia, M.R.; Alonso, C.; Bonay, P.; et al. Cross-Protective Effect of a Combined L5 plus L3 Leishmania Major Ribosomal Protein Based Vaccine Combined with a Th1 Adjuvant in Murine Cutaneous and Visceral Leishmaniasis. *Parasites and Vectors* **2014**, *7*, 1–11.

276. Coler, R.N.; Duthie, M.S.; Hofmeyer, K.A.; Guderian, J.; Jayashankar, L.; Vergara, J.; Rolf, T.; Misquith, A.; Laurance, J.D.; Raman, V.S.; et al. From Mouse to Man: Safety, Immunogenicity and Efficacy of a Candidate Leishmaniasis Vaccine LEISH-F3+GLA-SE. *Clin. Transl. Immunol.* **2015**, *4*, e35.

277. Duthie, M.S.; Favila, M.; Hofmeyer, K.A.; Tutterrow, Y.L.; Reed, S.J.; Laurance, J.D.; Picone, A.; Guderian, J.; Bailor, H.R.; Vallur, A.C.; et al. Strategic Evaluation of Vaccine Candidate Antigens for the Prevention of Visceral Leishmaniasis. *Vaccine* **2016**, *34*, 2779–2786.

278. Sukumaran, B.; Tewary, P.; Saxena, S.; Madhubala, R. Vaccination with DNA Encoding ORFF Antigen Confers Protective Immunity in Mice Infected with *Leishmania Donovani*. *Vaccine* **2003**, *21*, 1292–1299.

279. Yam, K.K.; Hugentobler, F.; Pouliot, P.; Stern, A.M.; Lalande, J.D.; Matlashewski, G.; Olivier, M.; Cousineau, B. Generation and Evaluation of A2-Expressing *Lactococcus Lactis* Live Vaccines against *Leishmania Donovani* in BALB/c Mice. *J. Med. Microbiol.* **2011**, *60*, 1248–1260.

280. Sinha, S.; Kumar, A.; Sundaram, S. A Comprehensive Analysis of LACK (*Leishmania* Homologue of Receptors for Activated C Kinase) in the Context of Visceral Leishmaniasis. *Bioinformation* **2013**, *9*, 832–837.

281. Santos, D.M.; Carneiro, M.W.; de Moura, T.R.; Fukutani, K.; Clarencio, J.; Soto, M.; Espuelas, S.; Brodskyn, C.; Barral, A.; Barral-Netto, M.; et al. Towards Development of Novel Immunization Strategies against

Leishmaniasis Using PLGA Nanoparticles Loaded with Kinetoplastid Membrane Protein-11. *Int. J. Nanomedicine* **2012**, *7*, 2115–2127.

282. Gamboa-León, R.; Paraguai de Souza, E.; Borja-Cabrera, G.P.; Santos, F.N.; Myashiro, L.M.; Pinheiro, R.O.; Dumonteil, E.; Palatnik-de-Sousa, C.B. Immunotherapy against Visceral Leishmaniasis with the Nucleoside Hydrolase-DNA Vaccine of *Leishmania Donovani*. *Vaccine* **2006**, *24*, 4863–4873.

283. Ramos, I.; Alonso, A.; Marcen, J.M.; Peris, A.; Castillo, J.A.; Colmenares, M.; Larraga, V. Heterologous Prime-Boost Vaccination with a Non-Replicative Vaccinia Recombinant Vector Expressing LACK Confers Protection against Canine Visceral Leishmaniasis with a Predominant Th1-Specific Immune Response. *Vaccine* **2008**, *26*, 333–344.

284. Rafati, S.; Zahedifard, F.; Nazgouee, F. Prime-Boost Vaccination Using Cysteine Proteinases Type I and II of *Leishmania Infantum* Confers Protective Immunity in Murine Visceral Leishmaniasis. *Vaccine* **2006**, *24*, 2169–2175.

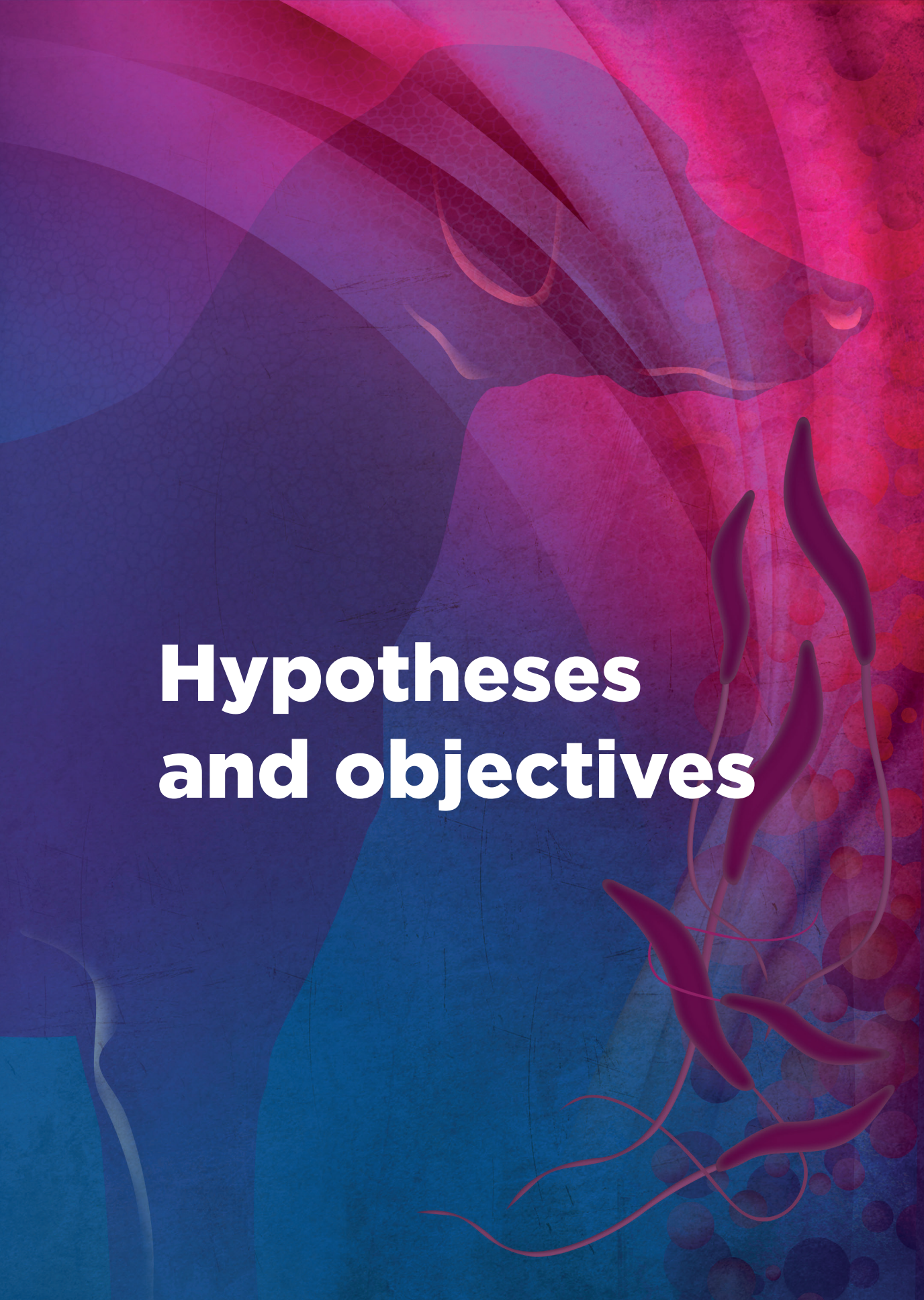
285. Khoshgoo, N.; Zahedifard, F.; Azizi, H.; Taslimi, Y.; Alonso, M.J.; Rafati, S. Cysteine Proteinase Type III Is Protective against *Leishmania Infantum* Infection in BALB/c Mice and Highly Antigenic in Visceral Leishmaniasis Individuals. *Vaccine* **2008**, *26*, 5822–5829.

286. Guha, R.; Das, S.; Ghosh, J.; Naskar, K.; Mandala, A.; Sundar, S.; Dujardin, J.C.; Roy, S. Heterologous Priming-Boosting with DNA and Vaccinia Virus Expressing Kinetoplastid Membrane Protein-11 Induces Potent Cellular Immune Response and Confers Protection against Infection with Antimony Resistant and Sensitive Strains of *Leishmania* (*Leishmania*) *Donovani*. *Vaccine* **2013**, *31*, 1905–1915.

287. Saljoughian, N.; Taheri, T.; Zahedifard, F.; Taslimi, Y.; Doustdari, F.; Bolhassani, A.; Doroud, D.; Azizi, H.; Heidari, K.; Vasei, M.; et al. Development of Novel Prime-Boost Strategies Based on a Tri-Gene Fusion Recombinant L. Tarentolae Vaccine against Experimental Murine Visceral Leishmaniasis. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2174.

288. Masih, S.; Arora, S.K.; Vasishta, R.K. Efficacy of *Leishmania Donovani* Ribosomal P1 Gene as DNA Vaccine in Experimental Visceral Leishmaniasis. *Exp. Parasitol.* **2011**, *129*, 55–64.

289. Carson, C.; Antoniou, M.; Ruiz-Argüello, M.B.; Alcami, A.; Christodoulou, V.; Messaritakis, I.; Blackwell, J.M.; Courtenay, O. A Prime/Boost DNA/Modified Vaccinia Virus Ankara Vaccine Expressing Recombinant *Leishmania* DNA Encoding TRYP Is Safe and Immunogenic in Outbred Dogs, the Reservoir of Zoonotic Visceral Leishmaniasis. *Vaccine* **2009**, *27*, 1080–1086.



Hypotheses and objectives

Hypothesis

As previously described in the introduction and in the justification, the hypotheses of this doctoral thesis are described below.

Hypothesis 1.a)

Topical administration of polyhexamethylene biguanide (PHMB) used alone or in combination with a TLR4 agonist in patients with papular dermatitis leads to a faster resolution of clinical signs and no significant adverse effects.

Hypothesis 1.b)

Topical administration of meglumine antimoniate in patients with papular dermatitis leads to a faster resolution of clinical signs and no significant adverse effects.

Hypothesis 1.c)

Dogs with papular dermatitis show a strong cellular immune response and a weak humoral immune response.

Hypothesis 2.a)

Healthy seropositive infected dogs or dogs with mild disease (stage I-papular dermatitis) show a higher proportion of activated neutrophils in peripheral blood than dogs in moderate to severe diseases (stages II-III).

Hypothesis 2.b)

IFN-γ is positively associated to activated neutrophils in peripheral blood of dogs with *L. infantum* infection as well as inversely related to antibody levels.

Hypothesis 3.a)

A candidate vaccine for *L. infantum* induces a protective immune response characterized by a strong cellular immune response and a mild specific humoral response as well as a good safety profile when administered to healthy beagles.

Objectives

The general aim of this thesis was to investigate new tools for prevention and treatment as well as enhancing knowledge regarding innate and adaptive immunity for canine *L. infantum* infection.

Objective 1.a)

To evaluate the safety and clinical efficacy of topical administration of polyhexamethylene biguanide (PHMB) used alone or in combination with a TLR4 agonist in the resolution of cutaneous lesions in dogs with papular dermatitis due to *L. infantum* infection.

- To evaluate the occurrence of local or systemic side effects of topical administration of PHMB used alone or in combination with a TLR4 agonist (by performing a complete physical and clinical exam during the drug's administration and comparing the results with placebo administration.
- To evaluate clinical efficacy of topical administration of PHMB used alone or in combination with a TLR4 agonist in dogs with papular dermatitis due to *L. infantum* by performing a physical exam and lesion follow-up at days 15, 30, 60, 90, 180, and 365 and registering the evolution of the lesions and possible relapses and comparing the results with placebo administration.

Objective 1.b)

To evaluate of the safety and efficacy of topical administration of meglumine antimoniate in the resolution of cutaneous lesions in dogs with papular dermatitis due to *L. infantum* infection.

- To evaluate the occurrence of local or systemic side effects of meglumine antimoniate by performing a complete physical and clinical exam during the drug's administration and comparing the results with placebo administration.
- To evaluate clinical efficacy of topical administration of meglumine antimoniate in dogs with papular dermatitis due to *L. infantum* by performing a physical exam and lesion follow-up at days 15, 30, 60, 90, 180, and 365 and registering the evolution of the lesions and possible relapses and comparing the results with placebo administration.

Objective 1.c)

To evaluate parasite-specific humoral and cellular immunity as well as parasitemia at the time of diagnosis and during the follow-up period of dogs with papular dermatitis throughout treatment with topical administration of meglumine antimoniate or PHMB used alone or in combination with a TLR4 agonist.

- To evaluate parasite-specific humoral response by measuring *L. infantum* antibody levels by quantitative serology using an *in-house* Enzyme-Linked ImmunoSorbent Assay (ELISA) at the time of diagnosis and at days 15, 30, 60, 90, 180, and 365.
- To evaluate the cellular immunity by measuring IFN-γ concentration with cytokine release whole blood assay at the time of diagnosis and at days 30, 90, 180, and 365, and measuring IL-17a concentrations with the same procedure at days 0, 30, and 365.
- To evaluate parasitemia by performing blood DNA extraction and *Leishmania* real-time PCR from blood samples taken at days 0, 30, and 6 or 12 months.
- To evaluate neutrophils oxidative activity by performing nitroblue tetrazolium reduction test (NBT) from blood samples taken at days 0, 30 and 360.

Objective 2.a)

To assess the variations in peripheral blood neutrophil oxidative activity in dogs with different stages of *L. infantum* infection by means of NBT rate.

- To assess and compare the NBT rate from peripheral blood in healthy seronegative dogs, *Leishmania*-seropositive but clinically healthy dogs, dogs with mild disease-LeishVet Stage I, dogs with moderate disease-LeishVet Stage II and dogs with severe disease-LeishVet stage III and very severe disease-LeishVet stage IV.

Objective 2.b)

To investigate the potential association between neutrophil oxidative activity and IFN-γ concentrations and antibody levels in dogs in different states of *L. infantum* infection.

- To assess IFN-γ concentration by performing cytokine release whole blood assay in healthy seronegative dogs, *Leishmania*-seropositive but clinically healthy dogs, dogs with mild disease-LeishVet Stage I, dogs with moderate disease-LeishVet Stage II and dogs with severe disease-LeishVet stage III and very severe disease-LeishVet stage IV and to compare these results with the NBT rate and antibody production detected by an *in-house* ELISA both performed at the same timepoint.

Objective 3.a)

To assess the safety and humoral and cellular immune response of a candidate vaccine for *L. infantum* in dogs.

- To evaluate the occurrence of short-term adverse effects by keeping the dogs under veterinary surveillance after each vaccine dose, performing clinical examinations and evaluate local adverse effects at the injection site and systemic adverse effects.
- To evaluate the occurrence of long-term adverse effects by performing a complete physical examination prior to prime vaccination (day 0) and before every booster (day 15, 30, 190 and 470), performing a complete blood count and serum biochemistry panel at day 0, 45, 190, 200 and 470 and recording the body weight two weeks after each booster.
- To evaluate parasite-specific humoral response by measuring *L. infantum* antibody levels by quantitative serology using an *in-house* ELISA at the time of prime vaccination and at days 10, 15, 30, 45, 60, 75, 90, 105, 126, 141, 190, 200, 215, 230, 245, 260, 275, 290, 330, 360, 410, 440, 470, 480, 485, 507, 570, 640, 745 and 836.
- To evaluate the cellular immunity by measuring IFN-γ concentration with cytokine release whole blood assay at the time of prime vaccination and at days 10, 30, 45, 60, 75, 90, 105, 126, 141, 190, 200, 215, 230, 245, 260, 275, 290, 330, 360, 410, 440, 470, 480, 485, 507, 570, 640, 745 and 836, and measuring IL-17a concentrations with the same procedure at days 0, 10, 30, 45, 60, 190, 200, 470, 485 and day 836.

Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis due to *Leishmania infantum* in Dogs

Icíar Martínez-Flórez[1], Maria Jose Guerrero[2], Annabel Dalmau[3], Maria Cabré[1,4], Maria Magdalena Alcover[5], Diana Berenguer[5], Liam Good[6], Roser Fisa[5], Cristina Riera[5], Laura Ordeix[1,4] and Laia Solano-Gallego[1,*]

[1]Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, 08193, Spain; imflorez.vet@gmail.com (I.M.-F.); mariacabregil@gmail.com (M.C.); laura.ordeix@uab.cat (L.O.); laia.solano@uab.cat (L.S.-G.)

[2]Hospital Veterinario Alhaurín el Grande, Alhaurín el Grande, 29120, Spain; marjoxgr@gmail.com

[3]Mediterrani Veterinaris, Reus, 43204, Spain; annabeldalmaulopez@gmail.com

[4]Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, 08193, Spain

[5]Departament de Biologia, Sanitat i Medi Ambient, Secció de Parasitologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, 08028, Spain

[6]mmagdalenaalcoveramengual@ub.edu (M.M.A.); berenguer.diana@gmail.com (D.B.); rfisa@ub.edu (R.F.); mcriera@ub.edu (C.R.)

[7]Department of Pathobiology and Population Sciences, The Royal Veterinary College, University of London, London, NW1 0TU, UK; lgood@rvc.ac.uk

*Correspondence: laia.solano@uab.cat

Published in: **Pathogens**
https://doi.org/10.3390/pathogens12060821

Abstract

Papular dermatitis is a cutaneous manifestation of canine *Leishmania infantum* infection associated with mild disease. Although it is a typical presentation, nowadays, there is still no established treatment. This study evaluated the safety and clinical efficacy of local meglumine antimoniate, locally administered polyhexamethylene biguanide (PHMB) alone or PHMB in combination with a Toll-like receptor 4 agonist (TLR4a) for the treatment of papular dermatitis due to *L. infantum* and assessed parasitological and immunological markers in this disease. Twenty-eight dogs with papular dermatitis were divided randomly into four different groups; three of them were considered treatment groups: PHMB (n = 5), PHMB + TLR4a (n = 4), and meglumine antimoniate (n = 10), and the remaining were considered the placebo group (n=9), which was further subdivided into two sub-groups: diluent (n = 5) and TLR4a (n = 4). Dogs were treated locally every 12 hours for four weeks. Compared to placebo, local administration of PHMB (alone or with TLR4a) showed a higher tendency towards resolution of papular dermatitis due to *L. infantum* infection at day 15 (χ^2 = 5.78; df = 2, p = 0.06) and day 30 (χ^2 = 4.; df = 2, p = 0.12), while local meglumine antimoniate administration demonstrated the fastest clinical resolution after 15 (χ^2= 12.58; df = 2, p = 0.002) and 30 days post-treatment (χ^2 = 9.47; df = 2, p = 0.009). Meglumine antimoniate showed a higher tendency towards resolution at day 30 when compared with PHMB (alone or with TLR4a) (χ^2 = 4.74; df = 2, p = 0.09). In conclusion, the local administration of meglumine antimoniate appears to be safe and clinically efficient for the treatment of canine papular dermatitis due to *L. infantum* infection.

Keywords: canine; cutaneous lesions; immunotherapy; leishmaniosis; local treatment; toll-like agonist.

1. Introduction

Canine leishmaniosis (CanL) is a vector-borne zoonotic disease with a worldwide distribution caused by the protozoan parasite *Leishmania infantum*. It is considered an endemic disease in the Mediterranean basin, Portugal, Latin America, and southern Asia [1]. Female phlebotomine sand flies are the biological vector, and dogs are considered the main reservoir [2].

Canine leishmaniosis is manifested by a broad spectrum of clinical signs and a wide range of disease severity that is classified into four stages: mild (stage I), moderate (stage II), severe (stage III), or very severe disease (stage IV) based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies [1]. Cutaneous manifestations are the most common clinical signs, and among them, papular dermatitis is considered a typical presentation in endemic areas and is associated with Leishvet stage I-mild leishmaniosis [1,3]. This cutaneous form is associated with an effective Th1 predominant parasite-specific cellular immunity and low humoral immune response, and thus, it is characterized by the absence of laboratory abnormalities and lack of systemic clinical signs. Self-healing of the skin lesions may occur at some point; however, it usually takes three to six months [4].

Clinical signs and outcomes of CanL depend on the interactions between the parasite and the host's innate and adaptive immune responses [5–7]. The adaptive response characterized by T-helper 1 (Th1) activation produces cytokines such as interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α), which help to control the infection. In contrast, the immune response mediated by Th2 induces the production of anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β) and is associated with disease progression [7,8]. Interleukin-17a (IL-17a) produced by several cells such as Th17, natural killer cells (NK), and macrophages, among others [9], plays a role in blocking parasite growth by the activation of inducible nitric oxide synthase (iNOS), among others [10, 11]. Therefore, IL-17a is associated with resistance against *L. infantum* infection or disease in humans [10] and dogs [12], acting synergistically with IFN-γ.

Regarding the Th1 response, disease control requires a balanced interaction between a proinflammatory immune response mediated by T helper 1 type (Th1) CD4+ T cells, with the aim of controlling parasite replication, and a regulatory immune response mediated by T regulatory 1 cells, which are necessary to avoid a self-damaging overactivation of the immune system [13]. B cells also play a key role in the progression of CanL. They induce a humoral immune response and act as antigen-presenting cells modulating the activation of CD4+ T cells, which in turn activate B cells to produce antibodies [13]. As the disease progresses, the

production of specific and non-specific IgG antibodies increases, reflected as hypergammaglobulinemia, and binds to *Leishmania* antigen creating immune complexes, which may contribute to disease progression [14–16].

One of the factors that regulate the activation of Th1 or Th2 immunity is pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which recognize molecules found in the promastigote that act as pathogen-associated molecular patterns (PAMPs) [17]. TLRs are located on the membrane or intracellular compartments of different types of cells, including T and B lymphocytes, macrophages, dendritic cells (DCs), and NK [18]. After binding their ligand, TLRs trigger the activation of the immune activity through diverse events such as the induction of inflammatory cytokines such as TNF-α and IFN-γ [19–22].

The innate immune response has an essential role in avoiding *Leishmania* survival. Cells such as neutrophils, macrophages, and DCs can kill the parasite via phagocytosis or facilitate their elimination by producing cytokines [7]. Neutrophils are activated following *L. infantum* inoculation and initiate several defense mechanisms, such as the production of reactive oxygen species (ROS) [23]. The nitroblue tetrazolium (NBT) reduction test is used to detect activated neutrophils in peripheral blood. NBT (soluble and colorless) is transformed into formazan (insoluble and blue-grey) in activated neutrophils, shown to be directly related to ROS production [24,25].

Treatment regarding Leishvet stage I has been scientifically neglected. Short-term treatment with one or two conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine and/or allopurinol) in dogs classified as Leishvet stage I has been described, as well as immune-potentiating treatments (domperidone or nucleotides plus- active hexose correlated compound (AHCC)) alone or in combination with the previously mentioned drugs [1,26]. Alternatively, monitoring without treatment can also be considered in some cases. There is limited evidence for treatment outcomes for dogs in this stage, and therefore, the clinical efficacy of these treatment options remains unknown [27].

Similarly, there is no universally applicable treatment for human cutaneous leishmaniasis (CL) [28]. Local treatment for CL in humans has been recommended as an alternative treatment to systemic therapy by the World Health Organization and the Pan American Health Organization in patients with at least four lesions smaller than 4 cm in diameter, especially if the face and joints are not affected [29]. Local treatments are described as easy to use and with a lower risk, toxicity, and cost compared to traditional therapies, and they include intralesional antimonial injections, cryotherapy (with liquid nitrogen), thermotherapy (use of localized current field radiofrequency heat), and topical formulations such as paromomycin ointment [28,30–32]. However, local treatment has not been investigated in stage I-papular dermatitis due to canine *L. infantum* infection.

Polyhexamethylene biguanide (PHMB) is a synthetic cationic polymer with antimicrobial activity commonly used as a first-line treatment for locally infected wounds [33]. Furthermore, PHMB has been reported to have antileishmanial activity by inducing disruption of the parasite´s membrane integrity and causing chromosomal damage [34]. On the other hand, TLR4 activation induces a protective immune response against *Leishmania*, and will also regulate iNOS leading to the death of the parasite [22]. PHMB alone or in combination with a TLR4 agonist (TLR4a) has been reported to induce lower percentages of *Leishmania* infection in canine DH-82 cells *in vitro* [22].

The main aim of this randomized, controlled, double-blinded study was to evaluate the safety and clinical efficacy of locally administered PHMB alone or in combination with TLR4a and the local administration of meglumine antimoniate in the treatment of papular dermatitis due to *L. infantum* infection in dogs. The secondary objectives were to assess parasite-specific humoral and cellular immunity as well as parasitemia and to evaluate neutrophil function based on NBT at the time of diagnosis and during the follow-up period.

2. Materials and Methods

2.1. Dogs and Study Design

This study was designed as a multicentric randomized double-blinded controlled study with a follow-up of one year and involving five veterinary centers from Spain: Hospital Veterinario Alhaurín El Grande (Málaga), Hospital Mediterrani Veterinaris (Reus, Tarragona), Clínica veterinaria Paws Patas (Mula, Murcia), Hospital Veterinari Canis (Palma, Mallorca), and Fundació Hospital Clínic Veterinari (UAB, Bellaterra, Barcelona).

Dogs visited at the mentioned centers from 2019 to 2022 were considered for inclusion in this study. Inclusion criteria were a diagnosis of mild leishmaniosis (LeishVet stage I) characterized by the absence of laboratory abnormalities, negative or low antibody levels, and papular dermatitis as the only clinical sign at the time of diagnosis [1]. Withdrawal criteria were the development of systemic disease based on clinical signs and clinicopathological abnormalities, treatment with conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine, or allopurinol), or highly increased antibody levels. A signed informed consent was obtained from all dog owners. Ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (CEAAH 4526, November 2018) and by "Generalitat de Catalunya" (FUE-2018-00944112 i ID KSHYD6LVR, April 2019).

A complete physical examination, complete blood count (CBC), and biochemistry panel, including at least creatinine, urea, total proteins (TP), and alanine transaminase (ALT), were performed in all dogs to assess the clinical

status. In some dogs, serum electrophoresis and urinalysis with urinary protein/creatinine ratio were also performed. Cutaneous lesions were photographed, borders around each lesion were measured to document size, and a cytology examination was performed from the lesions to diagnose *L. infantum* infection.

2.2. Treatment and Follow-Up

Five different products were manufactured as follows: PHMB alone (3 mg/mL PHMB in 30% ethanol), a combination of PHMB and TLR4a (1.5 µg/mL TLR4 agonist + 3 mg/mL PHMB in 30% ethanol), TLR4a alone (1.5 µg/mL TLR4 agonist in 30 % ethanol), meglumine antimoniate (30% meglumine antimoniate + 70% pluronic F-127), and diluent (30% ethanol in nuclease-free water). PHMB, PHMB+TLR4a, TLR4a, and diluent (30% ethanol in nuclease-free water) were formulated as liquid spray formulations. It was strongly advised to gently invert 20 times the sprayer vial before every application and to keep it upright when spraying. Meglumine antimoniate was a lotion spray formulation and was stored in the refrigerator. It was recommended to remove the remaining product over the patient's skin from the last application before a new administration [35].

Dogs were randomly divided into four groups: three of them were considered treatment groups, PHMB (n = 5), PHMB + TLR4a (n = 4), and meglumine antimoniate (n = 10), and the remaining was considered the placebo group (n = 9), which was further subdivided into two sub-groups (diluent (n = 5) and TLR4a (n = 4)). TLR4a showed no statistical differences when compared to a diluent, and therefore, both groups were considered as placebo. Each group was treated locally over the papules every 12 h for four weeks, and a physical exam and lesion follow-up were performed on days 15, 30, 60, 90, 180, and 365. Clinical efficacy was defined as partial or total resolution of the lesions at days 15 and 30, respectively **(Figure 4)**.

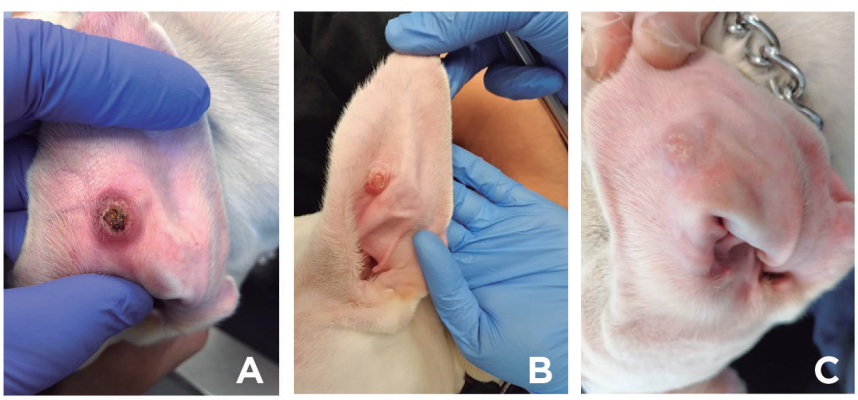


Figure 4. Clinical efficacy of local PHMB on papular dermatitis due to *L. infantum* at day 0 **(A)**, day 15 **(B)**, showing partial remission, and day 30 (**C**) full remission. Courtesy of Isaac Carrasco.

All the procedures performed on each dog at the time of diagnosis and during follow-up are described at **Figure 5**.

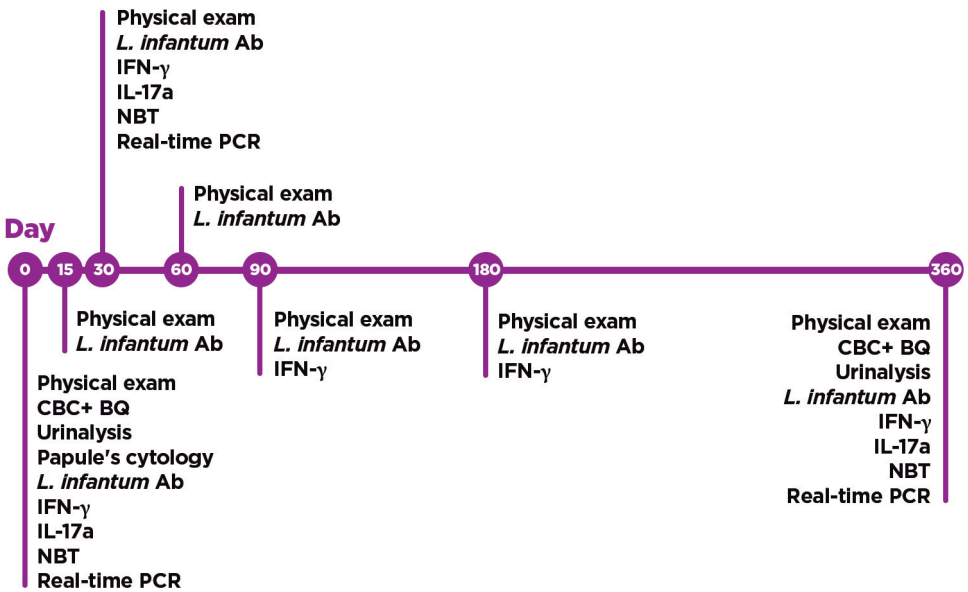


Figure 5. Timeline of the procedures performed on each dog at the time of diagnosis and during follow-up. CBC, complete blood count; **BQ**, biochemistry panel; **Ab**, antibody; **IFN-γ**, Interferon-gamma; **IL-17a**, Interleukin-17a; **LSA**, soluble *L. infantum* antigen; **NBT**, Nitroblue tetrazolium reduction test.

Persistent papules after sixty days from the beginning of the study or the appearance of new papules during the treatment were considered as a lack of response to treatment and relapse, respectively, and a rescue therapy (topical administration of 30% meglumine antimoniate + 70% pluronic F-127) was administered.

2.3. Safety Assessment

Drug safety assessment was performed based on a complete physical exam on days 15, 30, and 60 by veterinarians. Local side effects of the topical treatments at the application site, such as erythema and swelling, were recorded and graded (0-4) (Supplementary material 1). Local pruritus and pain were also recorded. Systemic signs such as lymphadenomegaly or increased temperature, among others, were also registered. This information was recorded in the data collection form of both the veterinarian and the owner or caregiver of the dog (Supplementary material 1). Furthermore, photographs of the application site were also reviewed by the first author to check for local reactions on days 15, 30, and 60.

2.4. ELISA for specific *L. infantum* antibody detection

Leishmania infantum antibody levels were measured by quantitative serology using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA) at the time of diagnosis and repeated on days 15, 30, 60, 90, 180, and 365 [5].

The in-house ELISA was performed on the sera of all dogs as previously described [36]. Briefly, the samples were diluted to 1:800 in phosphate buffer solution (PBS) with Tween 20 and 1% dry milk and incubated at 37° for 1 h. Then, the plates were washed three times with PBS-Tween and once with PBS and incubated with Protein A conjugated to horseradish peroxidase (Peroxidase Conjugate Protein A; Merck KGaA, Darmstadt, Germany) at 1:30,000 dilution for 1 h at 37 °C. After incubation, the plates were washed again, as described before. Then, o-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA, Darmstadt, Germany) was added to the plates, and the reaction was finally stopped with 5 M H2SO4. Absorbance values were read at 492 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China), and the results were quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and set at 100 EU.

The cut-off of the serum in-house ELISA was already determined to be 35 EU using the ELISA results of 80 dogs from a non-endemic area, as previously described [37]. Cut-off was established by the standard deviation (SD) method, consisting of multiplying the SD of the results by four and adding up the mean of the results obtained by the ELISA (mean + 4 SD). Serum was classified as high positive when the result was ≥300 EU, medium positive when the result was ≥150 EU and <300 EU, low positive when the result was ≥35 EU and <150 EU, and negative when the result was <35 EU [37]. All samples from each animal were analyzed on the same plate.

2.5. Cytokine Release Whole Blood Assay and Determination of Canine IFN-γ and IL-17a

IFN-γ concentration was measured at admission in all dogs and repeated at days 30, 90, 180, and 365, while IL-17a was performed at days 0, 30, and 365.

A heparinized whole blood cytokine release assay was performed as described elsewhere [5]. Briefly, whole blood was separately incubated with three different conditions: (i) medium alone (unstimulated); (ii) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 µg/mL; and (iii) medium with the mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 µg/mL. Blood cultures were collected after five days at 37 °C in 5% of CO_2 and were centrifugated at 300 g for 10 min. Supernatants were collected and stored at -80 °C until tested. IFN-γ and IL-17a were determined in

all samples by a commercial sandwich ELISA (DuoSet® ELISA by Development System R&DTM, Abingdon, UK) [5,6]. The standard curve for IFN-γ and IL-17a was calculated using a computer-generated four-parameter logistic curve fit with the program MyAssays (http://www.myassays.com/) [5].

Dogs were classified as IFN-γ producers when *L. infantum*-specific IFN-γ concentration was ≥110 pg/mL after subtracting the medium alone [38]. In the case of IL-17a, dogs were classified as producers when *L. infantum*-specific IL-17a concentration was ≥62.5 pg/mL after subtracting the medium alone [5].

2.6. Nitroblue Tetrazolium Reduction Test

Hematocrit capillary microtubes were filled with blood samples collected in EDTA tubes from all dogs and centrifugated at 2910 g for 5 min. Then, the buffy coat from each microtube was placed in an Eppendorf tube with the same volume of 0.1% NBT solution (1:1) (N6876, Sigma–Aldrich Co., St. Louis, USA). The Eppendorf tube was mildly agitated and incubated for 15 min at 37 °C and then for another 15 min at room temperature. Two blood smears from each Eppendorf tube were obtained by placing 3 µL of NBT-stained samples on each slide. The slides were stained with Diff-Quick, and the NBT reduction rate was assessed by light microscopy after counting 300 neutrophils. The percentage was calculated as the number of activated neutrophils, defined by those containing blue-black formazan deposits, divided by the total number of neutrophils counted, multiplying the result by 100.

2.7. Blood DNA Extraction and *Leishmania* Real-Time PCR

Real-time PCR was carried out from blood samples at day 0, day 30, and 6 or 12 months.

DNA was isolated from blood samples collected in EDTA tubes using MagMax CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) using an automated system (KingFisher Flex Purification System, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions for a simple workflow with whole blood samples. Briefly, 10 µL of proteinase K solution and 20 µL of magnetic beads were added to 100 µL of each sample. DNA was extracted by the robot after adding 700 µL of a mix of the binding and lysis solution in all the samples [36].

Leishmania real-time PCR was performed as previously described [39]. Each DNA sequence amplification for PCR was performed in triplicate. The mix reaction was prepared with 1x iTaq supermix with Rox (Bio-Rad), 0.2 µmoL of direct primer (5'-CTT TTC TGG TCC TCC GGG TAGG-3'), 0.2 µmoL of reverse primer (5'-CCA CCC GGC CCT ATT TTA CAC CAA -3'), 20 nmol of the labeled TaqMan probe (FAM- TTT TCG CAG AAC GCC CCT ACC CGC

TAMRA), 0.5 µL of H20, and 2.5 µL of sample DNA. Positive and negative controls were also included in each plate. A 10-fold dilution series of standard DNA from *L. infantum* promastigotes (CATB101) was used as a calibrator (serial dilution from 10^5 parasites/ml to 10^{-3} parasites/mL), allowing for the plotting of a standard curve. Cycling was performed using the QuantStudio™ 7 Pro (Thermo Fisher Scientific, Foster City, California, USA) at 95 °C/55 °C for 45 cycles. The PCR was considered positive for *Leishmania* when the quantification cycle (Cq) was <40, and the amplification was detected in all the replicates.

2.8. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 8.0.1 for Windows software (GraphPad Software, San Diego, California, USA). First, different normality tests were performed to know if the different variables (age, ELISA results, real-time PCR, LSA IFN-γ, ConA IFN-γ, LSA IL-17a, ConA IL-17a, and NBT rate) had a normal distribution at the time of diagnosis and follow-up. For each variable, the following tests were performed: Anderson–Darling, D'Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov. A p-value < 0.05 was considered statistically significant, meaning all variables did not follow a normal distribution except for the NBT rate. Therefore, non-parametric tests were used unless the comparison was between NBT rate groups, in which case, a parametric test was performed (paired t-test). A non-parametric Wilcoxon matched-pairs signed rank test was used for quantitative variables to compare two groups if the samples were paired. If samples were unpaired, a Mann–Whitney U-test was used. A Friedman test was used to compare more than two groups, and afterward, Dunn's multiple comparisons test was performed. The chi-square test or Fisher´s exact test was used to determine whether there was a significant association between two categorical variables. The Spearman Correlation Coefficient was used to evaluate differences in cytokine production of the dogs studied. Differences were considered significant, with a 5% significance level (p-value < 0.05).

3. Results

3.1. Dog Characteristics at the Time of Diagnosis

Twenty-eight dogs were included in this study **(Figure 6)** and were randomly divided into the aforementioned groups as follows: PHMB (n = 5), PHMB + TLR4a (n = 4), TLR4a-placebo (n = 4), meglumine antimoniate (n = 10), and diluent-placebo (n = 5). Unfortunately, complete blood samples and data were only obtained from 24, including 9 females and 15 males (37.5% and 62.5%, respectively).

Fifty-four percent of dogs were crossbred (n = 13); other represented breeds were Belgian Malinois (n = 4), German shepherd (n = 2), American bully (n = 1), Border collie (n = 1), Chihuahua (n = 1), Labrador retriever (n = 1), and Rottweiler (n = 1). Their ages ranged from 3 to 84 months, with a median of 6 months, with 91.7% being less than 12 months old and 45.8% less than 6 months old.

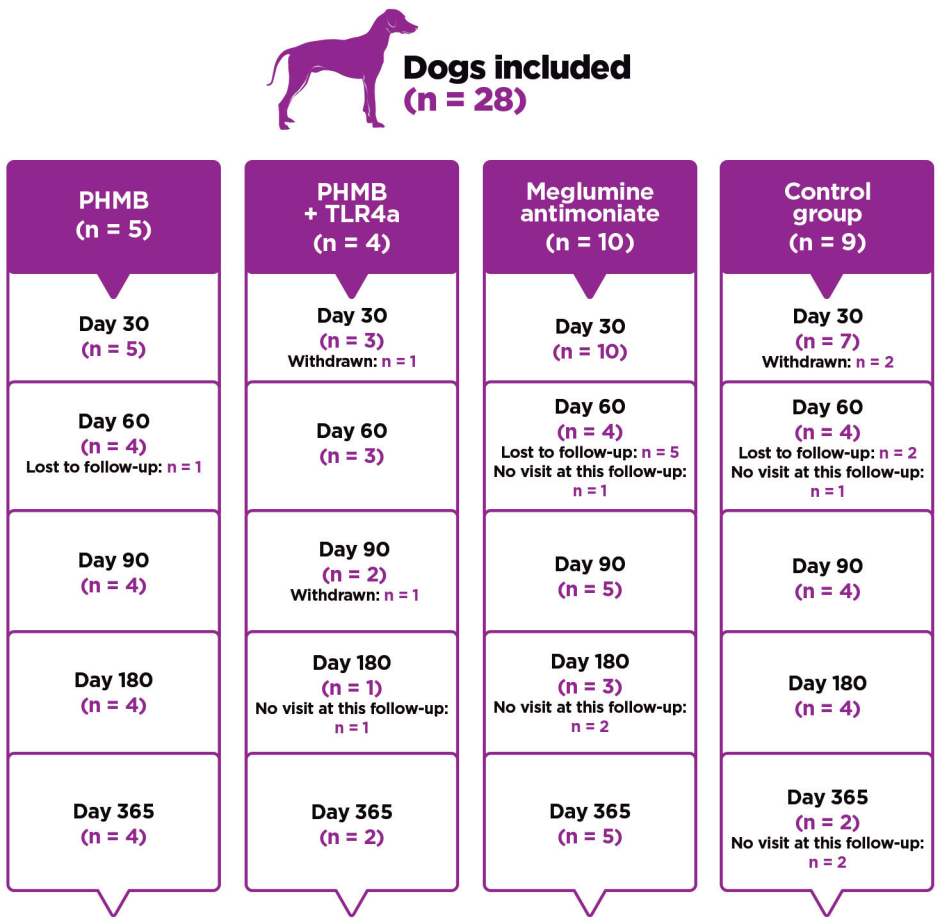


Figure 6. Flowchart displaying the number of dogs included at the time of diagnosis, treatment groups, as well as withdrawn dogs and lost to follow-up during the follow-up period. PHMB, polyhexamethylene biguanide; **TLR4a**, Toll-like receptor 4 agonist; **Control group** (n = 9): diluent (n = 5) + TLR4a (n = 4). **Lost to follow-up**, owners did not want to participate in the study anymore. **No visit at this follow-up**, owners did not show up at the Veterinary clinic that day. **Withdrawn**, dog was withdrawn of the study due to the development of systemic disease based on clinical signs and clinicopathological abnormalities, treatment with conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine, or allopurinol), or highly increased antibody levels.

On physical examination, solitary (n = 1) or multiple (n = 23) erythematous non-pruritic papules were observed in non-haired skin areas **(Table 8)**.

Table 8. Distribution of papules.

Site	Number of Lesions	%
Inner surface of the pinna	**15**	46.9
Abdomen	**6**	18.8
Eyelid	**3**	9.4
Lips	**3**	9.4
Foreskin	**2**	6.3
Elbow	**1**	3.1
Bridge of the nose	**1**	3.1
Nose	**1**	3.1

The onset of clinical lesions was mainly in autumn (70.8%), followed by winter (16.7%), spring (8.3%), and summer (4.2%) **(Table 9)**.

Table 9. The onset of clinical lesions analysed by season, sex, and median age.

	Total number of dogs	%	Median Age (Minimum-Maximum Months)
Autumn	**17**	**70.8**	**6 (3–84)**
Female	**5**	**20.8**	**7 (3–84)**
Male	**12**	**50**	**6 (4–6)**
Winter	**4**	**16.7**	**6 (6–10)**
Female	**3**	**12.5**	**6 (6–10)**
Male	**1**	**4.2**	**5**
Spring	**2**	**8.3**	**3.5 (3–4)**
Male	**2**	**8.3**	**3.5 (3–4)**
Summer	**1**	**4.2**	**5**
Female	**1**	**4.2**	**5**
Total	**24**	**100**	**6**

No other abnormalities were found on physical examination, except a mild lymphadenomegaly of regional lymph nodes in two dogs.

Most dogs showed no laboratory abnormalities except for one dog with a mild increase in TP and three dogs with a mild decrease in TP.

Clinical characteristics and laboratory tests according to different treatments at the time of diagnosis are displayed in **Table 10**.

Table 10. Clinical characteristics and laboratory tests at the time of diagnosis before starting local treatment.

Qualitative characteristics		PHMB + PHMB with TLR4a (n = 9)	Meglumine Antimoniate (n = 6/10)*	Control Group (n = 9)	*p*-Value (Fisher's Exact Test)
Bred	Crossbred	7 (78%)	3 (50%)	3 (33%)	0.162
	Purebred	2 (22%)	3 (50%)	6 (67%)	
Sex	Male	7 (78%)	2 (33%)	5 (56%)	0.227
	Female	2 (22%)	4 (67%)	4 (44%)	
ELISA at day 0	Low positive	3 (33%)	0 (0%)	3 (33%)	0.264
	Negative	6 (67%)	6 (100%)	6 (67%)	
Leishmania real-time PCR	Positive	5 (56%)	3 (50%)	5 (56%)	0.972
	Negative	4 (44%)	3 (50%)	4 (44%)	
IFN-γ	Producers	7 (78%)	3 (50%)	2 (22%)	0.1
	Non-prod.	2 (22%)	3 (50%)	7 (78%)	
IL-17a	Producers	8 (89%)	1 (17%)	1 (11%)	0.7
	Non-prod.	1 (11%)	5 (83%)	8 (89%)	

Qualitative characteristics	Median (min-max)	Median (min-max)	Median (min-max)	*p*-Value (Kruskal-Wallis test)
Age (months)	6 (3–8)	5.5 (4–84)	6 (3–36)	0.875
IFN-γ (pg/mL)	768.6 (11.6-3997.5)	140.4 (0-762.4)	23.6 (0–309)	0.011**
IL-17a (pg/mL)	768.6 (11.6-3997.5)	11.9 (0.148)	0 (0–71.5)	0.005**
ELISA units	26 (7.1–227.1)	5.7 (2.8–73.5)	22.3 (4.8–100.9)	0.11

Qualitative characteristics	Mean ± SD	Mean ± SD	Mean ± SD	*p*-Value (Ordinary one-way ANOVA)
Cq PCR	35.3 ± 1.9	35 ± 1.4	33.9 ± 2.7	0.58

PHMB, polyhexamethylene biguanide; **TLR4a**, Toll-like receptor 4 agonist; **IFN-γ**, Interferon-gamma; **IL-17a**, Interleukin-17a; **Cq**, Quantification cycle; **Non-prod**, non-producers.

*Complete data were only obtained from 6 out of 10 dogs in this group.

**Significant difference was observed between PHMB+PHMB with TLR4a and control groups but not between PHMB+PHMB with TLR4 and meglumine antimoniate groups and between meglumine antimoniate and control groups.

3.2. Safety and Clinical Efficacy of Treatments

All treatments studied were considered safe. No side effects (local or systemic) were observed during the treatment period in any formulations studied.

Regarding the PHMB formulations, there were no statistically significant differences between the PHMB alone group and the PHMB with TLR4a at day 15 (chi-square: $\chi^2 = 0.9$; df = 2, $p = 0.64$) and day 30 (chi-square: $\chi^2 = 3.6$; df = 2, $p = 0.17$). On the other hand, although differences between local administration of PHMB alone or in combination with TLR4a when compared to the placebo administration were not statistically significant, and thus it is unclear if this would reflect on a large canine population, the results suggested a higher tendency towards resolution (both partial and total) at day 15 (chi-square: $\chi^2 = 5.78$; df = 2, $p = 0.06$) and day 30 post-treatment (chi-square: $\chi^2 = 4.$; df = 2, $p = 0.12$). Conversely, dogs treated with local administration of meglumine antimoniate had a statistically significant fastest resolution compared with placebo treatment at 15 (chi-square: $\chi^2 = 12.58$; df = 2, $p = 0.002$) and 30 days post-treatment (chi-square: $\chi^2 = 9.47$; df = 2, $p = 0.009$). Although no statistically significant differences were observed between the administration of meglumine antimoniate and the administration of PHMB alone or in combination with TLR4a at 15 days post-treatment (chi-square: $\chi^2 = 2.57$; df = 2, $p = 0.27$), there was a higher tendency towards resolution at 30 days post-treatment (chi-square: $\chi^2 = 4.74$; df = 2, $p = 0.09$) when meglumine antimoniate was administered **(Table 11)**.

Table 11. Clinical resolution of papules according to the different treatments (PHMB alone, PHMB in combination with TLR4a, and meglumine antimoniate).

Local treatment (number of dogs)	Clinical Resolution (Number of Dogs/Total, %)					
	15 Days Post-Treatment			30 Days Post-Treatment		
	Without Resolution	Partial	Total	Without Resolution	Partial	Total
PHMB alone (n = 5)	1/5, 20%	3/5, 60%	1/5, 20%	1/5, 20%	2/5, 40%	2/5, 40%
PHMB+ TLR4a (n = 4)	1/4, 25%	3/4, 75%	0/4	0/4	4/4, 100%	0/4
Meglumine antimoniate (n = 10)	0/10	8/10, 80%	2/10, 20%	0/10	3/10, 30%	7/10, 70%
Control group (n = 9): diluent (n = 5) + TLR4a (n = 4)	7/9, 78%	2/9, 22%	0/9	5/9, 56%	3/9, 33%	1/9, 11%
Total (n = 28)	9	16	3	6	12	10

PHMB, polyhexamethylene biguanide; **TLR4a**, Toll-like receptor 4 agonist.

Regarding the dogs included in the control group (n = 9), some showed a total resolution of the papules at days 30 (n = 1/9), 90 (n = 2/9), and 180 (n = 1/9), two were excluded from the study at day 30 because of clinical worsening, and they were treated with meglumine antimoniate subcutaneously (2/9). The other three dogs (3/9) showed a partial resolution at day 60, but they were lost to follow-up **(Figure 6)**.

3.3. Clinical Worsening (Withdrawal) and Lost to Follow-Up

Some dogs were lost to follow-up or withdrawn from the study for several reasons **(Figure 6)**. In four (two from the control group and two from the PHMB + TLR4a group), other clinical signs such as loss of body weight, generalized lymphadenomegaly, and decreased appetite were observed during the follow-up, and *L. infantum*-specific antibody levels increased. They were treated with meglumine antimoniate subcutaneously and withdrawn from the study from that point. Samples from several dogs could not be collected during the lockdown caused by SARS-CoV-2, but they were collected again in further follow-ups **(Figure 6)**. In one dog treated with PHMB, initially, clinical resolution of papules was observed, although new papules appeared during the treatment. This was considered a relapse, and a local rescue therapy (30% meglumine antimoniate + 70% pluronic F-127) was instituted. In this case, papules were all resolved on day 15 after the rescue therapy was started.

3.4. *Leishmania infantum*-Specific Antibody Levels at the Time of Diagnosis and Follow-Up

The results of *L. infantum*-specific antibody levels at the time of diagnosis and during treatment follow-up are displayed in **Table 12**.

At the time of diagnosis, all dogs presented negative or low antibody levels except one dog (n = 24, median: 21.4, ranging from 2.8 to 227.1 EU). At day 0, six dogs were positive against *L. infantum* antigen, five of which were low positive, and one was medium positive. The rest of the dogs (n = 18) were seronegative. At day 15 (n = 23, median: 17.8, ranging from 3.5 to 167.4 EU), seven dogs were low positive, one dog was medium positive, and the rest were seronegative. At day 30 (n = 20, median: 13.8, ranging from 2.9 to 121.6 EU), six dogs were low positive, and the rest were seronegative. At day 60 (n = 14, median: 13.3, ranging from 3.8 to 115.5 EU), four dogs were low positive, and the rest were seronegative. At day 90 (n=15, median: 12.4, ranging from 2.8 to 161.6 EU), only one dog was medium positive, and the rest were seronegative. At day 180 (n = 13, median: 3, ranging from 2.8 to 26 EU), all dogs were seronegative. At day 365 (n = 13, median: 16.3, ranging from 2.4 to 213.7 EU), one dog was medium positive, and the rest were seronegative; this dog had not been tested at day 180 due to lack of compliance of the owner.

No significant differences were observed when comparing *L. infantum* antibody levels at day 0 with the follow-up at any time ($p = 0.98$). Regarding the follow-up, only in 10 dogs (41.7%) was it possible to complete it as planned (days 0, 15, 30, 60, 90, 180, and 365). At days 180 and 365, thirteen dogs out of a total of 24 (54.2%) were tested.

Twelve dogs remained seronegative, while only one dog remained seropositive during all the follow-ups. Interestingly, four dogs experimented with a seroreversion since the antibody levels changed from low positive to negative. In contrast, no dogs showed seroconversion during the follow-up period. One dog treated with PHMB + TLR4a changed from low positive to medium positive.

3.5. IFN-γ Concentration at the Time of Diagnosis and During Treatment Follow-Up

The results of IFN-γ concentration at the time of diagnosis and during treatment follow-up are displayed in **Table 12**.

At day 0, 12 of 24 dogs were considered IFN-γ producers. Supernatants from LSA-stimulated whole blood of IFN-γ producer dogs presented significantly higher concentrations of IFN-γ (median: 607.8, ranging from 171.9 to 3997.5 pg/mL) when compared with IFN-γ non-producers (median: 10.6, ranging from 0 to 84 pg/mL) ($p < 0.0001$) **(Figure 7a)**. However, the concentration of IFN-γ of ConA stimulated blood from IFN-γ non-producers (median: 1287, ranging from 153.8 to 4254.2 pg/mL) did not show statistically significant differences ($p = 0.442$) with the IFN-γ producers' group (median: 1287.5, ranging from 459.3 to 5959.9 pg/mL).

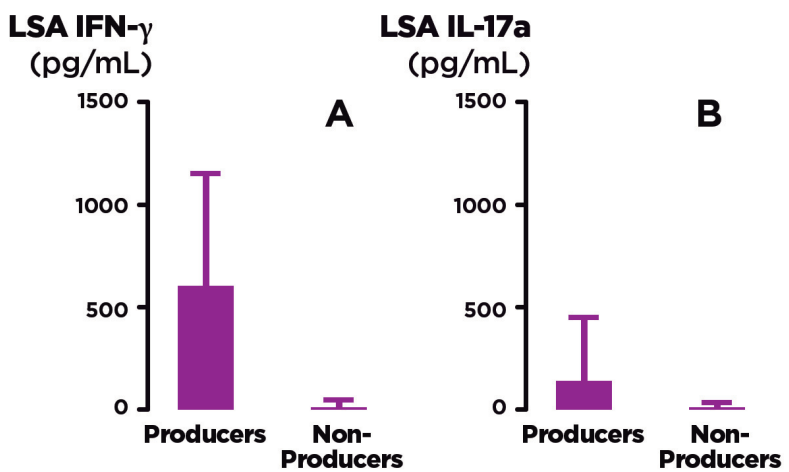


Figure 7. Median concentration with 95% CI **(A)** IFN-γ and **(B)** IL-17a concentration after LSA stimulation in cytokine producers and non-producers dogs at the time of diagnosis and prior to administration of local treatment.

Table 12. Serological, molecular, IFN-γ and IL-17a concentrations and NBT results of dogs at diagnosis and follow-ups.

	Number of seropositive dogs (%)	Median ELISA units (min-max)	Number of LSA IFN-γ producers (%)	Median LSA IFN-γ (pg/mL) (min-max)	Number of LSA IL-17a producers (%)	Median LSA IL-17a (pg/mL) (min-max)	PCR positives (%)	Mean Cq±SD	Mean NBT rate ±SD (%)
Day 0	6/24 (25%)	21.4 (2.8–227.1)	12/24 (50%)	127.9 (0–3998)	10/24 (42%)	23.2 (0–1933)	13 (54.2)	34.7 ± 2.1	18 ± 6%
Day 15	8/23 (34.8%)	17.8 (3.5–167.4)	–	–	–	–			–
Day 30	6/20 (30%)	13.8 (2.9–121.6)	9/20 (45%)	61.7 (0-3614)	11/20 (55%)	85.2 (0–630.3)	4 (20)	37.1 ± 0.5	19± 5%
Day 60	4/14 (28.6%)	13.3 (3.8–115.5)	–	–	–	–			–
Day 90	1/15 (6.7%)	12.4 (2.8–161.6)	9/12 (75%)	444.1 (4.6–6002.9)					
Day 180	0/13 (0%)	9.58 (3–26)	5/11 (45.5%)	41.6 (0–2660)					
Day 365	1/13 (8%)	16.3 (2.4–213.7)	5/12 (41.7%)	1250.2 (0–6947.7)			12 (92.3)	34.7 ± 1.2	

Cq, Quantification cycle; **IFN-γ**, Interferon-gamma; **IL-17a**, Interleukin-17a; **LSA**, aoluble *L. infantum* antigen; **NBT**, Nitroblue tetrazolium reduction test; **min**, minimum; **max**, maximum.

At day 30, 9 of the 20 dogs analyzed were classified as IFN-γ producers. Supernatants from LSA-stimulated whole blood of IFN-γ producer dogs showed higher concentrations of IFN-γ (median: 668.9, ranging from 233.9 to 3614 pg/mL) when compared with the IFN-γ non-producers' group (median: 23.6, ranging from 0 to 62.7 pg/mL) ($p = 0.0017$). Regarding the concentration of IFN-γ of ConA stimulated blood from IFN-γ producers (median: 2503.7, ranging from 357.3 to 5073.2 pg/mL), there were no significant differences ($p = 0.14$) with the IFN-γ non-producers (median: 1295.6, ranging from 293.8 to 3576 pg/mL).

Concerning the rest of the follow-up, 9 dogs out of a total of 12 were considered IFN-γ producers at day 90, 5 out of 11 dogs were producers at day 180, while 5 dogs out of 12 remained IFN-γ producers at day 365.

Additionally, no statistically significant differences were observed when comparing IFN-γ concentrations in supernatants of blood stimulated with LSA from all dogs at day 0 and day 30 ($p = 0.35$) nor when comparing IFN-γ concentration after ConA stimulation at day 0 and day 30 ($p = 0.81$).

3.6. IL-17a Concentration at Time of Diagnosis and During Treatment Follow-Up

The results of IL-17a concentration at the time of diagnosis and during treatment follow-up are displayed in **Table 12**.

At diagnosis, 10 of 24 dogs were classified as IL-17a producers. Supernatants from LSA-stimulated whole blood of IL-17a producer dogs presented significantly higher concentrations of IL-17a (median: 149.5, ranging from 69.4 to 1933.3 pg/mL) when comparing with IL-17a non-producers (median: 0, ranging from 0 to 32.1 pg/mL) ($p < 0.0001$) **(Fig. 7b)**. However, concentrations of IL-17a of ConA stimulated blood from IL-17a non-producers (median: 6466.3, ranging from 721.3 to 23050.2 pg/mL) did not show statistically significant differences ($p = 0.34$) with the IL-17a producers' group (median: 9228.7 ranging from 3363.5 to 18143.3 pg/mL).

On day 30, 11 dogs were considered IL-17a producers out of 20 dogs. IL-17a concentration after LSA stimulation of IL-17a producer dogs demonstrated higher concentrations of IL-17a (median: 155.7 ranging from 74 to 630.3 pg/mL) when comparing with the IL-17a non-producers' group (median: 2.8 ranging from 0 to 40 pg/mL) ($p < 0.0001$). IL-17a concentration after ConA stimulation was not statistically different ($p = 0.56$) between IL-17a producers (median: 14088.6 ranging from 1082.1 to 41390 pg/mL) and IL-17a non-producers (median: 12425.3 ranging from 2637 to 31150 pg/mL) dogs.

Furthermore, no statistically significant differences were observed when comparing IL-17a concentrations in supernatants of blood stimulated with LSA

from all dogs at day 0 and day 30 (p = 0.08). However, significant differences were found when comparing IL-17a concentration after ConA stimulation at day 0 and day 30 (p = 0.01).

3.7. Correlation between Parameters Studied

Considering all dogs studied (n = 24), a correlation was not found between IFN-γ concentration after LSA stimulation and *L. infantum* specific antibody levels (Spearman r: 0.31, p = 0.15) neither between IL-17a after LSA stimulation and antibody levels (Spearman r: 0.25, p = 0.24). Moreover, there was no correlation between the age of the dogs and antibody levels (Spearman r: -0.28, p = 0.18).

No correlation was found between PCR Cq and *L. infantum* specific antibody levels (Spearman r: -0.07, p =0.8) nor PCR Cq and IFN-γ concentration after LSA stimulation (Spearman r: 0.41, p = 0.09) nor between PCR Cq and IL-17a after LSA stimulation (Spearman r: 0.46, p = 0.06).

IFN-γ concentration after LSA stimulation was significantly positively correlated with IL-17a concentration after LSA stimulation (Spearman r: 0.59, p < 0.0001) **(Figure 8)**.

Similarly, there was a significant correlation between IFN-γ concentration after Con A stimulation and IL-17a concentration after Con A stimulation (Spearman r: 0.54, p = 0.0002).

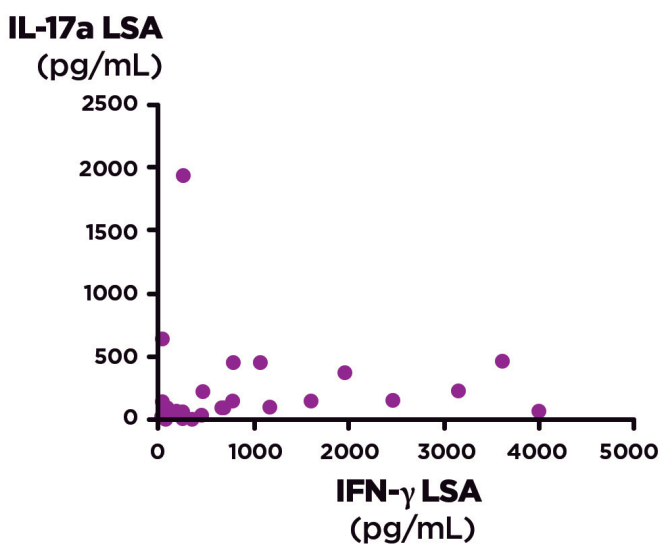


Figure 8. Correlation between IL-17a (pg/mL) and IFN-γ (pg/mL) after LSA stimulation in the total of dogs studied at the time of diagnosis and follow-up. Spearman r: 0.59, p < 0.0001.

3.8. Nitroblue Tetrazolium Reduction Test

There were no statistically significant differences when comparing the NBT reduction rate at day 0 (mean ± SD: 18 ± 6 %) with the NBT reduction rate at day 30 (mean ± SD: 19 ± 5 %) (p=0.53).

3.9. Blood PCR

The *Leishmania* real-time PCR results at the time of diagnosis and during the follow-up period are depicted in **Table 12**.

At the time of diagnosis, 13 out of a total of 24 (54.2%) dogs were PCR positive.

At day 30, only 4 dogs were PCR positive from a total of 20 (20%) dogs.

Interestingly, 2 dogs remained positive from day 0 to day 30. The other 2 negative dogs at day 0 were PCR positive at day 30. In contrast, 8 dogs that were PCR positive at day 0 were negative at day 30. Unfortunately, 4 dogs were lost to follow-up on day 30.

Regarding the last PCR performed from each dog, one was at day 60 and was positive, while it was negative at days 0 and 30.

On day 180, 3 dogs were tested, and all were PCR positive.

Finally, at day 365, 12 out of 13 dogs were PCR positive (92%). Two dogs remained always positive from day 0, only one remained negative from day 0, and the others changed depending on the time.

Regarding the proportion of positive dogs, significant differences were found when comparing day 0 to day 30 (p = 0.03), day 0 to day 365 (p = 0.02), and day 30 to day 365 (p = <0.0001). Statistically significant differences were observed when comparing PCR Cq results at day 0 (mean ± SD: 34.7 ± 2.1) and day 30 (mean ± SD: 37.1 ± 0.5) (p = 0.04), and when comparing PCR Cq results at day 30 and day 365 (mean ± SD: 34.7 ± 1.2) (p = 0.0023). However, no differences were observed when PCR Cq results at day 0 and day 365 were compared (p = 0.93).

4. Discussion

To the author's best knowledge, there are few studies evaluating the effectiveness of local treatment in canine cutaneous leishmaniosis (CCL), but no studies so far have evaluated local treatment of papular dermatitis due to *L. infantum* infection [40–42]. In the present randomized controlled study, 70% of dogs treated with meglumine antimoniate and 40% of dogs treated with PHMB alone showed a complete response after 30 days of treatment, demonstrating a much faster response than the median of 98 ± 5 days observed in a previous

observational study. However, it is difficult to compare these results as we only included dogs with stage I-mild disease-papular dermatitis, and in the previous study, all dogs had ulcerative lesions in which self-healing was not expected [1,40]. Moreover, no adverse effects were observed throughout the course of the study in any of the dogs. Therefore, we might conclude that all the studied substances were safe.

Although meglumine antimoniate is widely described as a systemic treatment for *L. infantum* infection both in humans and dogs [36], to the author's best knowledge, there is limited information regarding the efficacy of its topical use in dogs with either cutaneous leishmaniosis or papular dermatitis. Interestingly, a recent study demonstrated the efficacy of an intralesional meglumine antimoniate compared with a placebo for the treatment of CCL due to *L. braziliensis* [41]. Dogs with localized cutaneous ulcers were enrolled in this study and showed a higher and faster healing rate when they were treated with intralesional meglumine antimoniate [41]. In human medicine, meglumine antimoniate is commonly used as a local treatment through intralesional administration and is considered appropriate for the treatment of simple cutaneous lesions due to *L. panamensis* and *L. mexicana* [43]. Local application of meglumine antimoniate has also been tested in murine leishmaniosis models [44–47]. The main obstacle to the local administration of meglumine antimoniate is that it is considered a water-soluble molecule, and thus, it has limited interaction with lipophilic skin structures [29].

In murine models, different drug delivery systems have been evaluated. Moosavian Kalat et al. (2014) tested a liposome containing 22.5% meglumine antimoniate as a local treatment on infected BALB/c mice and observed a reduction in lesion size and a lower spleen parasite burden in treated mice compared to the control group [44]. The same group, in a further study (2019), tested a similar mixture by adding stearylamine, which improved liposome performance with good clinical improvement [45]. In another study published in 2013, meglumine antimoniate administered using a liposomal formulation showed a reduction in lesion size and amastigote counts, although it had no significant therapeutic difference compared to the control group [46]. Finally, Horoiwaone et al. (2020) tested the local application of meglumine antimoniate contained in maltodextrin polymeric colloidal nanocarriers in a murine leishmaniasis model, which showed similar efficacy to the intraperitoneal injection regarding parasite titer reduction and superior healing activity in terms of collagen area deposition [47]. Other delivery systems have also been tested for systemic administration of meglumine antimoniate, which could be considered for topical administration in future studies, including polymeric nanoformulations such as aqueous-core poly-L-lactide nanocapsules, which have shown a great antileishmanial activity on mice infected with *L. infantum*

[48]. In addition, this new formulation promotes meglumine antimoniate uptake within the macrophages, increasing its efficacy and decreasing its cellular-negative outcome [48].

In the present study, the administration of meglumine antimoniate with pluronic F-127 as a delivery system was tested, which is a non-ionic detergent used to facilitate the solubilization of water-insoluble materials in physiological media. A significant improvement was observed in our patients when compared to the control group, and no adverse effects were detected during the length of administration nor the posterior follow-ups, which could mean that pluronic F-127 is a safe and adequate delivery system and that locally administered meglumine antimoniate could be considered as a viable treatment option in dogs with localized papular dermatitis due to *L. infantum*. This new local formulation of meglumine antimoniate has shown to be safe for its use in humans, and it has been previously tested *in vitro* in three cell lines, *ex vivo* using human skin samples, and *in vivo* testing on human volunteers [35].

Regarding PHMB, to the author's best knowledge, there are no previous *in vivo* studies reporting its use as a topical formulation for the treatment of papules due to *L. infantum* infection, neither in dogs nor in humans. On the other hand, it has been described in previous publications as a topical antimicrobial for locally infected wounds in dogs [49,50], and there is a study about the safety of its use in combination with other drugs as an ear flush in dogs [51]. In humans, PHMB has been used as a first-line treatment for infected wounds [52,53] and as a treatment for *Acanthamoeba* keratitis [54].

As mentioned previously, in the present study, dogs treated with topical PHMB showed a higher tendency toward resolution compared with the control group, although the results were not statistically significant. PHMB has been shown to have antileishmanial properties, such as disruption of the parasite´s membrane integrity and chromosomal damage, in previous *in vitro* studies [22,34]. We also evaluated its combination with TLR4a, as according to a previous study, TLR4 activation leads to the parasite's death by inducing a protective immune response against *Leishmania* and regulating iNOS [22]. According to our results, no statistically significant differences were observed between the PHMB alone group and the PHMB combined with TLR4a. This lack of difference could be due to a lack of effectiveness regarding the TLR4a stimulation, insufficient dosage, or due to complications such as lack of absorption of the TLR4a. However, a small number of dogs were included in these groups, and thus a significant difference could have been observed if more dogs were included.

To the author's best knowledge, there are only two published studies in which a follow-up over dogs with papular dermatitis was performed [4,55]. In one of them, 15 out of 17 dogs were treated with subcutaneous meglumine antimoniate

for 25–30 days, showing complete resolution of lesions by day 25 in all dogs [4]. The remaining dog received no treatment and was only revisited four months later, showing only partial remission [4]. In the other study, 3 out of 8 dogs were treated twice a day with meglumine antimoniate subcutaneously (50 mg/kg SC BID) and allopurinol orally (10 mg/kg PO BID), showing total resolution by 10 days of treatment [55]. The other four dogs received no treatment, showing improvement only after 3 to 5 months [55]. Similarly, in the present study, all dogs treated with topical meglumine antimoniate showed improvement one month after starting treatment, although only 70% had a complete resolution of lesions. However, taking into account the mean of administration (much less painful and stressful for the dog and the owner) and the lower risk of adverse effects, we can consider topical treatment as an advantageous alternative.

As mentioned previously, self-healing of the skin lesions may occur between three and six months in previous studies [4,55]. This fact is similar to the results of the present study, in which some dogs included in the control group showed a total resolution of the papules at days 90 (22%) and 180 (11%) or partial resolution at day 60 (11%), although one showed total resolution by day 30 (11%).

The immune response was also evaluated in this study. IFN-γ is one of the cytokines expressed by a Th1 response (considered an effective immune response). In the present study, 50% (12/24) of dogs at day 0 and 45% (9/20) at day 30 were considered IFN- γ producers. These findings are similar to previous studies using IFN-γ release whole blood assay in dogs with stage I and papular dermatitis. In one study [5], dogs in stage I (25.7%) and IIa (48.5%) showed a higher production of IFN-γ than other dogs classified as stage IIb (8.5%), III (14.2%), and IV (2.8%). In another study, 15 of 19 dogs in stage I (79%) were IFN-γ producers, whereas only 6 out of 15 dogs (40%) were in stage II-III [6]. Interestingly, in this study, the dogs that were followed up at different time points presented a production of parasite-specific IFN-γ that ranged from 41 to 75% of the dogs studied.

The results of IFN-γ concentration showed a positive correlation with IL-17a concentration after LSA and Con A stimulation. This correlation was expected regarding previous studies where it was demonstrated that IL-17a played a synergic role with IFN-γ by blocking parasite growth after checking IFN-γ and IL-17a levels in infected mice [10]. In another study, mRNA expression for iNOS and IFN- γ was positively correlated with IL-17a gene transcription in dogs [11]. However, another study concluded that SNPs located in analogous regions of canine IL-17a gene promoter did not show an association with *Leishmania spp.* resistance [56]. Further studies on the role played by IL-17a in *L. infantum* infection are required to determine if it might be used as a prognostic marker.

In addition, it is important to highlight that, similarly to previous studies, the

majority of these dogs were seronegative or presented very low positive antibody levels throughout the study period, and four dogs even showed seroreversion [6]. Therefore, even if dogs were only treated with local treatment or no treatment, most of them did not have relapses or worsening of the infection. Regarding this, only one dog treated with PHMB alone showed relapse of papular dermatitis, another dog treated with PHMB + TLR4a demonstrated an increase in serology levels through treatment, and four dogs were withdrawn from the study due to worsening of clinical signs, two associated to the control group (2/4) and two to PHMB + TLR4a group (2/4). Conversely, no dogs treated with local meglumine antimoniate showed worsening or relapse of clinical signs throughout the study period, nor an increase in antibody levels, and the dog that had relapsed while PHMB was being administrated, did show complete resolution after changing to local meglumine antimoniate, demonstrating, in these cases, a good efficacy not only in the treatment of clinical signs but also to avoid relapses or worsening of the disease.

According to previous papers, in this study, there was no association between sex or age and NBT reduction rate [24,25]. In a previous study, the NBT reduction rate of 40 healthy dogs (4.57 ± 1.72 %), 20 dogs in Stage-I (34 ± 10.05%), and 20 dogs in Stage-IV (3.7 ± 2.03 %) was compared, showing that NBT reduction rate was significantly higher in dogs in Stage-I [24]. In another study, the NBT reduction rate was compared between ten healthy dogs treated with 0.5 mg/kg oral (PO) of domperidone once a day (SID) for 30 days and ten non-treated healthy dogs [25]. In this previous study, the results from the non-treated group showed similar means to the first study (5.9 ± 1.6%) [25]. According to these previous papers, our results of NBT reduction rate at day 0 (mean ± SD: 18 ± 6%) and day 30 (mean ± SD: 19 ± 5%) were closer to the Stage-I results [24,25].

Concerning real-time PCR, the results of the present study are in agreement with previous studies where dogs with mild leishmaniosis showed a lower proportion of positive cases than dogs in more advanced stages [5,6,57]. It is well known that sick dogs with high antibody levels also show high parasitemia levels [57]. It is worth noticing that a difference was observed between the proportion of PCR-positive dogs at day 0 (54.2%) and day 30 (20%) ($p = 0.03$), and when comparing dogs at day 30 and day 365 (92.3%) ($p = 0.0001$), showing a significantly lower result at day 30 and a significantly higher result at day 365. This could be interpreted as a signal of infection recrudescence. However, it was not associated with clinical or analytical worsening, and the parasitemia (PCR Cq) was the same at day 365 (34.7 ± 1.2) and at day 0 (34.7 ± 2.1), indicating a possible clinical cure with infection persistence or progression. Nevertheless, it should be taken into account that in this study, dogs were changing between PCR positive or negative depending on the day of the follow-up. Intermittent or transitory parasitemia has been commonly observed in previous studies in dogs

with *Leishmania* infection and is the most likely explanation for the variability of the results throughout the follow-up [58]. Moreover, the number of dogs that were available for PCR testing on day 365 was much lower than initially, and this could affect the results. It should also be pointed out that PCR performed on DNA extracted from whole blood samples is a less sensitive technique than PCR performed on bone marrow, lymph node, spleen, or skin, which could be regarded as a limitation of this study [59,60]. Invasive sampling for PCR was considered not ethical in this scenario.

Regarding the onset of clinical signs, 87.5% of the papules appeared in autumn and winter, as previously described [4,61]. This period belongs to the end of the sandfly season, which could mean a delay between *Leishmania* inoculation and the development of papular dermatitis. This might be explained by a period of parasite amplification, as it was described in mice [62] and dogs [63]. The majority of dogs in this study were young, 91.7% were below 12 months of age, and 45.8% were below 6 months of age, as was observed in previous studies [4,61]. This could mean that papular dermatitis might be more common at the time of the first contact of the parasite with the host's immune system. In the same way, the distribution of papular lesions was similar to these previous studies, 46.9% were located on the inner surface of the pinna, and 18.8% were on the abdomen [4,61].

5. Conclusions

In conclusion, the results of this study showed that dogs with papular dermatitis treated with local meglumine antimoniate healed faster than dogs treated with a placebo. Furthermore, no dog treated with topical meglumine antimoniate showed worsening clinical signs or relapse during the one-year follow-up. On the other hand, PHMB (alone or in combination with TLR4a) showed a higher tendency towards resolution when compared to placebo, although results were considered non-significant. Moreover, no adverse effects were observed in any of the drugs throughout the study in any of the dogs. Therefore, we might conclude that local meglumine antimoniate is a safe and effective alternative for the treatment of papular dermatitis in dogs with *L. infantum* infection. The present study also showed that most dogs presented a protective immune response at the time of diagnosis and during a year follow-up period without clinical failure.

Author Contributions: Conceptualization, L.S.-G.; formal analysis, I.M.-F.; investigation, I.M.-F., M.J.G., A.D., M.C., L.O., and M.M.A.; resources; L.S.-G., M.M.A., D.B., L.G., R.F., and C.R.; data curation, I.M.-F.; writing—original draft preparation, I.M.-F.; writing—review and editing, L.S.-G. and L.O.; visualization, L.S.-G.; supervision,

L.S.-G.; project administration, L.S.-G.; funding acquisition, L.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish ministry grant, *Ministerio de Economía y competitividad,* and *Fondo Europeo de Desarrollo Regional* (FEDER, EU) (AGL2015-68477).

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of *Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona* (CEAAH 4526, November 2018) and by *Generalitat de Catalunya* (FUE-2018-00944112 i ID KSHYD6LVR, April 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting reported results can be obtained by emailing the corresponding author.

Acknowledgments: Authors would like to acknowledge Isaac Carrasco Rivero and Amalia Melendreras Montesinos for their contribution to clinical cases, Lourdes Alarcón Sánchez for her priceless technical help and support, Marta Baxarias Canals for her considerable assistance with the study, and Carles Blasi Brugué for his valuable work reviewing the article.

Conflicts of Interest: The authors declare no conflict of interest.

6. References

1. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet guidelines for the practical management of canine leishmaniosis. *Parasites Vectors* **2011**, *4*, 1–16.
2. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine leishmaniosis—New concepts and insights on an expanding zoonosis: Part one. *Trends Parasitol.* **2008**, *24*, 324–330.
3. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological findings and detection of Toll-like receptor 2 in cutaneous lesions of canine leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163.
4. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular dermatitis due to *Leishmania infantum* infection in seventeen dogs: Diagnostic features, extent of the infection and treatment outcome. *Parasites Vectors* **2014**, *7*, 1–11.

5. Solano-Gallego, L.; Montserrrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-specific production of IFN-γ and IL-10 in stimulated blood from dogs with clinical leishmaniosis. *Parasites Vectors* **2016**, *9*.

6. Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P.; Solano-Gallego, L. Parasite specific antibody levels, interferon-γ and TLR2 and TLR4 transcripts in blood from dogs with different clinical stages of leishmaniosis. *Vet. Sci.* **2018**, *5*, 31.

7. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on adaptive and innate immunity in canine leishmaniosis. *Parasitology* **2017**, *144*, 95–115.

8. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania infantum*. V*et. Immunol. Immunopathol.* **2015**, *163*, 94–102.

9. Hernandez-Bures, A.; Gillen, J.; Apostolidis, K.; Saridomichelakis, M.; Di Loria, A.; Santoro, D. Evaluation of the cutaneous inflammatory cells in dogs with leishmaniosis and in dogs without the disease that were naturally infected by *Leishmania infantum* (syn. *L. chagasi*). *Vet. Dermatol.* **2021**, *32*, 99–e19.

10. Nascimento, M.S.L.; Carregaro, V.; Lima-Júnior, D.S.; Costa, D.L.; Ryffel, B.; Duthie, M.S.; de Jesus, A.; de Almeida, R.P.; da Silva, J.S. Interleukin 17A acts synergistically with interferon γ to promote protection against *Leishmania infantum* infection. *J. Infect. Dis.* **2015**, *211*, 1015–1026.

11. Nascimento, M.S.L.; Albuquerque, T.D.R.; Nascimento, A.F.S. Impairment of Interleukin-17A Expression in Canine Visceral Leishmaniosis is Correlated with Reduced Interferon-γ and Inducible Nitric Oxide Synthase Expression. *J. Comp. Pathol.* **2015**, *153*, 197–205.

12. Rebech, G.T.; Venturin, G.L.; Siqueira Ito, L.T.; Bragato, J.P.; de Carvalho Fonseca, B.S.; Melo, L.M.; Costa, S.F.; de Rezende Eugênio, F.; Dos Santos, P.S.P.; de Lima, V.M.F. PD-1 regulates leishmanicidal activity and IL-17 in dogs with leishmaniasis. *Vet. Immunol. Immunopathol.* **2020**, *219*, 109970.

13. Toepp, A.J.; Petersen, C.A. The balancing act: Immunology of leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25.

14. Esch, K.J.; Schaut, R.G.; Lamb, I.M.; Clay, G.; Lima, L.M.; Nascimento, R.P.; Whitley, E.M.; Jeronimo, S.M.; Sutterwala, F.S.; Haynes, J.S.; Petersen, C.A. Activation of Autophagy and Nucleotide-Binding Domain Leucine-Rich Repeat e Containing-Like Receptor Family, Pyrin Domain e Containing 3 Inflammasome during *Leishmania infantum* e Associated Glomerulonephritis. *Am. J. Pathol.* **2015**, *185*, 2105–2117.

15. Gibson-Corley, K.N.; Hostetter, J.M.; Hostetter, S.J.; Mullin, K.; Ramer-Tait, A.E.; Boggiatto, P.M.; Petersen, C.A. Disseminated *Leishmania infantum* infection in two sibling foxhounds due to possible vertical transmission. *Can. Vet. J.* **2008**, *49*, 1005–1008.

16. Gibson-corley, K.N.; Boggiatto, P.M.; Mukbel, R.M.; Petersen, C.A.; Jones, D.E. A deficiency in the B cell response of C57BL / 6 mice correlates with loss of macrophage-mediated killing of *Leishmania amazonensis*. *Int. J. Parasitol.* **2010**, *40*, 157–161.

17. Pereira-Fonseca, D.C.M.; Oliveira-Rovai, F.M.; Rodas, L.A.C.; Beloti, C.A.C.; Torrecilha, R.B.P.; Ito, P.K.R.K.; Avanço, S.V.; Cipriano, R.S.; Utsunomiya, Y.T.; Hiramoto, R.M.; Calvo-Bado, L.; Courtenay, O.; Machado, G.F.; Lima, V.M.F.; Nunes, C.M. Dog skin parasite load, TLR-2, IL-10 and TNF-α expression and infectiousness. *Parasite Immunol.* **2017**, *39*, 11.

18. Carpenter, S.; O'Neill, L.A.J. How important are Toll-like receptors for antimicrobial responses? *Cell Microbiol.* **2007**, *9*, 1891–1901.

19. Kumar, H.; Kawai, T.; Akira, S. Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* **2011**, *30*, 16–34.

20. Kawai, T.; Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **2011**, *34*, 637–650.

21. Ordeix, L.; Montserrat-Sangrà, S.; Martínez-Orellana, P.; Baxarias, M.; Solano-Gallego, L. Toll-like receptors 2, 4 and 7, interferon-gamma and interleukin 10, and programmed death ligand 1 transcripts in skin from dogs of different clinical stages of leishmaniosis. *Parasit. Vectors.* **2019**, *12*, 575.

22. Martínez-Orellana, P.; Baxarias, M.; Good, L.; Solano-Gallego, L. The effects of polyhexamethylene biguanide (PHMB) and TLR agonists alone or as polyplex nanoparticles against *Leishmania infantum* promastigotes and amastigotes. *Vet. Sci.* **2020**, *7*, 179.

23. Winterbourn, C.C.; Kettle, A.J.; Hampton, M.B. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **2016**, *85*, 765–792.

24. Gómez-Ochoa, P.; Lara, A.; Couto, G.; Marcen, J.M.; Peris, A.; Gascón, M.; Castillo, J.A. The nitroblue tetrazolium reduction test in canine leishmaniosis. *Vet. Parasitol.* **2010**, *172*, 135–138.

25. Gómez-Ochoa, P.; Sabate, D.; Homedes, J.; Ferrer, L. Use of the nitroblue tetrazolium reduction test for the evaluation of Domperidone effects on the neutrophilic function of healthy dogs. *Vet. Immunol. Immunopathol.* **2012**, *146*, 97–99.

26. Dea-Ayuela, M.A.; Segarra, S.; Serrano, D.R.; Bolás-Fernández, F. Nucleotides and AHCC Enhance Th1 Responses in Vitro in *Leishmania*-Stimulated/Infected Murine Cells. *Molecules* **2020**, *25,* 3918.

27. Ordeix, L.; Rodríguez, A.; Martínez-Orellana Pamela Montserrat Sangrà, S.; Solano-Gallego, L. Clinical follow up of a series of dogs with papular dermatitis due to *Leishmania infantum*. In Proceedings of the 29th Annual Congress of the ESVD-ECVD, Lausanne, Switzerland, 14 September 2017.

28. Aronson, N.; Herwaldt, B.L.; Libman, M.; Pearson, R.; Lopez-Velez, R.; Weina, P.; Carvalho, E.; Ephros, M.; Jeronimo, S.; Magill, A. Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin. Infect. Dis.* **2016**, *63*, 1539–1557.

29. Garza-Tovar, T.F.; Sacriste-Hernández, M.I.; Juárez-Durán, E.R.; Arenas, R. An overview of the treatment of cutaneous leishmaniasis. *Fac. Rev.* **2020**, *9*, 28.

30. Burza, S.; Croft, S.L.; Boelaert, M. Leishmaniasis. *Lancet* **2018**, *392*, 951–970.

31. Nassif, P.W.; França, T.; Mello, P.D.E.; Navasconi, T.R.; Mota, C.A.; Demarchi, I.G.; Aristides, S.M.A.; Lonardoni, M.V.C.; Teixeira, J.J.V.; Silveira, T.G.V. Safety and efficacy of current alternatives in the topical treatment of cutaneous leishmaniasis : A systematic review. *Parasitology* **2017**, *144*, 995–1004.

32. Uribe-Restrepo, A.F.; Prieto, M.D.; Cossio, A.; Desai, M.M.; Del Mar Castro, M. Eligibility for Local Therapies in Adolescents and Adults with Cutaneous Leishmaniasis from Southwestern Colombia: A Cross-Sectional Study. *Am. J. Trop. Med. Hyg.* **2019**, *100*, 306–310.

33. Mulder, G.; Cavorsi, J.; Lee, D. Polyhexamethylene Biguanide (PHMB): An Addendum to Current Topical Antimicrobials. *Wounds* **2007**, *19*, 173–182.

34. Firdessa, R.; Good, L.; Amstalden, M.C.; Chindera, K.; Kamaruzzaman, N.F.; Schultheis, M.; Röger, B.; Hecht, N.; Oelschlaeger, T.A.; Meinel, L.; Lühmann, T.; Moll, H. Pathogen- and Host-Directed Antileishmanial Effects Mediated by Polyhexanide (PHMB). *PLoS Negl. Trop. Dis.* **2015**, *9*, e0004041.

35. Berenguer, D.; Sosa, L.; Alcover, M.; Sessa, M.; Halbaut, L.; Guillén, C.; Fisa, R.; Calpena-Campmany, A.C.; Riera, C. Development and characterization of a semi-solid dosage form of meglumine antimoniate for topical treatment of cutaneous leishmaniasis. *Pharmaceutics* **2019**, *11*, 613.

36. Solano-Gallego, L.; Di Filippo, L.; Ordeix, L.; Planellas, M.; Roura, X.; Altet, L.; Martínez-Orellana, P.; Montserrat, S. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. *Parasit Vectors* **2016**, *9*, 1–9.

37. Solano-Gallego, L.; Villanueva-Saz, S.; Carbonell, M.; Trotta, M.; Furlanello, T.; Natale, A. Serological diagnosis of canine leishmaniosis: Comparison of three commercial ELISA tests (Leiscan®, ID Screen® and *Leishmania* 96®), a rapid test (Speed Leish K®) and an in-house IFAT. *Parasit. Vectors* **2014**, *7*, 1–10.

38. Martínez-Orellana, P.; González, N.; Baldassarre, A.; Álvarez-Fernández, A.; Ordeix, L.; Paradies, P.; Soto, M.; Solano-Gallego, L. Humoral

Responses and *Ex Vivo* IFN-γ Production after Canine Whole Blood Stimulation with *Leishmania infantum* Antigen or KMP11 Recombinant Protein. *Vet. Sci.* **2022**, *9*, 116.

39. Martín-Ezquerra, G.; Fisa, R.; Riera, C.; Rocamora, V.; Fernández-Casado, A.; Barranco, C.; Serra, T.; Baró, T.; Pujol, R. Role of *Leishmania spp.* infestation in nondiagnostic cutaneous granulomatous lesions: Report of a series of patients from a Western Mediterranean area. *Br. J. Dermatol.* **2009**, *161*, 320–325.
40. Piragauta, S.P.; Higuita-Castro, J.L.; Arbeláez, N.; Restrepo, A.M.; Archbold, R.; Quiñones, W.; Torres, F.; Echeverri, F.; Escobar, G.; Vélez, I.D.; et al. Utility of the combination of hederagenin glucoside saponins and chromane hydrazone in the topical treatment of canine cutaneous leishmaniasis. An observational study. *Parasitol. Res.* **2022**, *121*, 1419–1428.
41. Lago, J.; Fraga, D.; Guimarães, L.H.; Lago, T.; Santos, Y.; Lago, E.; Werneck, G.L.; Bacellar, O.; Carvalho, E.M. Efficacy of intralesional meglumine antimoniate in the treatment of canine tegumentary leishmaniasis: A Randomized controlled trial. *PLOS Negl. Trop. Dis.* **2023**, *17*, e0011064.
42. Barbosa Santos, E.G.O.; Marzochi, M.C.A.; Conceição, N.F.; Brito, C.M.M.; Barroso, J.A.; Pacheco, R.S. N-methylglucamine antimonate (SbV+): Intralesional canine tegumentary leishmaniasis therapy. *Parasite* **1998**, *5*, 175–180.
43. Aronson, N.E.; Joya, C.A. Cutaneous Leishmaniasis: Updates in Diagnosis and Management. *Infect. Dis. Clin. N Am.* **2019**, *33*, 101–117.
44. Moosavian Kalat, S.A.; Khamesipour, A.; Bavarsad, N.; Fallah, M.; Khashyarmanesha, Z.; Feizi, E.; Neghabi, K.; Abbasi, A.; Jaafari, M. Use of topical liposomes containing meglumine antimoniate (Glucantime) for the treatment of *L. major* lesion in BALB/c mice. *Exp. Parasitol.* **2014**, *143*, 5–10.
45. Moosavian, S.A.; Fallah, M.; Jaafari, M.R. The activity of encapsulated meglumine antimoniate in stearylamine-bearing liposomes against cutaneous leishmaniasis in BALB/c mice. *Exp. Parasitol.* **2019**, *200*, 30–35.
46. Momeni, A.; Rasoolian, M.; Momeni, A.; Navaei, A.; Emami, S.; Shaker, Z.; Mohebali, M.; Khoshdel, A. Development of liposomes loaded with anti-leishmanial drugs for the treatment of cutaneous leishmaniasis. *J. Liposome Res.* **2013**, *23*, 134–144.
47. Aragão Horoiwa, T.; Cortez, M.; Sauter, I.P.; Migotto, A.; Bandeira, C.L.; Cerize, N.N.P.; de Oliveira, A.M. Sugar-based colloidal nanocarriers for topical meglumine antimoniate application to cutaneous leishmaniasis treatment: *Ex vivo* cutaneous retention and *in vivo* evaluation. *Eur. J. Pharm. Sci.* **2020**, *147*, 105295.
48. Cosco, D.; Bruno, F.; Castelli, G.; Puleio, R.; Bonacci, S.; Procopio, A.; Britti, D.; Fresta, M.; Vitale, F.; Paolino, D. Meglumine antimoniate-loaded aqueous-core PLA nanocapsules: Old drug, new formulation against *Leishmania*-related diseases. *Macromol. Biosci.* **2021**, *21,* e2100046.

49. Nolff, M.C.; Winter, S.; Reese, S.; Meyer-Lindenberg, A. Comparison of polyhexanide, cold atmospheric plasma and saline in the treatment of canine bite wounds. *J. Small Anim. Pract.* **2019**, *60*, 348–355.

50. Llorens, E.; Calderón, S.; Del Valle, L.J.; Puiggalí, J. Polybiguanide (PHMB) loaded in PLA scaffolds displaying high hydrophobic, biocompatibility and antibacterial properties. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2015**, *50*, 74–84.

51. Mills, P.C.; Ahlstrom, L.; Wilson, W.J. Ototoxicity and tolerance assessment of a TrisEDTA and polyhexamethylene biguanide ear flush formulation in dogs. *J. Vet. Pharmacol. Ther.* **2005**, *28*, 391–397.

52. Barrigah-Benissan, K.; Ory, J.; Sotto, A.; Salipante, F.; Lavigne, J.-P.; Loubet, P. Antiseptic Agents for Chronic Wounds: A Systematic Review. *Antibiotics* **2022**, *11,* 350.

53. Worsley, A.; Vassileva, K.; Tsui, J.; Song, W.; Good, L. Polyhexamethylene Biguanide_Polyurethane Blend Nanofibrous Membranes for Wound Infection Control. *Polymers* **2019**, *11*, 915.

54. Bagga, B.; Sharma, S.; Gour, R.P.S.; Mohamed, A.; Joseph, J.; MRathi, V.; Garg, P. A randomized masked pilot clinical trial to compare the efficacy of topical 1% voriconazole ophthalmic solution as monotherapy with combination therapy of topical 0.02% polyhexamethylene biguanide and 0.02% chlorhexidine in the treatment of *Acanthamoeba* keratitis. *Eye* **2021**, *35*, 1326–1333.

55. Bottero, E.; Poggi, M.; Viglione, M. Lesioni papulari indotte da *Leishmania* spp. in 8 cani giovani. *Veterinaria* **2006**, *20*, 33–36.

56. Gonçalves-de-albuquerque, C.; Gomes, L.; Sousa-paula LCDe Gaud, K.; Sales, S.; Boegel, A. Exploring IL-17 gene promoter polymorphisms in canine leishmaniasis. *Acta Trop.* **2022**, *232*, 106452.

57. Rodríguez-Cortés, A.; Ojeda, A.; López-Fuertes, L.; Timón, M.; Altet, L.; Solano-Gallego, L.; Sánchez-Robert, E.; Francino, O.; Alberola, J. A long term experimental study of canine visceral leishmaniasis. *Int. J. Parasitol.* **2007**, *37*, 683–693.

58. Pietro SDi Crinò, C.; Falcone, A.; Crupi, R.; Francaviglia, F.; Vitale, F.; Giudice, E. Parasitemia and its daily variation in canine leishmaniasis. *Parasitol. Res.* **2020**, *119*, 3541–3548.

59. Solano-Gallego, L.; Rodriguez-Cortes, A.; Trotta, M.; Zampieron, C.; Razia, L.; Furlanello, T.; Caldin, M.; Roura, X.; Alberola, J.. Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniosis. *Vet. Parasitol.* **2007**, *147*, 315–319.

60. Solano-Gallego, L.; Koutinas, A.; Miró, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet. Parasitol.* **2009**, *165*, 1–18.

61. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular dermatitis due to *Leishmania spp.* infection in dogs with parasite-specific cellular immune responses. *Vet. Dermatol.* **2005**, *16*, 187–191.

62. Belkaid, Y.; Mendez, S.; Lira, R.; Kadambi, N.; Milon, G.; Sacks, D. A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* **2000**, *165*, 969–977.

63. Aslan, H.; Oliveira, F.; Meneses, C.; Castrovinci, P.; Gomes, R.; Teixeira, C.; Derenge, C.A.; Orandle, M.; Gradoni, L.; Oliva, G.; et al. New insights into the transmissibility of *Leishmania infantum* from dogs to sand flies: Experimental vector-transmission reveals persistent parasite depots at bite sites. *J. Infect. Dis.* **2016**, *213*, 1752–1761.

Exploring the Relationship Between Neutrophil Activation and Different States of Canine *L. infantum* Infection: Nitroblue Tetrazolium Test and IFN-γ

Carles Blasi-Brugué[†], Icíar Martínez-Flórez[†], Marta Baxarias, Joan del Rio-Velasco and Laia Solano-Gallego[*]

Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; carles.blasi@uab.cat (C.B.-B.); iciar.martinez@uab.cat (I.M.-F.); martabaxcan@gmail.com (M.B.); rio.joan@gmail.com (J.dR.)

[*] Correspondence: laia.solano@uab.cat

[†] These authors contributed equally to this work

Published in: **Veterinary sciences**
https://doi.org/10.3390/vetsci10090572

Simple Summary

This study aimed to understand the role of neutrophils in canine leishmaniosis (CanL) by assessing neutrophil activation and its relationship with different states of *Leishmania infantum* infection and antibody and IFN-γ production. The results showed that sick dogs in stage I-mild disease had significantly higher neutrophil activation compared to healthy seronegative and seropositive dogs and sick dogs in advanced stages (II, III–IV). Healthy seropositive dogs exhibited higher neutrophil activation compared to all other groups except sick dogs in stage I. Dogs in advanced disease stages (II, III–IV) did not show significant differences in neutrophil activation compared to healthy seronegative dogs. Furthermore, dogs in stage I had significantly higher IFN-γ concentrations compared to healthy seronegative and sick dogs in advanced disease stages. Dogs in stage II showed higher IFN-γ concentrations compared to healthy seronegative dogs, while no significant differences were observed in dogs in stage III–IV. Healthy seropositive dogs had elevated IFN-γ concentrations compared to healthy seronegative dogs and dogs in stage III–IV. These findings indicate that neutrophil activation is predominant in dogs with mild disease and healthy seropositive dogs with an association with potent IFN-γ production.

Abstract

This study aimed to investigate the role of neutrophils in canine leishmaniosis by assessing neutrophil activation and its relationship with different states of *L. infantum* infection and antibody and IFN-γ production. Dogs were categorized into five groups: healthy-seronegative (n = 25), healthy-seropositive (n = 21), LeishVet-stage I (n = 25), Leishvet-stage II (n = 41), and LeishVet-stage III–IV (n = 16). Results of the nitroblue tetrazolium reduction test (NBT) showed significantly higher neutrophil activation in stage I (median:17.17, range: [7.33–31.50] %) compared to in healthy-seronegative (4.10 [1.20–18.00]%), healthy-seropositive (7.65 [3.98–21.74]%), stage II (6.50 [1.50–28.70]%), and stage III–IV (7.50 [3.00–16.75]%) groups ($p < 0.0001$). Healthy-seropositive dogs also displayed higher values than all groups except stage I. Stages II and III–IV did not show significant differences compared to healthy-seronegative. Regarding IFN-γ, stage I dogs had higher concentrations (median:127.90, range: [0–3998.00] pg/mL) than healthy-seronegative (0 [0–109.50] pg/mL) ($p = 0.0002$), stage II (9.00 [0–5086.00] pg/mL) ($p = 0.045$), and stage III–IV (3.50 [80.00–548.80] pg/mL) ($p = 0.02$) dogs. Stage II dogs showed increased IFN-γ compared to healthy-seronegative dogs ($p = 0.015$), while stage III–IV dogs had no significant differences compared to healthy-seronegative dogs ($p = 0.12$). Healthy-seropositive dogs had elevated IFN-γ concentrations compared to healthy-seronegative dogs ($p = 0.001$) and dogs in stage III–IV ($p = 0.03$). In conclusion, neutrophil activation was higher in dogs with mild disease and healthy-seropositive dogs, and a relationship between neutrophil activation and the production of IFN-γ was found

Keywords: NBT; dog; immunity; interferon-gamma; neutrophil; leishmaniosis; oxidative burst; oxidative metabolism.

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic vector-borne disease caused by the protozoan parasite *Leishmania infantum* and primarily transmitted through the bite of female Phlebotomine sand flies [1]. It has a worldwide distribution and is considered endemic in regions, such as the Mediterranean basin, Latin America, and southern Asia [1]. Dogs serve as the main reservoir for this infection. *Leishmania infantum* is an obligate intracellular parasite that infects and survives within various myeloid lineage cells, including monocytes, macrophages, dendritic cells, and neutrophils. Its presence in the body has unique implications for the immune system during the course of the infection [2].

Clinical manifestations of CanL can vary widely, ranging from subclinical infection to severe systemic disease [1]. The severity of the disease is classified into four stages: mild disease-LeishVet stage I, characterized by mild clinical signs like papular dermatitis, exhibiting no noteworthy clinicopathological abnormalities, and displaying antibody levels that range from negative to low; moderate disease-LeishVet stage II, characterized by diffuse clinical manifestations, like extensive skin lesions, widespread lymph node enlargement or weight loss, clinicopathological abnormalities without a rise in creatinine concentrations, and antibody levels varying from weakly positive to strongly positive; severe disease-LeishVet stage III, defined by extensive clinical manifestations along with clinical signs linked to the deposition of immune complexes (like uveitis or glomerulonephritis), clinicopathological abnormalities, including either IRIS Stage 1 proteinuric or IRIS Stage 2 chronic kidney disease (CKD), accompanied by antibody levels ranging from moderate to high positivity; and very severe disease-LeishVet stage IV, determined by diffuse clinical signs, clinicopathological abnormalities, and advanced CKD stage 3 or 4, accompanied by antibody levels ranging from moderate to high positivity [1]. The interplay between the host's innate and adaptive immune responses in response to the parasite infection plays a crucial role in determining the clinical signs and overall outcome of the disease [3,4]. Dogs with a predominant cellular immune response are associated with disease resistance or subclinical forms, while dogs exhibiting a predominantly humoral response are associated with clinical illness [2,5]. Adaptive T-cell immunity has been widely investigated to play a crucial role in disease development and resistance. The T helper 1 (Th1)-mediated immune response, characterized by the secretion of cytokines, such as interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α), is associated with immune protection [4,6]. Conversely, the Th2-mediated immune response leads to the production of cytokines, like interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β), which are linked to a predominantly humoral immune response

and disease progression [4,6]. A balanced response between proinflammatory Th1 CD4+ T cells that inhibit parasite replication and an immune regulatory response mediated by T regulatory 1 (Tr1) cells is crucial for disease control [2]. The former stimulates macrophage activity through IFN-γ production, promoting parasite elimination [7]. However, prolonged exposure to antigens can lead to T cell exhaustion, resulting in reduced T cell proliferation and IFN-γ production. This, in turn, allows for parasite survival and expansion within macrophages [7].

In recent years, growing evidence has highlighted the significant role of the innate immune response in controlling *Leishmania* infection. Among the components of the innate immune system, neutrophils have emerged as key players, particularly in the early stages of *Leishmania* infection [8]. While macrophages are considered the primary host target cells for the replication of *L. infantum*, neutrophils also play a distinct role in the control or progression of leishmaniosis, thanks to their ability to kill parasites and produce interleukins [9,10]. As the first line of defense, neutrophils are the initial cells that migrate to the site of infection and internalize the *Leishmania* promastigote [11–14]. Upon phagocytosis, neutrophils activate their defense mechanisms, including the production of reactive oxygen species (ROS), which is a crucial antileishmanial mechanism produced in phagocytes (oxidative burst) [15–18]. The phagocyte's NADPH oxidase generates the superoxide anion (O_2^-), which serves as a precursor to hydrogen peroxide and other ROS [15–17,19–21]. Subsequently, the neutrophil releases these ROS within the phagosomes containing *L. infantum* amastigotes, effectively killing the parasite [19]. In addition to the oxidative burst, neutrophils that ingest *Leishmania* amastigotes initiate further defensive strategies. These include the secretion of TNF-α and IFN-**γ**, which aid in stimulating macrophage activation and recruitment to the infection site [22,23] and the release of neutrophil extracellular traps, which also potentially play a significant role in combatting protozoal infections, such as *Leishmania* [24].

However, *Leishmania* amastigotes have the ability to deactivate the oxidative activity of polymorphonuclear leukocytes, allowing them to survive within neutrophils and exploit them as a means to hide from the immune system while silently infecting the final host cell, the macrophage [10,22,25]. Alternatively, if the oxidative burst is successful and limits parasite growth within cells, the infection can be controlled [26,27]. Therefore, the ability or predisposition of neutrophils to activate the oxidative burst can have a significant impact on disease progression or containment.

Interferons (IFNs) are a family of proteins that exhibit strong antiviral and antibacterial effects and play crucial roles in regulating effector cells within

both innate and adaptive immune responses [28–31]. Among these, IFN-γ stands out for having the most diverse and potent effects [32]. While much of the research on IFN-γ has focused on its effects on adaptive immunity, recent studies have revealed its ability to modulate myeloid cell activity, including neutrophils, in diverse ways. These include enhancing NADPH oxidase activity and the oxidative burst, promoting nitric oxide (NO) production, increasing the expression of MHCII proteins, altering cytokine and chemokine production, inhibiting chemotaxis, and suppressing apoptosis [33]. Although these effects have been predominantly observed in vitro based on mature neutrophils, studies suggest that more substantial changes occur when IFN-γ interacts with maturing myeloid cells [34]. Thus, IFN-γ could potentially have an impact on the capacity of neutrophils to eliminate phagocyted *Leishmania* amastigotes and promastigotes by stimulating the cellular immune response and activating phagosomes to induce an oxidative burst. Therefore, a positive relationship within IFN-γ and the oxidative metabolism of neutrophils could be suspected.

The nitroblue tetrazolium reduction test (NBT) is a diagnostic assay that utilizes the ability of neutrophils and macrophages to generate free radicals (oxidative burst) during the process of phagocytosis [35,36]. This test relies on the reduction of nitroblue tetrazolium, a colorless compound, to formazan, a blue compound, by the NADPH oxidase present in activated neutrophils within the phagocytic vacuole. As a result, the cytoplasm of the cells undergoes a color change, indicating the production of ROS [36]. The NBT reaction provides an assessment of the ROS-generating activity in the cytoplasm of cells and has been shown to correlate strongly with the levels of ROS produced during the oxidative burst [37,38]. By employing conventional light microscopy, the rate of NBT can be determined by calculating the percentage of neutrophils with formazan present in their cytoplasm, reflecting the activation status of neutrophils and monocytes in the peripheral blood [35]. The primary objective of this study was to assess the variations in peripheral blood neutrophil ROS generation in dogs with different stages of *L. infantum* infection by means of the NBT. Additionally, we aimed to investigate the potential association between neutrophil ROS generation and IFN-γ concentrations, as well as antibody levels, in dogs with different states of *L. infantum* infection.

2. Materials and Methods

All dogs included in this study belonged to volunteer owners who had granted permission to a physical examination and blood collection from their dogs through a signed consent form.

2.1. Dogs and Clinical Data

Dogs of different breeds from an endemic area were selected to enter the study. A complete blood count, serum biochemistry profile (at least containing creatinine, alanine aminotransferase, and albumin), urinalysis, serum electrophoresis, and ELISA serology were performed for all dogs, as well as cytology of the lesions in dogs in stage I.

A total of 128 dogs were enrolled in this study and divided into five groups according to the LeishVet staging system [1]: Group 1 included healthy seronegative dogs with no clinical signs or hematological or biochemical abnormalities (n = 25); Group 2 included *Leishmania*-seropositive but clinically healthy dogs with no clinical signs, hematological and serum and urinary biochemical parameters within normal limits, and presenting low to medium antibody levels (n = 21); Group 3 included dogs with mild disease-LeishVet stage I presenting papular dermatitis as the sole clinical sign [39], the identification of intralesional amastigotes via cytology, hematological, serum, and urinary biochemical parameters within normal limits (including creatinine < 1.4 mg/dL), and presenting negative to low antibody levels (n = 25); Group 4 included dogs with moderate disease-LeishVet stage II, including the characteristic clinical signs (diffuse clinical manifestations, like extensive skin lesions, widespread lymph node enlargement, or weight loss among others), hematological and serum biochemical abnormalities with normal creatinine values (<1.4 mg/dL), and medium-to-high antibody levels (n = 41); Group 5 included dogs with severe disease-LeishVet stage III and very severe disease-Leishvet stage IV, including the characteristic clinical signs, hematological, biochemical abnormalities (including creatinine values of 1.4–2.8 mg/dL or UPC > 1 for stage III and creatinine values > 2.8 for stage IV or UPC > 5), and presenting positive antibody levels (n = 16) [1]. The urine protein creatinine ratio was assessed in 12/25 dogs in stage I and in all dogs in stages II, III, and IV. *Leishmania infantum*-specific antibody levels were assessed in all dogs using an end point ELISA. As for IFN-γ, this test was conducted on most of the dogs, although technical limitations prevented its performance in all cases. IFN-γ measurements were obtained from 10 out of 25 healthy seronegative dogs, 15 out of 21 healthy seropositive dogs, 24 out of 25 stage I dogs, 34 out of 41 stage II dogs, and 13 out of 16 stage III/IV dogs. A follow-up of up to one year was available for all dogs with papular dermatitis without conventional anti-*Leishmania* systemic treatment instituted. Some dogs in this group received local treatment whit either topical meglumine antimoniate as a lotion spray (n = 6/25), polyhexamethylene biguanide alone or in combination with Toll like receptor 4 as a spray (n = 9/25), or placebo (n = 9/25), and one received no treatment at all [39].

2.2. Blood Collection

The blood was collected via jugular or cephalic venipuncture and placed in EDTA tubes for the hematologic analysis and the NBT test, into heparin tubes to perform cytokine release whole blood assay, and into serum tubes for the antileishmanial antibody quantification.

2.3. Antileishmanial Antibody Quantification via ELISA

Antibody levels against *L. infantum* were assessed using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA), as outlined in previous studies [3,40]. Dog sera were diluted to a ratio of 1:800 in a phosphate buffer solution (PBS) containing Tween 20 and 1% dry milk. This mixture was then incubated in plates previously coated overnight with sonicated promastigotes of *L. infantum* (MHOM/MON-1/LEM-75) obtained from an infected dog at a concentration of 20 µg/mL, and the incubation took place at 37 °C for 1 h [41]. Following this, the plates underwent a series of washes using PBS-Tween and PBS. Next, Protein A conjugated to horseradish peroxidase (Peroxidase Conjugate Protein A; Merck KGaA, Darmstadt, Germany) was added to the plates at a dilution of 1:30,000 and incubated for 1 h at 37 °C. After another round of washing, the plates were treated with o-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA, Darmstadt, Germany). The reaction was terminated using 5 M H_2SO_4. Absorbance readings were taken at 492 nm using a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd., Shenzhen, China). The quantification of results was performed in terms of ELISA units (EUs), normalized against positive canine serum utilized as a calibrator and set at 100 EU.

The threshold was established at 35 EU [39]. Serum samples were categorized as negative when their value fell below 35 EU. If the result ranged from 35 EU up to but not exceeding 150 EU, it was considered low positive. Similarly, if the result fell between 150 EU and 300 EU, it was categorized as medium positive. Finally, if the value equaled or surpassed 300 EU, it was classified as high positive.

For a more in-depth examination of samples identified as medium or high positive, an ELISA involving a two-fold serial dilution was conducted. This process was initiated at a dilution of 1:800 and continued with an additional 7 to 11 dilutions. Quantification was predicated on arbitrary units (EU) in relation to a calibrator set at 100 EU, which corresponded to an optical density (OD) value of 1 at the 1:800 dilution. The mean values of dilutions displaying an OD approximate to one were chosen for calculating the EU. This computation was executed using the following formula: (sample OD/calibrator OD) × 100 × dilution factor [40].

2.4. Nitroblue Tetrazolium Reduction Test

For each EDTA tube, blood was drawn and filled into three hematocrit capillary microtubes (Servoprax, Wiesel, Germany). These microtubes were then subjected to centrifugation at 2910 × *g* for 5 min (Fugevet+ GDC005, Nahita International Ltd., London, UK) to isolate the buffy coat. The obtained buffy coat was transferred to an Eppendorf tube, where it was mixed with an equal volume of 0.1% NBT solution (1:1) (N6876, Sigma-Aldrich Co., St. Louis, MO, USA). The mixture was gently agitated and then placed in a heater at 37.5 °C for a duration of 15 min (INB 200, Memmert GmbH + Co. KG, Schwabach, Germany), followed by an additional 15 min at room temperature. Subsequently, two blood smears were prepared from each Eppendorf tube, with 3 µL of NBT-stained blood being placed on each glass slide. These slides were further stained using the Diff-Quik method. To assess the NBT rate, a total of 300 neutrophils displaying a clear morphology were counted on each slide using standard light microscopy. Neutrophils that were aggregated or damaged were excluded from the count. The NBT rate was calculated by determining the percentage of activated neutrophils, characterized by the presence of blue-black formazan deposits, among the total counted neutrophils [42].

Dogs were considered to have an increased NBT rate when the NBT value exceeded the cut-off. This cut-off was calculated by considering the NBT values of the 25 healthy seronegative dogs as the control group. A high sensitivity was desired for this cut-off. The standard deviation was added to the mean value of this group, resulting in a cut-off of 10.80%. With this cut-off, considering the healthy seronegative dogs as negative and the dogs with papular dermatitis as positive, the sensitivity was 92% and the specificity 84%.

2.5. Cytokine Release Whole Blood Assay and Determination of Canine IFN-γ

The heparinized cytokine release whole blood assay was conducted following established procedures [3]. To outline the process, the assay involved incubating whole blood separately under three distinct conditions: (i) medium alone (unstimulated); (ii) medium containing *L. infantum* soluble antigen (LSA) at a concentration of 10 µg/mL [43]; and (iii) medium supplemented with the mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 µg/mL. LSA was obtained through a process involving triple cycles of freezing and thawing of cultured *L. infantum* promastigotes (MHOM/MON-1/LEM-75) suspended at a concentration of 1×10^9 cells/mL in PBS. Subsequently, the supernatant was harvested following centrifugation (8.000 × *g*, 20 min, 4 °C), adhering closely to the methodology outlined in Carrillo et al., 2015, albeit with minor adjustments [44]. Following five days of incubation at 37 °C in an environment containing 5% CO_2, supernatants were

obtained through centrifugation at 300 × *g* for a duration of 10 min. These supernatants were then collected and stored at −80 °C until the testing phase. The measurement of IFN-γ was carried out for all samples utilizing a commercially available sandwich ELISA (Du-oSet® ELISA by Development System R&DTM, Abingdon, UK) using a canine IFN-γ antibody. A standard curve for canine IFN-γ was established through a computer-generated four-parameter logistic curve-fit using the MyAssays program (http://www.myassays.com/) [3]. Dogs were categorized as IFN-γ producers if the concentration of *L. infantum*-specific IFN-γ exceeded or was equal to 110 pg/mL after accounting for the medium-alone baseline [43].

2.6. Statistical Analysis

The statistical analysis of data was performed using GraphPad Prism 8.0.1 for Windows software (GraphPad Software, San Diego, CA, USA).

Several normality tests (Anderson–Darling, D'Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov) were performed to assess the normality of the different variables (NBT rate, serological results, and IFN-γ concentration). No variables followed a normal distribution except for the NBT rate in stage I and stage III–IV considering a p-value < 0.05 as statistically significant. Therefore, non-parametric tests were used to assess the data, except for the unpaired *t*-test (parametric test) performed between the parameters with a normal distribution. A non-parametric Mann–Whitney *U*-test was used for quantitative variables to compare two groups.

A Kruskal–Wallis test and a Dunn's multiple comparisons test were used to compare more than two groups when data did not follow a normal distribution. If data followed a normal distribution, an ordinary one-way ANOVA and a Tukey's multiple comparisons test were performed. The Chi-square test or Fisher's exact test was performed to determine if an association between categorical variables existed. The Spearman (non-normal distribution) or Pearson (normal distribution) correlation coefficient was used to evaluate if there were correlations among different parameters studied. Results were considered statistically significant at a p-value < 0.05.

3. Results

3.1. Dog Clinical Characteristics and Performed Tests

Dog clinical characteristics and results of performed tests are summarized and displayed in **Tables 13** and **14**. Forty-two percent of dogs were crossbred (n = 54), and the other most represented breeds were Beagle (n = 10), German shepherd (n = 8), and Greyhound (n = 5)

Table 13. Distribution of qualitative characteristics including breed, sex, positive/negative NBT, positive/negative serology, and productive/non-productive status for IFN-γ among dogs categorized within the five states of infection studied.

Qualitative characteristics		Healthy sero-negative (n = 25)	Healthy sero-positive (n = 21)	Stage I (n = 25)	Stage II (n = 41)	Stage III-IV (n = 16)	*p*-value (Chi-square, df)
Bred (n = 128)	Crossbred (n = 54)	9 (36%)	11 (52.4%)	14 (56%)	16 (39%)	4 (25%)	0.25 (5.3, 4)
	Purebred (n = 74)	16 (64%)	10 (47.6%)	11 (44%)	25 (61%)	12 (75%)	
Sex (n = 128)	Male (n = 74)	12 (48%)	10 (47.6%)	16 (64%)	23 (56.1%)	13 (81.3%)	0.2 (5.9, 4)
	Female (n = 54)	13 (52%)	11 (52.4%)	9 (36%)	18 (43.9%)	3 (18.7%)	
NBT (n = 128)	Increased (n = 46)	4 (16%)	5 (23.8%)	23 (92%)	10 (24.4%)	3 (18.8%)	< 0.0001* (44.6, 4)
	WNL (n = 82)	21 (84%)	16 (76.2%)	2 (8%)	31 (75.6%)	13 (81.2%)	
Serology (n = 128)	Positive (n = 84)	0 (0%)	21 (100%)	6 (24%)	41 (100%)	16 (100%)	< 0.0001* (107.8, 4)
	Negative (n = 44)	25 (100%)	0 (0%)	19 (76%)	0 (0%)	0 (0%)	
IFN-γ (n = 97)	Producers (n = 34)	0 (0%)	9 (60%)	12 (50%)	9 (26.5%)	4 (30.8%)	0.01* (12.5, 4)
	Non-prod. (n = 63)	10 (100%)	6 (40%)	12 (50%)	25 (73.5%)	9 (69.2%)	

NBT, nitroblue tetrazolium reduction test; **WNL,** within normal limits; **IFN-γ,** Interferon-gamma; **Non-prod.,** non-producers; **ELISA,** Enzyme-Linked ImmunoSorbent Assay.
*Significant differences were observed between groups.

Table 14. Median, minimum, and maximum values for quantitative traits like age, NBT, serology, and IFN-γ within each group.

Qualitative characteristics Median (min-max)	Healthy sero-negative (n = 25)	Healthy sero-positive (n = 21)	Stage I (n = 25)	Stage II (n = 41)	Stage III-IV (n = 16)	*p*-value (Kruskal-Wallis test)
Age (years)	1.3 (1-1.6)	5 (1-13)	0.5 (0.25-7)	4 (1-13)	7.5 (1-11)	<0.0001*
NBT (%)	4.1 (1.2-18)	7.7 (4-21.7)	17.2 (7.3-31.5)	6.5 (1.5-28.7)	7.5 (3-16.75)	<0.0001*
IFN-γ (pg/mL)	0 (0-109.5)	204.1 (0-4763)	127.9 (0-3998)	9 (0-5086)	3.5 (80-548.8)	0.001*
Serology (ELISA units)	5.5 (0.3-28.5)	133.9 (75.8-956.3)	21.4 (2.8-227.1)	1894 (57.2-10293)	1857 (96.8-11114)	<0.0001*

NBT, nitroblue tetrazolium reduction test; **WNL,** within normal limits; **IFN-γ,** Interferon-gamma; **Non-prod.,** non-producers; **ELISA,** Enzyme-Linked ImmunoSorbent Assay.
*Significant differences were observed between groups.

3.2. Age-Stratified Analysis of NBT Rate in Dogs with Papular Dermatitis (Stage I): Insights from Younger and Older Canine Cohorts

Dogs within the stage I group were further subdivided between dogs younger than one year of age (n = 22) and dogs older than one year of age (n = 3) for a further statistical analysis. No statistical differences were found when comparing the NBT rate of dogs in both groups (p = 0.674).

Additionally, the statistical study comparing the stage I group with the other groups was repeated but only taking in consideration the dogs that were older than 1 year of age, showing similar results to the previous study performed. Stage I (older than 1 year of age) had a significantly higher NBT rate than dogs in the healthy seronegative group (p = 0.02), the healthy seropositive group (p = 0.011), the stage II group (p = 0.002), and the stage III–IV group ($p < 0.0001$).

3.3. Follow-up of Dogs with Papular Dermatitis (Stage I)

Over the following months after diagnosis, the majority of the dogs exhibited a notable improvement or the resolution of their cutaneous lesions, and the antibody levels demonstrated a declining trend. However, among the 25 dogs studied, three exhibited a deterioration in their cutaneous lesions, which progressed to the point of ulceration in two cases. Consequently, a systemic conventional anti-*Leishmania* treatment approach was initiated to address their condition.

For the purpose of conducting a statistical analysis, the three dogs that experienced a worsening of their clinical signs were categorized as belonging to group PD-B, and the remaining 22 dogs that displayed an improvement or the resolution of their clinical signs without the need for systemic treatment were designated as members of group PD-A. Detailed results pertaining to NBT, IFN-γ, and serology, both for these delineated groups and for each individual dog within group PD-B, can be found in **Table 15**.

Upon conducting a comparative analysis of the NBT, IFN-γ, and serology results between the two groups, no statistically significant differences were found (p = 0.074, p = 0.176, and p = 0.271, respectively). However, it is worth noting that the sample size for group PD-B was limited to only three dogs, which could have influenced the robustness of the statistical outcomes.

Interestingly, despite the limitations in sample size, a significant correlation was established between the number of dogs surpassing the NBT cut-off value and the achievement of a clinical improvement without the requirement for systemic treatment. This observation indicated that dogs in group PD-B (those experiencing worsening clinical signs) were more prone to exhibit NBT

results within the normal range, unlike dogs in group PD-A (demonstrating an improvement without systemic treatment). However, no corresponding link was identified between the various groups and the status of being an IFN-γ producer or having a positive serology.

Table 15. NBT and IFN-γ results for dogs presented with papular dermatitis and divided into Group PD-A (clinical improvement without systemic treatment) and Group PD-B (clinical worsening and need for systemic treatment).

	NBT (%)	NBT status (cut-off = 10.8)	IFN-γ	IFN-γ status	Serology	Serological status	Clinical evolution
Group PD-A (n = 22)	Median: 17.17 (min: 9.7, max: 31.5)	23/23 Over cutoff	Median: 127.9 (min: 0, max: 1599)	10/10 Producers	Median: 18.21 (min: 2.76, max: 227.1)	5/22 Positive	Partial or total improvement
Group PD-B (n = 3)	Median: 8.8 (min: 7.3, max: 20.8)	2/3 WNL	Median: 7 (min: 0, max: 234.2)	1/3 Producer	Median: 27.41 (min: 22.34, max: 79.9)	1/3 Positive	–
Dog PD-B.1	8.8	WNL	7	Non-producer	79.9	Positive	Worsening of cutaneous clinical signs: diffuse worsening
Dog PD-B.2	7.3	WNL	234.2	Producer	27.41	Negative	Worsening of cutaneous clinical signs: ulcerative lesion at the nose bridge
Dog PD-B.3	20.8	Over cutoff	0	Non-producer	22.34	Positive	Worsening of cutaneous clinical signs: ulcerative lesion at the level of the right lower eyelid

Group PD-A, dogs diagnosed with papular dermatitis that showed improvement of clinical signs without systemic treatment; **Group PD-B,** dogs diagnosed with papular dermatitis that showed worsening of clinical signs and in which systemic treatment was considered necessary; **NBT,** nitroblue tetrazolium reduction test; **WNL,** within normal limits; **IFN-γ,** Interferon-gamma.

3.4. Total Leucocyte Concentration and Differential Leukocyte Concentration

The results from the total leucocyte concentration and the differential leukocyte concentration analysis within the different groups studied are displayed in **Table 16**. Upon scrutinizing the outcomes across different groups, no statistically significant differences were identified in terms of total leukocyte, neutrophil, and eosinophil concentrations. However, significant differences were observed in relation to the lymphocyte concentration. Specifically, healthy seronegative dogs exhibited notably higher lymphocyte concentrations in comparison to the healthy seropositive dogs ($p = 0.028$), as well as to dogs in stage II ($p = 0.048$) and stage III–IV ($p = 0.001$). Interestingly, no substantial variance was found

when comparing the lymphocyte concentration between healthy seronegative dogs and dogs in the stage I group. Furthermore, regarding the lymphocyte concentration within the stage I group, significantly higher values were also recorded in contrast to healthy seropositive dogs ($p = 0.022$), dogs in stage II ($p = 0.033$), and dogs in stage III–IV ($p = 0.001$). Notably, no significant differences were evident when comparing the stage II group with the stage III–IV group.

Regarding the monocyte concentration, statistically significant differences were observed between healthy seropositive dogs and those in stage I ($p = 0.004$), as well as between dogs in stage II and those in stage I ($p = 0.04$). In both cases, the stage I group exhibited markedly higher values.

When exploring potential correlations between the NBT rate and both neutrophil and lymphocyte concentrations, a comprehensive analysis encompassing all of the dogs collectively revealed no significant correlations ($p = 0.491$ and $p = 0.199$, respectively). This pattern persisted when conducting separate evaluations within distinct groups, which included healthy seronegative ($p = 0.682$ and $p = 0.811$, respectively), healthy seropositive ($p = 0.766$ and $p = 0.144$, respectively), stage I ($p = 0.187$ and $p = 0.507$, respectively), stage II ($p = 0.408$ and $p = 0.494$, respectively), and stage III–IV ($p = 0.625$ and $p = 0.525$, respectively) groups.

Regarding the monocyte concentration, a significant negative correlation with the NBT was observed exclusively within the stage I group ($p = 0.005$). In contrast, no such correlations emerged when examining the healthy seronegative ($p = 0.785$), healthy seropositive ($p = 0.128$), stage II ($p = 0.451$), and stage III–IV ($p = 0.73$) groups separately. Similarly, no overall correlation was identified when considering all dogs collectively ($p = 0.159$).

Table 16. Median (min-max) results for the leukogram and differential leukocyte concentration in each group.

Median (min-max)	Healthy sero-negative	Healthy sero-positive	Stage I	Stage II	Stage III-IV	*p*-value
Leucocytes (cell/µl)	11330 (6390-15020)	9710 (3360-16920)	11180 (6200-20540)	10470 (3990-19430)	9650 (3420-13630)	0.115
Neutrophiles (cell/µl)	6675 (3706-9913)	5889 (2755-11844)	6939 (3300-17860)	71781 (3032-14378)	6482 (2633-10223)	0.936
Lymphocytes (cell/µl)	3006 (1968-3930)	1711 (235-3849)	3590 (900-5800)	1921 (518-4696)	1472 (616-2385)	<0.0001*
Eosinophils (cell/µl)	409 (300-934)	514 (63-2369)	500 (20-1100)	479 (0-2176)	386 (0-1204)	0.541
Monocytes (cell/µl)	668 (313-1502)	413 (34-1157)	750 (100-1310)	517 (129-1269)	560 (171-1204)	0.003*

*Significant differences were observed between groups.

3.5. Nitroblue Tetrazolium Reduction Test

The results of NBT are depicted in **Table 13**, **Table 14**, and **Figure 9**. A significantly lower median NBT rate was observed in the healthy seronegative group (median: 4.13% range: [1.15–17.96]%) when comparing this to the to the healthy seropositive group (7.65% [3.98–21.74]%) (p = 0.006) and stage I group (17.17% [7.33–31.50]%) ($p < 0.0001$). However, no significant differences were found between the healthy seronegative group and stage II (6.50% [1.50–28.70]%) (p = 0.08) or stage III–IV group (7.50% [3.00–16.75]%) (p = 0.09).

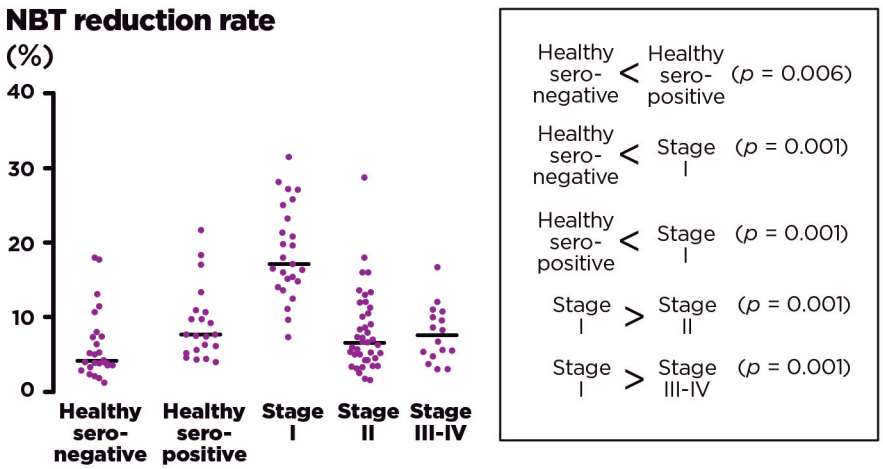


Figure 9. **NBT reduction rate results (median, minimum and maximum values) in different *L. infantum* states of infection.**

Regarding healthy seropositive dogs, a significantly lower median NBT rate was also observed when comparing this group to stage I ($p < 0.0001$). However, no significant differences were found with the other groups: stage II (p = 0.23) and stage III–IV (p = 0.53). A significantly higher median NBT rate was found when comparing stage I to both stage II ($p < 0.0001$) and stage III–IV ($p < 0.0001$). Conversely, there were no significant differences between stage II and stage III–IV (p = 0.8).

Regarding the number of dogs over the cut-off value for NBT, there was a significantly higher proportion of dogs that surpassed the cut-off in the stage I group (92.00%) when compared to seronegative (16.00%) ($p < 0.0001$), healthy seropositive (23.8%) ($p < 0.0001$), stage II (24.40%) ($p < 0.0001$), and stage III–IV dogs (18.75%) ($p < 0.0001$). No additional significant differences were found when comparing the other groups **(Table 13)**.

3.6. *Leishmania infantum*-Specific Antibody Levels

All dogs in the healthy seronegative group were considered negative (median: 5.50, range: [0.30–28.49] EU). Among the healthy seropositive group (median: 133.90, range: [75.79–956.30] EU), 76.19% of dogs were classified as low positive, 14.29% as medium positive, and 9.52% exhibited high antibody levels. In the stage I group (median: 21.42, range: [2.76–227.10] EU), all dogs had negative (76.00%) or low (20.00%) antibody levels, except for one dog (4.00%), which was considered medium positive.

For the stage II group (median: 1894, range: [57.16–10,293.00] EU), 21.95% of dogs were classified as low positive, 34.14% as medium positive, and 43.90% as high positive. In the stage III–IV group (median: 1857, range: [96.79–11,114.00] EU), 37.50% of dogs were classified as medium positive, another 37.50% as high positive, and 25.00% fell into the low positive category.

Statistically significant lower values were found when comparing the healthy seronegative group to the healthy seropositive group ($p < 0.0001$), stage I ($p = 0.0003$), stage II ($p < 0.0001$), and stage III–IV ($p < 0.0001$) groups. The healthy seropositive group showed significantly higher values when compared to stage I ($p < 0.0001$) and significantly lower values when compared to stage II ($p < 0.0001$) and stage III–IV ($p = 0.0001$) groups. Significantly lower values were also observed when comparing stage I to stage II ($p < 0.0001$) and stage III–IV ($p < 0.0001$) groups. No statistically significant differences were observed between the stage II and stage III–IV groups ($p = 0.93$).

3.7. IFN-γ Concentration

The results of the IFN-γ concentration are depicted in **Tables 13** and **14** and **Figure 10**. In the healthy seronegative group, all tested dogs (10/10) were classified as IFN-γ-non-producers. Among the healthy seropositive group, 9 out of 15 dogs (60.00%) were considered as IFN-γ-producers. In the stage I group, 50.00% of dogs (12/24) were classified as IFN-γ-producers. In stage II, a total of 9 out of 34 dogs (26.47%) were classified as IFN-γ-producers, while the majority, 73.53%, were non-producers. Similarly, in stage III–IV, only 4 out of 13 dogs (30.77%) were identified as IFN-γ-producers, with 69.23% classified as non-producers.

Supernatants from LSA-stimulated whole blood of healthy seronegative dogs (median: 0, range: [0–109.50] pg/mL) showed significantly lower concentrations of IFN-γ compared to healthy seropositive dogs (median: 204.10, range: [0–4763.00] pg/mL) ($p = 0.001$), dogs in stage I (median: 127.90 pg/mL, range: [0–3998.00] pg/mL) ($p = 0.0002$), and dogs in stage II (median: 9.00 pg/mL, range: [0–5086.00] pg/mL) ($p = 0.015$). However, no differences were observed when comparing IFN-γ concentration between healthy seronegative dogs and dogs in stage III–IV (median: 3.48 pg/mL, range [0–548.80] pg/mL) ($p = 0.12$).

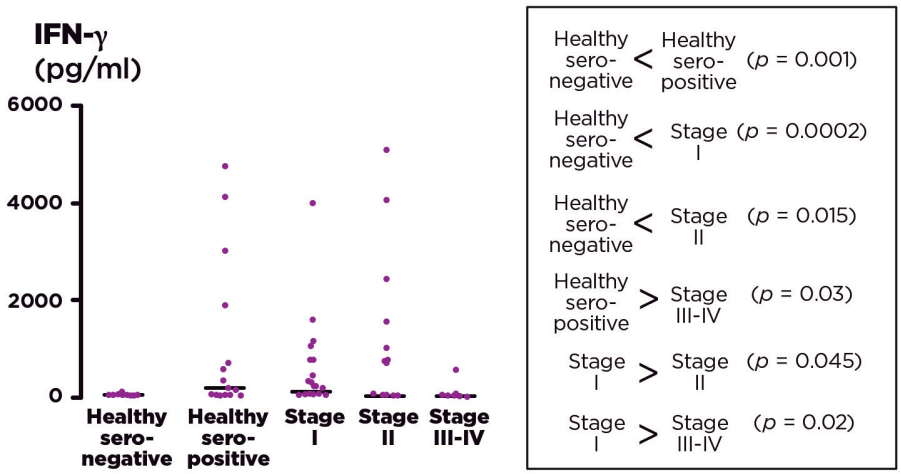


Figure 10. IFN-γ concentrations (median, minimum and maximum values) after stimulation with LSA in different *L. infantum* states of infection.
IFN-γ, Interferon-gamma; **LSA,** *L. infantum* soluble antigen.

Regarding healthy seropositive dogs, no significant differences were found when comparing their supernatants from LSA-stimulated whole blood results to dogs in stage I (p = 0.6) and stage II (p = 0.06). However, statistically significant higher values were observed in healthy seropositive dogs when comparing them to dogs in stage III–IV (p = 0.03).

Concentrations of IFN-γ from dogs in stage I were significantly higher than concentrations of IFN-γ from dogs in stage II (p = 0.045) and stage III–IV (p = 0.02). No statistically significant differences were found between IFN-γ concentrations from dogs in stage II and dogs in stage III–IV (p = 0.5).

3.8. Correlation Between Parameters Studied

No significant correlation was observed between the concentration of IFN-γ after LSA stimulation and *L. infantum*-specific antibody levels (p = 0.107) when considering all the groups. Similarly, there was no correlation between the NBT rate and antibody levels (p = 0.088). Additionally, no correlation was found between the NBT rate and the concentration of IFN-γ after LSA stimulation (p = 0.087).

When examining the correlation within specific groups, no significant correlation was observed between the concentration of IFN-γ and *L. infantum*-specific antibody levels in the healthy seronegative group (p = 0.2) and in the stage I group (p = 0.146). Conversely, a significant negative correlation was observed between IFN-γ and *L. infantum*-specific antibody levels when evaluating the results in the healthy seropositive (p = 0.028), the stage II (p = 0.001), and stage III–IV groups (p = 0.002).

No significant correlation was found between the NBT results and *L. infantum*-specific antibody levels when comparing different groups, including healthy seronegative dogs ($p = 0.778$), the healthy seropositive group ($p = 0.938$), stage I ($p = 0.663$), stage II ($p = 0.9$), and stage III–IV ($p = 0.164$).

Furthermore, no correlation was observed when comparing the NBT results and the concentration of IFN-γ after LSA stimulation within specific groups, including healthy seronegative dogs ($p = 0.8$), healthy seropositive group ($p = 0.609$), stage I ($p = 0.94$), stage II ($p = 0.13$), and stage III–IV ($p = 0.16$).

In terms of qualitative values, a significant positive association was found between IFN-γ-producers and increased NBT results when evaluating all dogs ($p = 0.009$) **(Figure 11)**. Additionally, there was a negative association between the proportion of increased NBT rate and ELISA seropositivity when evaluating all dogs ($p = 0.035$) **(Figure 12)**. A negative association was also found between the IFN-γ-producer status and ELISA positivity ($p = 0.04$). These associations were not found when evaluating the dogs separately by groups.

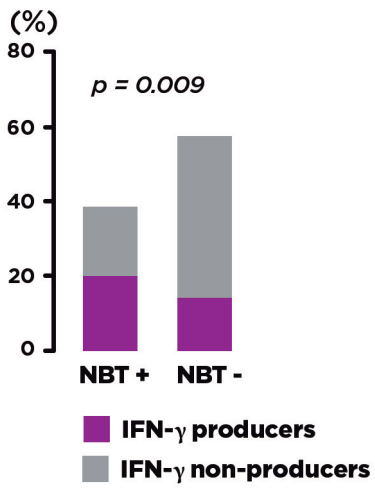


Figure 11. **NBT association to IFN-γ producer status.**
NBT+, increased nitroblue tetrazolium reduction test; **NBT-,** nitroblue tetrazolium reduction test within normal limits; **IFN-γ,** Interferon-gamma.

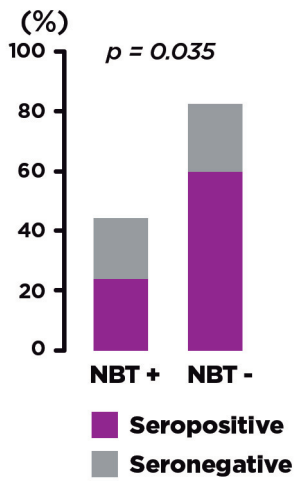


Figure 12. **NBT association to serological status.**
NBT+, increased nitroblue tetrazolium reduction test; **NBT-,** nitroblue tetrazolium reduction test within normal limits.

4. Discussion

Neutrophils are suspected to be a key factor for disease development in CanL; however, their role remains poorly understood [2,8]. The objective of this study was to assess neutrophil activation and its relationship with different clinical manifestations ranging from subclinical infection to advanced disease

and antibody production and to evaluate if the ability of IFN-γ to stimulate neutrophils correlates with the activation of its oxidative metabolism through NBT measurements. This is the first study to comprehensively evaluate the NBT across all states of this infection, including healthy seropositive and seronegative dogs, and to establish a relationship between antibody levels and the IFN-γ production status.

Regarding the assessment of oxidative metabolism in different clinical stages of leishmaniosis, the present findings revealed that dogs in the mild disease LeishVet stage I exhibited significantly higher NBT values compared to healthy seronegative and seropositive dogs, as well as to dogs in the moderate and severe disease stages. This indicates elevated oxidative metabolism in neutrophils specifically in dogs with mild, self-limiting disease, whereas this increase was not observed in dogs with moderate-to-very severe disease [37]. Additionally, although the healthy seropositive group showed a significantly lower NBT rate than the stage I group, it still displayed significantly higher values compared to all other groups. These results provide support for the hypothesis that neutrophil activation is closely linked to an effective immune response against leishmaniosis. This hypothesis is further supported by the observed association between the lack of an NBT rate increase and the deterioration of clinical signs during the follow-up of dogs with papular dermatitis. It is important to note that among the 25 dogs, only three exhibited worsening clinical signs necessitating systemic treatment, aligning with existing research portraying papular dermatitis as a predominantly self-resolving condition linked to a robust immune response [39,45–47]. Remarkably, two of these three dogs did not display an elevated NBT rate, while all dogs manifesting with self-limiting disease (22 out of 25) demonstrated NBT values surpassing the established threshold.

The diminished response observed in the healthy seropositive group, when compared to dogs with mild self-limiting disease (papular dermatitis), could potentially be attributed to different states of infection, resulting in varying levels of immune stimulation or activation. Thus, enhanced oxidative metabolism in neutrophils may serve as an indicator of an effective immune response against *Leishmania* infection, further supporting the significance of neutrophils in disease management.

However, it is important to consider that differences in neutrophil activity within the stage I group could potentially arise from variations in age between these dogs and those belonging to the other groups. Notably, dogs with papular dermatitis are markedly younger than their counterparts, boasting a median age of 6 months, with ages ranging from 3 months to 7 years. This is in line with previous studies, as papular dermatitis is consistently diagnosed in dogs under one year of age [39,48,49]. This has been theorized to be attributed to

the condition's propensity to manifest during the initial encounter between the parasite and the host's immune system, making it more prevalent in young dogs, particularly in areas where the parasite is endemic [39]. To mitigate the potential bias introduced by age, the statistical analysis was re-executed, focusing exclusively on dogs older than 12 months. This refined analysis yielded outcomes similar to those obtained when considering the entire dog population, despite the limited population of older dogs with papular dermatitis (three cases). Additionally, no significant differences were observed when comparing dogs aged 12 months and above with their younger counterparts within the stage I group. Consequently, the authors consider it unlikely for age to act as a confounding factor in neutrophil activation levels. An alternative hypothesis could be considered, suggesting that the neutrophilic response observed in patients at stage I could be linked to an early phase of the disease rather than a profile of resistance. However, this proposition seems less plausible given that, in the current study, every dog included within the stage I category exclusively displayed papular dermatitis as the solitary clinical manifestation. This particular clinical presentation is commonly associated with a robust and protective immune response. Notably, these dogs spontaneously resolve cutaneous lesions, as evidenced by long-term follow-up, which also indicates an absence of clinical relapse or the emergence of systemic clinical signs, and thus, they are more likely to be representative of an effective immune response and not just an early phase of the disease [39,45–47,49]. To further support this, the long-term follow-up in the current study showed that only three animals out of 25 were considered to get worse without systemic treatment, and the other 22 dogs resolved cutaneous lesions and remained healthy. Additionally, as mentioned previously, an association between the lack of an NBT rate increase and the deterioration of clinical signs was found.

In contrast to stage I and healthy seropositive dogs, dogs in stages II and III–IV did not exhibit significant differences compared to healthy seronegative dogs. This observation could indicate a correlation between the absence of neutrophil activation and the progression of clinical disease, mirroring the findings obtained while assessing dogs in stage I who exhibited a deterioration in clinical signs and reflecting a non-effective immune phenotype, including immunity exhaustion [7]. Previous studies have demonstrated an increased rate of neutrophil apoptosis in severe clinical stages of CanL, which may contribute to the reduction in the NBT rate [50]. While an initial increase in oxidative metabolism by neutrophils might be viewed as a protective response, if *Leishmania* infection is not controlled, prolonged neutrophil activation can lead to the generation of oxidative stress and the depletion of antioxidant defenses and ultimately trigger neutrophil apoptosis [51]. In addition, in dogs with LeishVet stage III–IV, chronic kidney disease may also contribute to

an increased apoptotic rate and reduced neutrophil superoxide production [52,53]. Regardless of the underlying cause, this negatively impacts neutrophil oxidative metabolism, which can significantly compromise the organism's primary defense mechanisms. This impairment ultimately results in a reduced ability to control CanL and likely increases the vulnerability to co-infections [50,52,54,55].

When assessing the total leukocyte and leukocyte differential concentrations, despite observing no distinctions in leukocyte, neutrophil, and eosinophil concentrations across the groups, some differences were found when evaluating the lymphocytes and monocytes. Specifically, healthy seropositive dogs and those with moderate-to-advanced leishmaniosis (stage II and stage III–IV) displayed significantly diminished lymphocyte concentrations compared to both healthy seronegative dogs and those in stage I. This partly concurs with earlier research linking clinical systemic CanL to a reduced lymphocyte concentration, consistent with the present observations in stage II and III-V cohorts [56,57]. This could be due to multifactorial mechanisms, and although the most likely explanation is stress-induced changes in the leukogram, other causes, like lymphocyte entrapment or hindered hematopoiesis attributed to bone marrow parasitism, could also contribute to lymphopenia [57]. It is also worth noting that dogs in the stage I group exhibited lymphocyte concentrations comparable to those of healthy seronegative dogs. This observation suggests the absence of a stress leukogram in dogs with mild self-healing disease. Furthermore, dogs in stage I showed a significant increase in the monocyte concentration compared to seropositive dogs and those in stage II. While elevated monocyte concentrations in infected dogs have been documented previously, particularly in dogs with positive splenic cultures for *L. infantum* [58], our study indicated a distinct pattern: solely dogs in stage I exhibited significantly elevated levels, particularly when contrasted against other infection statuses (healthy seropositive and stage II). This could be explained by the increased mobilization of monocytes linked to an effective cellular immune response. Additionally, a correlation between the NBT rate and leukocyte differential was explored, and while none was found when evaluating the neutrophil and lymphocyte concentrations, an inverse correlation surfaced when evaluating the monocyte concentration, though this was only observed within the stage I group. This negative correlation could be explained by the increased recruitment of monocytes to the infection site induced by activated neutrophils, consequently depleting the circulating monocyte pool [22,23].

Previous research has provided evidence for the crucial role of neutrophils in the early stages of *L. infantum* infection [8,11,59]. These cells form an essential part of the initial nonspecific immune response by internalizing the

promastigote and acting as a "trojan horse" to infect definitive host cells, such as macrophages [8,11,59]. However, this process could only occur if the parasite is able to evade or suppress the parasite-killing activity of polymorphonuclear cells, which has been observed to happen primarily through inhibiting or evading its oxidative damage capacity [10,59,60]. The findings of the current study support this hypothesis, as dogs with mild self-healing disease and seropositive healthy dogs exhibited a higher rate of NBT in their circulating white blood cells. This indicates increased neutrophil activity and enhanced parasite-killing capacity through the oxidative burst mechanism. This could explain why the infection was confined to the site of inoculation (papular dermatitis) and systemic disease did not develop [39].

Previous studies on neutrophil oxidative metabolism in CanL have reported conflicting results. Some studies suggested a decrease in oxidative metabolism in infected dogs, while others found increased superoxide production and oxidative metabolism [9,36,61,62]. However, these studies had limitations, such as small sample sizes and the lack of disease severity assessments.

Our study findings align with the conclusions drawn in two more recent publications, further demonstrating that neutrophil oxidative metabolism is dependent on the disease stage [36,50]. In line with the study by Gómez-Ochoa et al., our results indicate that dogs with mild disease-LeishVet stage I exhibited significantly higher neutrophil reactivity compared to both healthy dogs and dogs presenting with severe disease [36]. However, in contrast to the findings of Almeida et al., which reported a significantly increased NBT rate in stage II dogs when compared to healthy and stage IV dogs, our study revealed that both stage II and stages III–IV groups exhibited reduced oxidative metabolism, with no significant differences observed between them or the healthy seronegative group [50]. It is important to note that our study included a much larger population, with 41 dogs in stage II (compared to 15 in the previous study), which reduces the risk of population bias. Additionally, we employed a modified NBT technique that mitigates potential biases by evaluating a minimum of 300 cells per sample, whereas previous studies evaluated a minimum of 100 cells per sample, thus enhancing the reliability of our results in this specific aspect [36,50,62]. Furthermore, our study is the first to perform a direct comparison between dogs in stage I and stage II, revealing a significantly higher proportion of activated neutrophils in dogs with mild stage I disease, as well as to evaluate and compare healthy seropositive dogs with both healthy seronegative and diseased dogs. Interestingly, we found a mild yet significant increase in the NBT rate among healthy seropositive dogs when compared to the healthy seronegative group. Finally, this is also the first study to assess the clinical progression of dogs diagnosed with papular dermatitis and compare it to the NBT rate at diagnosis, showing an association

of clinical worsening with a lack of neutrophil activation. These findings highlight the distinct neutrophil activation patterns in different states of *L. infantum* infection in dogs and provide valuable insights into the immune responses in CanL.

Gómez-Ochoa et al. proposed a hypothesis suggesting that the variations in neutrophil oxidative metabolism and its augmentation during the early stages of *L. infantum* infection may be associated with the heightened production of chemotactic agents, such as cytokines, in healthy infected dogs, thereby promoting resistance against infection [36]. IFN-γ is a potent immunoregulatory cytokine that not only modulates the adaptive immune system but also plays a role in enhancing neutrophil functions [29,32]. In agreement with this, when evaluating our results, IFN-γ was found to be increased in dogs with mild self-limiting clinical leishmaniosis.

The production of *Leishmania*-specific IFN-γ in stimulated blood, being this a cytokine associated with a robust Th1 immune response, has been previously correlated with a resistant phenotype or a less-severe clinical presentation [2–4,63–66].

Previous studies demonstrated that different clinical stages of leishmaniosis in dogs were associated with different cytokine profiles in stimulated blood [3,66]. They reported that the IFN-γ concentration was significantly higher in dogs staged I and IIa when compared to more severe clinical stages, although results were not compared to healthy dogs [3]. Our study yielded similar findings, as dogs in stage I exhibited significantly higher concentrations of IFN-γ compared to healthy dogs, dogs in the moderate stage I,I, and dogs in the severe stage III–IV disease categories. Notably, dogs in stage II displayed a mild yet significant increase in IFN-γ concentrations compared to healthy seronegative dogs, whereas no significant differences were observed in dogs in stage III–IV when compared to healthy seronegative dogs. Additionally, the majority of dogs in stage II and stage III–IV were classified as IFN-γ-non-producers. Thus, in line with previous studies, dogs in more advanced clinical stages showed lower levels of IFN-γ, which could reflect a less effective immune response, away from a Th1 phenotype [7,67]. It has been previously reported that, as disease progresses, T cells develop certain unresponsiveness to *L. infantum* antigens, showing an impairment in CD4+ T cell proliferation and IFN-γ production, called T-cell exhaustion [7,67].

Interestingly, in our study, healthy seropositive dogs displayed elevated IFN-γ concentrations in comparison to both healthy seronegative dogs and dogs in stage III–IV, the latter of which aligns with a recent study performed in seropositive dogs [40]. However, the previous study did not compare the results to healthy seronegative dogs [40]. This observation could indicate the

presence of an effective Th1 response that effectively maintains the infection in a subclinical state.

In a recent study performed on humans, the administration of IFN-γ was found to have notable effects on circulating neutrophils, leading to significant changes in gene expression, protein expression, and overall function. These findings indicate an enhancement in neutrophil activity [32]. The results of that study further suggested that IFN-γ not only stimulates NADPH oxidase activity, thereby promoting the oxidative burst, but also impacts Fc receptor-mediated ingestion, pathogen recognition by innate immune receptors, antigen presentation (including MHCI and MHCII), the activation of GBPs (guanylate-binding proteins), the upregulation of NO production, and the augmentation of neutrophil release into circulation [32]. Therefore, it is plausible that IFN-γ has a direct correlation with NBT by stimulating the oxidative burst of white blood cells, which in turn may be associated with an effective immune response through neutrophil reactivity.

In our study, we discovered a significant association between IFN-γ production and an increased NBT rate. Moreover, both factors exhibited a substantial increase in dogs with subclinical or controlled infection (healthy seropositive), as well as dogs with mild self-limiting disease (stage I). These findings lend support to the notion that IFN-γ plays a role in promoting the oxidative metabolism of neutrophils, thereby contributing to an effective immune response [32]. This suggests that the modulation of neutrophil function through IFN-γ-mediated pathways could serve as one of the mechanisms by which the immune system achieves an efficient defense against the disease [32]. Furthermore, the reduction in IFN-γ associated with T-cell exhaustion could also contribute to the lack of an increase in the oxidative metabolism of neutrophils observed in advanced stages [7,32]. The findings of this investigation also revealed an inverse relationship between IFN-γ and levels of *L. infantum*-specific antibody levels across the healthy seropositive, stage II, and stage III–IV groups. Additionally, an association was found between the IFN-γ-producer status and negative ELISA results. This is in agreement with a previous article by Solano-Gallego et al. published in 2016 [3]. These negative correlations and associations could be explained by IFN-γ's role as a marker for an effective Th1 response, primarily characterized as cellular rather than humoral. Conversely, heightened antibody levels align with a robust humoral immune response, linked to other cytokines, such as IL-4, IL-10, and TGF-β [4,6,68]. Overall, these findings contribute to a better understanding of the complex immune response in CanL and emphasize the significance of neutrophil activation and IFN-γ production in disease management. Further research is needed to explore other underlying mechanisms and potential therapeutic targets for enhancing the immune response against *Leishmania* infection.

One limitation of this study was the lack of a sufficient number of dogs in stage IV, preventing a separate evaluation of this group. As a result, dogs in stage IV had to be combined with those in stage III in a sole group. However, the classification of dogs into healthy, mild disease, moderate disease, and severe disease categories still holds relevance. Another limitation is inherent to the NBT technique, which relies on a subjective measurement through light microscopy to assess activated neutrophils. To mitigate potential operator bias, a rigorous approach was adopted: a minimum of 300 cells was evaluated per sample, and at least 50% of the samples were independently reviewed by two different investigators.

5. Conclusions

In conclusion, neutrophil activation, as assessed using NBT, is significantly higher in dogs with mild self-limiting disease, followed by healthy seropositive dogs, indicating enhanced oxidative metabolism in neutrophils in these cases. Additionally, dogs in stage I exhibiting an NBT rate within normal limits were found to be linked to clinical deterioration when contrasted with dogs displaying an elevated NBT rate at the time of diagnosis. On the other hand, dogs in more advanced stages of the disease did not exhibit significant differences in neutrophil activation compared to healthy seronegative dogs. Furthermore, the study demonstrates a relationship between neutrophil activation and the production of IFN-γ, a key cytokine associated with an effective immune response against *Leishmania* infection. This study provides valuable insights into the role of neutrophil activation and its relationship with subclinical states and degrees of disease severity and suggests that neutrophils and oxidative metabolism could play a crucial role in the effective immune response against leishmaniosis.

Author Contributions: Conceptualization, C.B.-B., I.M.-F., and L.S.-G.; investigation, C.B.-B., I.M.-F., J.d.R.-V, and M.B.; formal analysis, I.M.-F.; writing—original draft preparation, C.B.-B. and I.M.-F.; writing—review and editing, M.B. and L.S.-G.; supervision, L.S.-G.; funding acquisition, L.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the Spanish ministry grant, Ministerio de Economía y competitividad, and Fondo Europeo de Desarrollo Regional (FEDER, EU) (AGL2015-68477). This research was also partially funded by Ecuphar veterinaria SLU with funding number CF617624.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Universitat Autònoma de Barcelona

(for dogs in group 1, ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (CEAAH 4526, November 2018) and by "Generalitat de Catalunya" (FUE-2018-00944112 i ID KSHYD6LVR, April 2019). For the 10 dogs purchased at Isoquimen S.L., ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (OH-CEEA100 i CEEA 4747, July 2019) and by "Generalitat de Catalunya" (10649, June 2021). For the rest of the dogs, study authorization was obtained from the Spanish authority Agencia Española de Medicamentos y Productos Sanitarios (AEMPS), with the authorization number 008/EPA-2383ESP.

Informed Consent Statement: All dogs included in this study belonged to volunteer owners who had granted permission to a physical examination and blood collection from their dogs through a signed consent form.

Data Availability Statement: Data of the study are available from the corresponding author upon reasonable request.

Acknowledgments: Authors would like to acknowledge Lourdes Alarcón Sánchez and Tamara Rivero Balsera for their priceless technical help and support.

Conflicts of Interest: The authors declare no conflict of interest.

6. References

1. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites Vectors* **2011**, 4, 86.
2. Toepp, A.J.; Petersen, C.A. The Balancing Act: Immunology of Leishmaniosis. *Res. Vet. Sci.* **2020**, 130, 19–25.
3. Solano-Gallego, L.; Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-Specific Production of IFN-γ and IL-10 in Stimulated Blood from Dogs with Clinical Leishmaniosis. *Parasites Vectors* **2016**, 9, 317.
4. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, 144, 95–115.
5. Alvar, J.; Cañavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine Leishmaniasis. *Adv. Parasitol.* **2004**, 57, 1–88.
6. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous Immune Mechanisms in Canine Leishmaniosis Due to *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2015**, 163, 94–102.

7. Boggiatto, P.M.; Ramer-Tait, A.E.; Metz, K.; Kramer, E.E.; Gibson-corley, K.; Mullin, K.; Hostetter, J.M.; Gallup, J.M.; Jones, D.E.; Petersen, C.A. Immunologic Indicators of Clinical Progression during Canine *Leishmania infantum* Infection. *Clin. Vaccine Immunol.* **2010**, 17, 267–273.

8. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, 64, 38–43.

9. Brandonisio, O.; Panunzio, M.; Faliero, S.M.; Ceci, L.; Fasanella, A.; Puccini, V. Evaluation of Polymorphonuclear Cell and Monocyte Functions in *Leishmania infantum*-Infected Dogs. *Vet. Immunol. Immunopathol.* **1996**, 53, 95–103.

10. Panaro, M.A.; Puccini, V.; Faliero, S.M.; Marzio, R.; Marangi, A.; Lisi, S.; Brandonisio, O. *Leishmania donovani* lipophosphoglycan (LPG) inhibits respiratory burst and chemotaxis of dog phagocytes. *New Microbiol.* **1996**, 19, 107–112.

11. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, 173, 6521–6525.

12. Mantovani, A.; Cassatella, M.A.; Costantini, C.; Jaillon, S. Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity. *Nat. Rev. Immunol.* **2011**, 11, 519–531.

13. Nauseef, W.M.; Borregaard, N. Neutrophils at Work. *Nat. Immunol.* **2014**, 15, 602–611.

14. Mócsai, A. Diverse Novel Functions of Neutrophils in Immunity, Inflammation, and Beyond. *J. Exp. Med.* **2013**, 210, 1289–1299.

15. Roos, D.; Van Bruggen, R.; Meischl, C. Oxidative Killing of Microbes by Neutrophils. *Microbes Infect.* **2003**, 5, 1307–1315.

16. Nauseef, W.M. How Human Neutrophils Kill and Degrade Microbes: An Integrated View. *Immunol. Rev.* **2007**, 219, 88–102.

17. Hampton, M.B.; Kettle, A.J.; Winterbourn, C.C. The undefined Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. Blood **1998**, 92, 3007–3017.

18. Underhill, D.M.; Ozinsky, A. Phagocytosis of Microbes: Complexity in Action. *Annu. Rev. Immunol.* **2002**, 20, 825–852.

19. Winterbourn, C.; Kettle, A.; Hampton, M. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **2016**, 85, 765–792.

20. Babior, B.M. NADPH Oxidase: An Update. *Blood* **1999**, 93, 1464–1476.

21. Babior, B.M. *The Respiratory Burst Oxidase*. **2006**, 65, 49–95.

22. Wardini, A.B.; Pinto-da-Silva, L.H.; Nadaes, N.R.; Nascimento, M.T.; Roatt, B.M.; Reis, A.B.; Viana, K.F.; Giunchetti, R.C.; Saraiva, E.M.

Neutrophil Properties in Healthy and L*eishmania infantum*-Naturally Infected Dogs. *Sci. Rep.* **2019**, 9, 6247.

23. Carlsen, E.D.; Liang, Y.; Shelite, T.R.; Walker, D.H.; Melby, P.C.; Soong, L. Permissive and Protective Roles for Neutrophils in Leishmaniasis. *Clin. Exp. Immunol.* **2015**, 182, 109–118.
24. Guimaraes-Costa, A.B.; Nascimento, M.T.; Froment, G.S.; Soares, R.P.; Morgado, F.N.; Conceicao-Silva, F.; Saraiva, E. *Leishmania amazonensis* Promastigotes Induce and Are Killed by Neutrophil Extracellular Traps. *Proc. Natl. Acad. Sci. USA* **2009**, 106, 6748–6753.
25. Pereira, M.; Valério-Bolas, A.; Santos-Mateus, D.; Alexandre-Pires, G.; Santos, M.; Rodrigues, A.; Rocha, H.; Santos, A.; Martins, C.; Tomas, A.; et al. Canine Neutrophils Activate Effector Mechanisms in Response to *Leishmania infantum*. *Vet. Parasitol.* **2017**, 248, 10–20.
26. Kaye, P.; Scott, P. Leishmaniasis: Complexity at the Host-Pathogen Interface. *Nat. Rev. Microbiol.* **2011**, 9, 604–615.
27. Terrazas, C.; Oghumu, S.; Varikuti, S.; Martinez-Saucedo, D.; Beverley, S.M.; Satoskar, A.R. Uncovering Leishmania-Macrophage Interplay Using Imaging Flow Cytometry. *J. Immunol. Methods* **2015**, 423, 93–98.
28. Pollard, K.M.; Cauvi, D.M.; Toomey, C.B.; Morris, K.V.; Kono, D.H. Interferon-γ and Systemic Autoimmunity. *Discov. Med.* **2013**, 16, 123.
29. Schoenborn, J.R.; Wilson, C.B. Regulation of Interferon-Gamma during Innate and Adaptive Immune Responses. *Adv. Immunol.* **2007**, 96, 41–101.
30. Hertzog, P.; Forster, S.; Samarajiwa, S. Systems Biology of Interferon Responses. *J. Interferon Cytokine Res.* **2011**, 31, 5–11.
31. Baccala, R.; Kono, D.H.; Theofilopoulos, A.N. Interferons as Pathogenic Effectors in Autoimmunity. *Immunol. Rev.* **2005**, 204, 9–26.
32. Ambruso, D.R.; Briones, N.J.; Baroffio, A.F.; Murphy, J.R.; Tran, A.D.; Gowan, K.; Sanford, B.; Ellison, M.; Jones, K.L. In Vivo Interferon-Gamma Induced Changes in Gene Expression Dramatically Alter Neutrophil Phenotype. *PLoS ONE* **2022**, 17, e0263370.
33. Ellis, T.N.; Beaman, B.L. Interferon-Gamma Activation of Polymorphonuclear Neutrophil Function. *Immunology* **2004**, 112, 2–12.
34. Ellison, M.A.; Gearheart, C.M.; Porter, C.C.; Ambruso, D.R. IFN-γ Alters the Expression of Diverse Immunity Related Genes in a Cell Culture Model Designed to Represent Maturing Neutrophils. *PLoS ONE* **2017**, 12, e0185956.
35. Gómez-Ochoa, P.; Sabate, D.; Homedes, J.; Ferrer, L. Use of the Nitroblue Tetrazolium Reduction Test for the Evaluation of Domperidone Effects on the Neutrophilic Function of Healthy Dogs. *Vet. Immunol. Immunopathol.* **2012**, 146, 97–99.
36. Gómez-Ochoa, P.; Lara, A.; Couto, G.; Marcen, J.M.; Peris, A.; Gascón,

M.; Castillo, J.A. The Nitroblue Tetrazolium Reduction Test in Canine Leishmaniosis. *Vet. Parasitol.* **2010**, 172, 135–138.

37. Esfandiari, N.; Sharma, R.K.; Saleh, R.A.; Thomas, A.J.; Agarwal, A. Utility of the Nitroblue Tetrazolium Reduction Test for Assessment of Reactive Oxygen Species Production by Seminal Leukocytes and Spermatozoa. *J. Androl.* **2003**, 24, 862–870.
38. Muniz-Junqueira, M.I.; de Paula-Coelho, V.N. Meglumine Antimonate Directly Increases Phagocytosis, Superoxide Anion and TNF-Alpha Production, but Only via TNF-Alpha It Indirectly Increases Nitric Oxide Production by Phagocytes of Healthy Individuals, in Vitro. *Int. Immunopharmacol.* **2008**, 8, 1633–1638.
39. Martínez-Flórez, I.; Guerrero, M.J.; Dalmau, A.; Cabré, M.; Alcover, M.M.; Berenguer, D.; Good, L.; Fisa, R.; Riera, C.; Ordeix, L.; et al. Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis Due to *Leishmania infantum* in Dogs. *Pathogens* **2023**, 12, 821.
40. Baxarias, M.; Jornet-Rius, O.; Donato, G.; Mateu, C.; Alcover, M.M.; Pennisi, M.G.; Solano-Gallego, L. Signalment, Immunological and Parasitological Status and Clinicopathological Findings of *Leishmania*-Seropositive Apparently Healthy Dogs. *Animals* **2023**, 13, 1649.
41. Riera, C.; Valladares, J.E.; Gállego, M.; Aisa, M.J.; Castillejo, S.; Fisa, R.; Ribas, N.; Carrió, J.; Alberola, J.; Arboix, M. Serological and Parasitological Follow-Up in Dogs Experimentally Infected with *Leishmania infantum* and Treated with Meglumine Antimoniate. *Vet. Parasitol.* **1999**, 84, 33–47.
42. Baxarias, M.; Solano-Gallego, L. Effect of Storage on Nitro Blue Tetrazolium Reduction Test in Dog Blood Samples. *Vet. Clin. Pathol.* **2023**, online ahead of print.
43. Martínez-Orellana, P.; González, N.; Baldassarre, A.; Álvarez-Fernández, A.; Ordeix, L.; Paradies, P.; Soto, M.; Solano-Gallego, L. Humoral Responses and Ex Vivo IFN-γ Production after Canine Whole Blood Stimulation with *Leishmania infantum* Antigen or KMP11 Recombinant Protein. *Vet. Sci.* **2022**, 9, 116.
44. Carrillo, E.; Carrasco-Antón, N.; López-Medrano, F.; Salto, E.; Fernández, L.; San Martín, J.V.; Alvar, J.; Aguado, J.M.; Moreno, J. Cytokine Release Assays as Tests for Exposure to *Leishmania*, and for Confirming Cure from Leishmaniasis, in Solid Organ Transplant Recipients. *PLoS Negl. Trop. Dis.* **2015**, 9, e0004179.
45. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular Dermatitis Due to *Leishmania infantum* Infection in Seventeen Dogs: Diagnostic Features, Extent of the Infection and Treatment Outcome. *Parasites Vectors* **2014**, 7, 120.

46. Ordeix, L.; Rodríguez, A.; Martínez-Orellana, P.; Montserrat-Sangrà, S.; Solano-Gallego, L. Clinical follow up of a series of dogs with papular dermatitis due to *Leishmania infantum*. In *Proceedings of the 29th Annual Congress of the ESVD-ECVD*, Lausanne, Switzerland, 7–9 September **2017**.

47. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular Dermatitis Due to *Leishmania Spp*. Infection in Dogs with Parasite-Specific Cellular Immune Responses. *Vet. Dermatol.* **2005**, 16, 187–191.

48. Bottero, E.; Poggi, M.; Viglione, M. Lesioni Papulari Indotte Da *Leishmania spp*. in 8 Cani Giovani. Veterinaria **2006**, 20, 33–36.

49. Noli, C.; Cornegliani, L. Leishmaniosi Bottoniforme. Descrizione Di Cinque Casi Italiani e Confronto Con La Letteratura. *Quad. Dermatol.* **2006**, 1, 23–26.

50. Almeida, B.F.M.; Narciso, L.G.; Bosco, A.M.; Pereira, P.P.; Braga, E.T.; Avanço, S.V.; Marcondes, M.; Ciarlini, P.C. Neutrophil Dysfunction Varies with the Stage of Canine Visceral Leishmaniosis. *Vet. Parasitol.* **2013**, 196, 6–12.

51. Anwar, S.; Whyte, M.K.B. Neutrophil Apoptosis in Infectious Disease. *Exp. Lung Res.* **2007**, 33, 519–528.

52. Silva, A.C.R.A.; de Almeida, B.F.M.; Soeiro, C.S.; Ferreira, W.L.; de Lima, V.M.F.; Ciarlini, P.C. Oxidative Stress, Superoxide Production, and Apoptosis of Neutrophils in Dogs with Chronic Kidney Disease. *Can. J. Vet. Res.* **2013**, 77, 136.

53. Barbosa, T.S.; Mori, C.K.; Ciarlini, P.C. Efeito Inibidor Do Soro Urêmico Sobre o Metabolismo Oxidativo Dos Neutrófilos de Cães. *Arq. Bras. Med. Veterinária Zootec.* **2010**, 62, 1352–1358.

54. Kannan, K.; Jain, S.K. Oxidative Stress and Apoptosis. *Pathophysiology* **2000**, 7, 153–163.

55. Kennedy, A.D.; Deleo, F.R. Neutrophil Apoptosis and the Resolution of Infection. *Immunol. Res.* **2009**, 43, 25–61.

56. Abbehusen, M.M.C.; Almeida, D.A.V.; Solcà, S.M.; Da Silva Pereira, L.; Costa, D.J.; Gil-Santana, L.; Bozza, P.T.; Fraga, D.B.M.; Veras, P.S.T.; Dos-Santos, W.L.C.; et al. Clinical and Immunopathological Findings during Long Term Follow-up in *Leishmania infantum* Experimentally Infected Dogs. *Sci. Rep.* **2017**, 7, 15914.

57. Reis, A.B.; Martins-Filho, O.A.; Teixeira-Carvalho, A.; Carvalho, M.G.; Mayrink, W.; França-Silva, J.C.; Giunchetti, R.C.; Genaro, O.; Corrêa-Oliveira, R. Parasite Density and Impaired Biochemical/Hematological Status Are Associated with Severe Clinical Aspects of Canine Visceral Leishmaniasis. *Res. Vet. Sci.* **2006**, 81, 68–75.

58. Almeida, V.; Lima, I.; Fraga, D.; Carrillo, E.; Moreno, J.; Dos-Santos, W.L.C. Hematological Changes in Dogs with Visceral Leishmaniasis Are

Associated with Increased Ifn-γ and Tnf Gene Expression Levels in the Bone Marrow. *Microorganisms* **2021**, 9, 1618.

59. Gueirard, P.; Laplante, A.; Rondeau, C.; Milon, G.; Desjardins, M. Trafficking of *Leishmania donovani* Promastigotes in Non-Lytic Compartments in Neutrophils Enables the Subsequent Transfer of Parasites to Macrophages. *Cell. Microbiol.* **2008**, 10, 100–111.
60. Brandonisio, O.; Panaro, M.A.; Marzio, R.; Marangi, A.; Faliero, S.M.; Jirillo, E. Impairment of the Human Phagocyte Oxidative Responses Caused by *Leishmania* Lipophosphoglycan (LPG): In Vitro Studies. FEMS Immunol. *Med. Microbiol.* **1994**, 8, 57–62.
61. Vuotto, M.L.; De Luna, R.; Ielpo, M.T.L.; De Sole, P.; Moscatiello, V.; Simeone, I.; Gradoni, L.; Mancino, D. Chemiluminescence Activity in Whole Blood Phagocytes of Dogs Naturally Infected with *Leishmania infantum*. Luminescence **2000**, 15, 251–255.
62. Ciarlini, P.C.; Valadares, T.C.; Ikeda-Garcia, F.A.; Marcondes, M.; De Lima, V.M.F. Leucograma E Metabolismo Oxidativo dos Neutrófilos de Cães com Leishmaniose Visceral Antes e Após O Tratamento com Antimoniato de Meglumina e Alopurinol. *Ciência Anim. Bras.* **2010**, 11, 369–375.
63. Santos-Gomes, G.M.; Rosa, R.; Leandro, C.; Cortes, S.; Romão, P.; Silveira, H. Cytokine Expression during the Outcome of Canine Experimental Infection by *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2002**, 88, 21–30.
64. do Nascimento, P.R.P.; Martins, D.R.A.; Monteiro, G.R.G.; Queiroz, P.V.; Freire-Neto, F.P.; Queiroz, J.W.; Morais Lima, Á.L.; Jeronimo, S.M.B. Association of Pro-Inflammatory Cytokines and Iron Regulatory Protein 2 (IRP2) with *Leishmania* Burden in Canine Visceral Leishmaniasis. *PLoS ONE* **2013**, 8, e73873.
65. Aslan, H.; Oliveira, F.; Meneses, C.; Castrovinci, P.; Gomes, R.; Teixeira, C.; Derenge, C.A.; Orandle, M.; Gradoni, L.; Oliva, G.; et al. New Insights Into the Transmissibility of *Leishmania infantum* from Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J. Infect. Dis.* **2016**, 213, 1752–1761.
66. Martínez-Orellana, P.; Marí-Martorell, D.; Montserrat-Sangrà, S.; Ordeix, L.; Baneth, G.; Solano-Gallego, L. *Leishmania infantum*-Specific IFN-γ Production in Stimulated Blood from Dogs with Clinical Leishmaniosis at Diagnosis and during Treatment. *Vet. Parasitol.* **2017**, 248, 39–47.
67. Esch, K.J.; Juelsgaard, R.; Martinez, P.A.; Jones, D.E.; Petersen, C.A. PD-1-Mediated T Cell Exhaustion during Visceral Leishmaniasis Impairs Phagocyte Function. *J. Immunol.* **2013**, 191, 5542.
68. Scott, P.; Riley, E.M. Acquired Immunity to Intracellular Protozoa. *Immune Response Infect.* **2014**, 24, 301–311.

Phase I trial on the safety and immunogenicity of a candidate *Leishmania infantum* vaccine in dogs

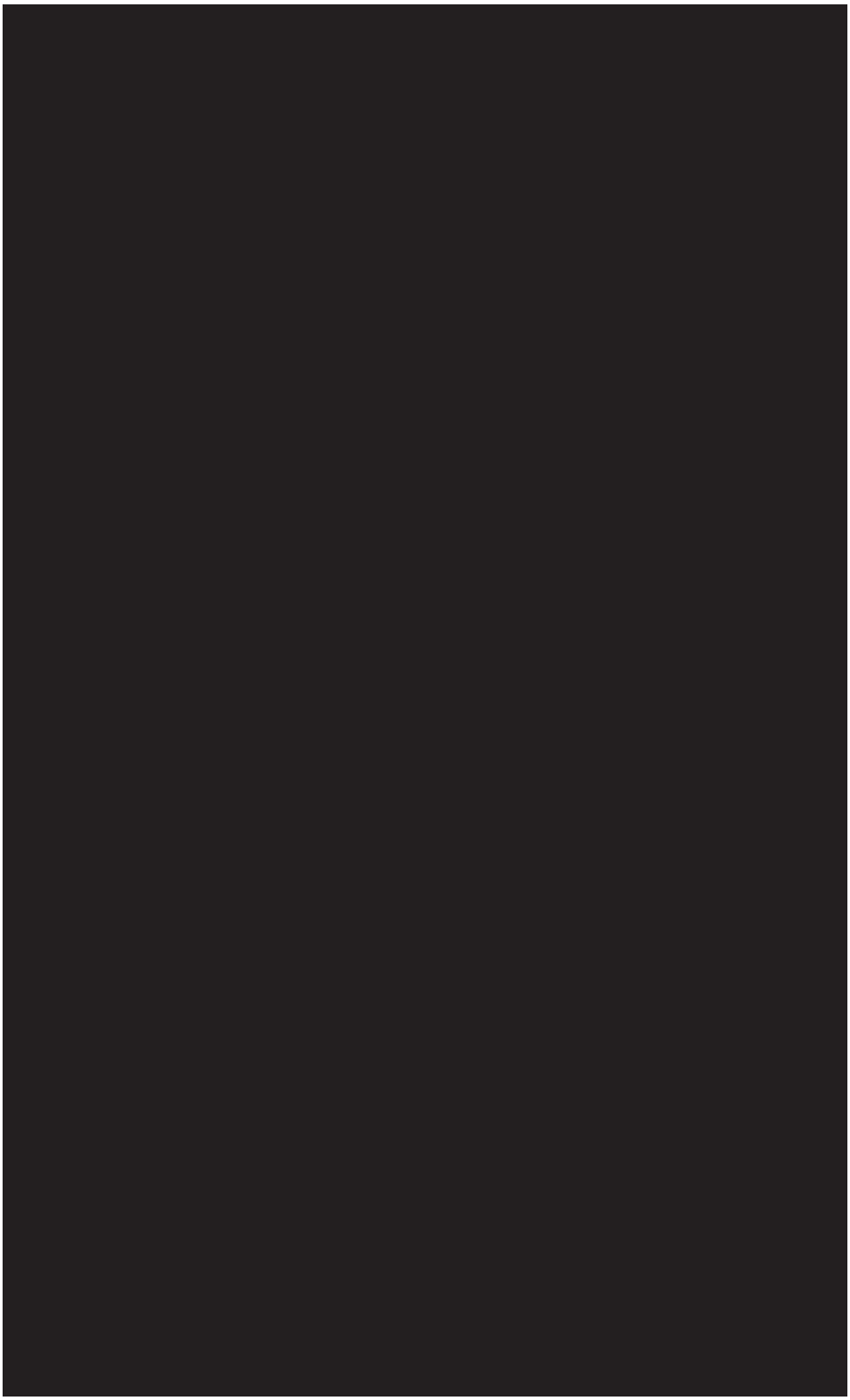
I.Martínez-Flórez[1], M. Margaroni[2], E. Karagouni[2], E. Tsanaktsidou[4], O. Kammona[3], C. Kiparissides[3, 4], C. Blasi-Brugué [1], L. Alarcón[1], L. Solano-Gallego[1]

[1]Department of Animal Medicine and Surgery, Autonomous University of Barcelona, Barcelona, Spain

[2]Immunology of Infection group, Department of Microbiology, Hellenic Pasteur Institute, Athens, Greece

[3]Chemical Process & Energy Resources Institute, Centre for Research and Technology Hellas, Thessaloniki, Greec

[4]Department of Chemical Engineering, Aristotle University of Thessaloniki, Thessaloniki, Greece



Discussion

The present PhD thesis aimed to investigate various aspects of canine leishmaniosis (CanL) with a focus on improving the understanding of the immune response, exploring new therapeutic options for some presentations (papular dermatitis) as well as exploring the immunogenicity and safety of a novel *Leishmania* vaccine candidate. Three independent studies were conducted, each contributing to a comprehensive understanding of different facets of CanL. In this general discussion, we will synthesize the findings from the three studies and discuss their implications for CanL research and future directions in the field.

New therapeutic options for dogs with Leish-Vet stage I CanL

The topical treatment of *Leishmania* dermatological lesions has garnered attention as a potential therapeutic approach for managing cutaneous leishmaniosis in both humans and dogs [1–4]. Nevertheless, until now, no studies evaluated topical treatment for papular dermatitis (mild disease-stage I) due to *L. infantum* infection in dogs, which is a common presentation of this disease [2–4]. Traditional treatment options for leishmaniosis often involve systemic medications, such as antimonials, amphotericin B, or miltefosine [5,6]. However, these systemic treatments can be associated with various side effects and require prolonged administration, and thus might be considered unsuitable for the treatment of papular dermatitis [6,7]. Therefore, exploring topical treatments offers potential advantages in terms of targeted delivery, reduced systemic exposure, and fewer adverse effects [8]. In humans, local and intralesional therapies have been endorsed as an alternative to systemic drugs for patients with at least four lesions smaller than 4 cm in diameter, particularly when the face or joints are unaffected (WHO, PAHO). Notably, intralesional therapy has been found to be effective and safe for localized forms of the disease [9–12].

The findings of the present study demonstrated that dogs with papular dermatitis who received local meglumine antimoniate experienced faster healing compared to those who received a placebo. Additionally, none of the dogs exhibited worsening of clinical signs during the one-year follow-up period.

Thus, meglumine antimoniate, a commonly used systemic treatment for leishmaniosis, has also shown promise as a local treatment option. Systemic meglumine antimoniate is widely accepted as a mainstay treatment for all types of leishmaniosis [5,6]. Nevertheless, even that systemic administration is associated to adverse effects and even local pain, its topical administration has never been evaluated for the treatment of localized cutaneous form of the infection (papular dermatitis) [13]. In humans, intralesional treatment has

been recommended as an acceptable therapeutic alternative for New World leishmaniosis by the WHO Expert Committee on *Leishmania*sis in 2010 and the PAHO Expert Committee on *Leishmania*sis in 2013 [1,9–12]. Intralesional infiltration of meglumine antimoniate in cases of cutaneous leishmaniosis has reported efficacy rates of 68-82% [7]. Similarly, in dogs a higher healing rate has been reported when treating localized cutaneous ulcerations due to *L. braziliensis* with intralesional meglumine antimoniate [11]. The main downside of this technique is that it requires infiltration of all cutaneous lesions to be effective, additionally, local irritation, pain, edema, erythema, and pruritus have been described as adverse effects [1,11,14]. These make this technique non-viable for most of the cases of canine papular dermatitis, as they show as multiple small papules, and infiltration would not be possible. When comparing this to the topical formulation described in our study, the latter shows clear advantages, including the application form and the reduced risk of adverse effects. This topical formulation has shown to be a useful and easy to apply treatment for dogs with papular dermatitis.

In the present study, PHMB, whether administered alone or in combination with TLR4a, exhibited a higher inclination toward resolution, although the results did not reach statistical significance. PHMB is a topical antimicrobial agent that has previously shown anti*Leishmania*l properties in *in vitro* studies by disrupting the parasite's membrane integrity and inducing chromosomal damage, although there was limited *in vivo* research on the topical use of PHMB for the treatment of leishmaniosis [15,16]. Nevertheless, meglumine antimoniate showed better results in the present study and thus, it should be considered as a better option.

In conclusion, topical treatment holds promise as an alternative approach for managing *Leishmania* dermatological lesions. The use of meglumine antimoniate has shown encouraging results in terms of faster resolution and improved clinical response. These topical treatment offers the potential advantages of targeted delivery, reduced systemic exposure, and fewer adverse effects. However, further research is needed to optimize treatment protocols and expand the understanding of topical treatment options for leishmaniosis in both human and veterinary medicine.

Neutrophil activation in different states of canine *L. infantum* infection: Nitroblue tetrazolium test and IFN-γ

This study aimed to provide valuable insights into the role of neutrophils and their activation in the context of CanL. The study aimed to comprehensively evaluate the relationship between neutrophil activation in different states of

canine *L. infantum* infection and its possible association to the production of IFN-γ, a key cytokine associated with an effective immune response against *Leishmania* infection [17–23]. The findings shed light on the complex immune response in CanL and highlight the significance of neutrophils and their oxidative metabolism in disease management.

The study's main objectives were effectively addressed through the assessment of neutrophil activation using the nitroblue tetrazolium test (NBT) across different states of infection, including healthy seropositive and seronegative dogs. The results revealed that dogs with mild, self-limiting disease (papular dermatitis) exhibited significantly higher NBT values, indicating an elevated oxidative metabolism of neutrophils in these cases. This finding supports the hypothesis that neutrophil activation is closely linked to an effective immune response against *Leishmania* infection, and that an enhanced oxidative metabolism in neutrophils may serve as an indicator of such a response [24–26].

Conversely, dogs in more advanced stages of the disease did not exhibit significant differences in neutrophil activation compared to healthy seronegative dogs. This observation could be attributed to a correlation between the absence of neutrophil activation and the progression of clinical disease, potentially reflecting a non-effective immune phenotype or a consequence of disease progression and immunity exhaustion [27,28]. Notably, previous studies have shown an increased rate of neutrophil apoptosis in severe stages of CanL, which could contribute to the reduction in NBT rate [29–32]. Prolonged neutrophil activation can lead to the generation of oxidative stress, depletion of antioxidant defenses, and ultimately trigger neutrophil's apoptosis, negatively impacting neutrophil's oxidative metabolism and compromising the organism's primary defense mechanisms [30].

The study also explored the role of IFN-γ, a potent immunoregulatory cytokine, in relation to neutrophil activation. IFN-γ was found to be increased in dogs with mild self-limiting CanL, and its production correlated with an increased NBT rate while in more advanced clinical stages, dogs showed lower levels of IFN-γ, possibly indicating a less effective immune response or T-lymphocytes exhaustion [19]. This suggests that IFN-γ could play a role in promoting the oxidative metabolism of neutrophils and contributes to an effective immune response. A recent study demonstrated that the administration of IFN-γ had significant effects on circulating neutrophils in humans, resulting in noteworthy changes in gene expression, protein expression, and overall function, indicating an enhancement in neutrophil activity [33]. The study's findings further suggested that IFN-γ not only stimulates NADPH oxidase activity to promote the oxidative burst but also influences Fc receptor-mediated ingestion, pathogen recognition by innate immune receptors, antigen presentation (including MHCI

and MHCII), activation of GBPs (guanylate-binding proteins), upregulation of nitric oxide (NO) production, and increase of neutrophil release into circulation [33]. Hence, it is likely that IFN-γ has a direct correlation with NBT by inducing the oxidative burst in white blood cells, potentially contributing to an effective immune response through neutrophil reactivity.

Overall, the study's findings provide valuable insights into the immune responses in CanL, particularly regarding the role of neutrophils and their activation, as well as the influence of IFN-γ production over this. This knowledge contributes to a better understanding of the complex immune response in CanL and highlights the importance of neutrophil activation and IFN-γ production in disease management.

Further research is needed to explore other underlying mechanisms and potential therapeutic targets for enhancing the immune response against *Leishmania* infection. Understanding the interactions between different cytokines, T-lymphocyte responses, and neutrophil functions such as neutrophil extracellular traps could lead to the development of more effective treatment strategies and contribute to the management of CanL [19,34]. The findings presented in this study contribute significantly to the body of knowledge on CanL and lay the groundwork for future investigations in this field.

Integration of findings

Collectively, the findings from the three studies contribute to our understanding of the immune response in CanL and its potential implications for disease management and vaccine development. The immune response in CanL is characterized by a complex interplay of CD4+ T lymphocyte subsets, with Th2 response predominating in active disease [17,48]. The transition from a protective Th1 response to a regulatory response and T cell exhaustion were identified as key factors in disease progression [27]. Neutrophil activation and oxidative metabolism were found to be associated with disease severity, highlighting the significance of neutrophils in the immune response against CanL [17,19]. Furthermore, the evaluation of a new topical treatment for dogs with mild cutaneous presentations (papular dermatitis) demonstrated that the use of meglumine antimoniate as topical treatment showed encouraging results in terms of faster resolution and improved clinical response, holding promise as an alternative approach for managing *Leishmania* dermatological lesions, offering potential advantages. Finally, research done on a novel vaccine formulation demonstrated promising immunogenicity and safety profiles, emphasizing the need for further studies to evaluate its efficacy in disease prevention.

Future Directions

Based on the findings from this PhD thesis, several avenues for future research can be suggested. First, exploring different protocols and drugs for topical treatment, and to initiate additional large scale field studies. Second to continue exploring the role of neutrophils and their potential modulation via IFN-γ stimulation or others, and its effect in disease progression and immune response. Additionally, to study strategies to restore the balance between Th1 and Th2 responses and alleviate T cell exhaustion could offer new therapeutic approaches for CanL management. Lastly, to further continue evaluating the efficacy of the *Leishmania* candidate vaccine studied here by conducting phase III clinical trials, as this vaccine hold promise for effective disease prevention and warrant additional efficacy studies in dogs.

References

1. Garza-Tovar, T.F.; Sacriste-Hernández, M.I.; Juárez-Durán, E.R.; Arenas, R. An Overview of the Treatment of Cutaneous *Leishmania*sis. *Fac. Rev.* **2020**, *9*, doi:10.12703/R/9-28.
2. Piragauta, S.P.; Higuita-Castro, · J L; Arbeláez, · N; Restrepo, · A M; Archbold, · R; Quiñones, · W; Torres, · F; Echeverri, · F; Escobar, · G; Vélez, · I D; et al. Utility of the Combination of Hederagenin Glucoside Saponins and Chromane Hydrazone in the Topical Treatment of Canine Cutaneous *Leishmania*sis. An Observational Study. *Parasitol. Res. 1*, 3, doi:10.1007/s00436-022-07467-x.
3. Lago, J.; Fraga, D.; Guimarães, L.H.; Lago, T.; Santos, Y.; Lago, E.; Werneck, G.L.; Bacellar, O.; Carvalho, E.M. Efficacy of Intralesional Meglumine Antimoniate in the Treatment of Canine Tegumentary *Leishmania*sis: A Randomized Controlled Trial. *PLoS Negl. Trop. Dis.* **2023**, *17*, e0011064, doi:10.1371/journal.pntd.0011064.
4. Barbosa Santos, E.G.O.; Marzochi, M.C.A.; Conceição, N.F.; Brito, C.M.M.; Barroso, J.A.; Pacheco, R.S. N-Methylglucamine Antimonate (SbV+): Intralesional Canine Tegumentary *Leishmania*sis Therapy. *Parasite* **1998**, *5*, 175–180, doi:10.1051/parasite/1998052175.
5. Morales-Yuste, M.; Martín-Sánchez, J.; Corpas-Lopez, V. Canine *Leishmania*sis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. *Vet. Sci.* **2022**, *9*, doi:10.3390/VETSCI9080387.
6. Solano-Gallego, L.; Mirá, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites and Vectors* **2011**, *4*, 1–16, doi:10.1186/1756-3305-4-86.
7. Noli, C.; Saridomichelakis, M.N. An Update on the Diagnosis and Treatment of Canine Leishmaniosis Caused by *Leishmania infantum* (Syn. L. Chagasi). *Vet. J.* **2014**, *202*, 425–435, doi:10.1016/J.TVJL.2014.09.002.
8. Garza-Tovar, T.F.; Sacriste-Hernández, M.I.; Juárez-Durán, E.R.; Arenas, R. An Overview of the Treatment of Cutaneous *Leishmania*sis. *Fac. Rev.* **2020**, *9*, doi:10.12703/R/9-28.
9. Uribe-Restrepo, A.F.; Prieto, M.D.; Cossio, A.; Desai, M.M.; Del Mar Castro, M. Eligibility for Local Therapies in Adolescents and Adults with Cutaneous *Leishmania*sis from Southwestern Colombia: A Cross-Sectional Study. *Am. J. Trop. Med. Hyg.* **2019**, *100*, 306–310.
10. Aronson, N.E.; Joya, C.A. Cutaneous *Leishmania*sis: Updates in Diagnosis and Management. *Infect. Dis. Clin. North Am.* **2019**, *33*, 101–117.
11. Nassif, P.W.; De Mello, T.F.P.; Navasconi, T.R.; Mota, C.A.; Demarchi, I.G.; Aristides, S.M.A.; Lonardoni, M.V.C.; Teixeira, J.J.V.; Silveira, T.G.V. Safety and Efficacy of Current Alternatives in the Topical Treatment of

Cutaneous *Leishmania*sis: A Systematic Review. *Parasitology* **2017**, *144*, 995–1004.

12. Solano-Gallego, L.; Di Filippo, L.; Ordeix, L.; Planellas, M.; Roura, X.; Altet, L.; Martínez-Orellana, P.; Montserrat, S. Early Reduction of *Leishmania infantum*-Specific Antibodies and Blood Parasitemia during Treatment in Dogs with Moderate or Severe Disease. *Parasites and Vectors* **2016**, *9*, 1–9.
13. Chakravarty, J.; Sundar, S. Current and Emerging Medications for the Treatment of *Leishmania*sis. *Expert Opin. Pharmacother.* **2019**, *20*, 1251–1265.
14. Firdessa, R.; Good, L.; Amstalden, M.C.; Chindera, K.; Kamaruzzaman, N.F.; Schultheis, M.; Röger, B.; Hecht, N.; Oelschlaeger, T.A.; Meinel, L.; et al. Pathogen- and Host-Directed Anti*Leishmania*l Effects Mediated by Polyhexanide (PHMB). *PLoS Negl. Trop. Dis.* **2015**, *9*, e000404.
15. Martínez-Orellana, P.; Baxarias, M.; Good, L.; Solano-Gallego, L. The Effects of Polyhexamethylene Biguanide (PHMB) and TLR Agonists Alone or as Polyplex Nanoparticles against *Leishmania infantum* Promastigotes and Amastigotes. *Vet. Sci.* **2020**, *7*, 1–14.
16. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, *144*, 95–115.
17. Santos-Gomes, G.M.; Rosa, R.; Leandro, C.; Cortes, S.; Romão, P.; Silveira, H. Cytokine Expression during the Outcome of Canine Experimental Infection by *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2002**, *88*, 21–30.
18. Toepp, A.J.; Petersen, C.A. The Balancing Act: Immunology of Leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25.
19. Nascimento, P.R.P. do; Martins, D.R.A.; Monteiro, G.R.G.; Queiroz, P.V.; Freire-Neto, F.P.; Queiroz, J.W.; Morais Lima, Á.L.; Jeronimo, S.M.B. Association of Pro-Inflammatory Cytokines and Iron Regulatory Protein 2 (IRP2) with *Leishmania* Burden in Canine Visceral *Leishmania*sis. *PLoS One* **2013**, *8*, e73873.
20. Aslan, H.; Oliveira, F.; Meneses, C.; Castrovinci, P.; Gomes, R.; Teixeira, C.; Derenge, C.A.; Orandle, M.; Gradoni, L.; Oliva, G.; et al. New Insights Into the Transmissibility of *Leishmania infantum* From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J. Infect. Dis.* **2016**, *213*, 1752–1761.
21. Solano-Gallego, L.; Montserrrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-Specific Production of IFN-γ and IL-10 in Stimulated Blood from Dogs with Clinical Leishmaniosis. *Parasites and Vectors* **2016**, *9*, 317.
22. Martínez-Orellana, P.; Marí-Martorell, D.; Montserrat-Sangrà, S.; Ordeix, L.; Baneth, G.; Solano-Gallego, L. *Leishmania infantum*-Specific IFN-γ

Production in Stimulated Blood from Dogs with Clinical Leishmaniosis at Diagnosis and during Treatment. *Vet. Parasitol.* **2017**, *248*, 39–47.

23. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, *173*, 6521–6525.
24. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, *64*, 38–43.
25. Gueirard, P.; Laplante, A.; Rondeau, C.; Milon, G.; Desjardins, M. Trafficking of *Leishmania* Donovani Promastigotes in Non-Lytic Compartments in Neutrophils Enables the Subsequent Transfer of Parasites to Macrophages. *Cell. Microbiol.* **2008**, *10*, 100–111.
26. Boggiatto, P.M.; Ramer-tait, A.E.; Metz, K.; Kramer, E.E.; Gibson-corley, K.; Mullin, K.; Hostetter, J.M.; Gallup, J.M.; Jones, D.E.; Petersen, C.A. Immunologic Indicators of Clinical Progression during Canine *Leishmania infantum* Infection ☒. **2010**, *17*, 267–273.
27. Esch, K.J.; Juelsgaard, R.; Martinez, P.A.; Jones, D.E.; Petersen, C.A. PD-1-Mediated T Cell Exhaustion during Visceral *Leishmania*sis Impairs Phagocyte Function. *J. Immunol.* **2013**, *191*, 5542.
28. Almeida, B.F.M.; Narciso, L.G.; Bosco, A.M.; Pereira, P.P.; Braga, E.T.; Avanço, S. V.; Marcondes, M.; Ciarlini, P.C. Neutrophil Dysfunction Varies with the Stage of Canine Visceral Leishmaniosis. *Vet. Parasitol.* **2013**, *196*, 6–12.
29. Anwar, S.; Whyte, M.K.B. Neutrophil Apoptosis in Infectious Disease. *Exp. Lung Res.* **2007**, *33*, 519–528.
30. Silva, A.C.R.A.; de Almeida, B.F.M.; Soeiro, C.S.; Ferreira, W.L.; de Lima, V.M.F.; Ciarlini, P.C. Oxidative Stress, Superoxide Production, and Apoptosis of Neutrophils in Dogs with Chronic Kidney Disease. *Can. J. Vet. Res.* **2013**, *77*, 136.
31. Barbosa, T.S.; Mori, C.K.; Ciarlini, P.C. Efeito Inibidor Do Soro Urêmico Sobre o Metabolismo Oxidativo Dos Neutrófilos de Cães. *Arq. Bras. Med. Veterinária e Zootec.* **2010**, *62*, 1352–1358.
32. Ambruso, D.R.; Briones, N.J.; Baroffio, A.F.; Murphy, J.R.; Tran, A.D.; Gowan, K.; Sanford, B.; Ellison, M.; Jones, K.L. In Vivo Interferon-Gamma Induced Changes in Gene Expression Dramatically Alter Neutrophil Phenotype. *PLoS One* **2022**, *17*, 1–22.
33. Gómez-Ochoa, P.; Lara, A.; Couto, G.; Marcen, J.M.; Peris, A.; Gascón, M.; Castillo, J.A. The Nitroblue Tetrazolium Reduction Test in Canine Leishmaniosis. *Vet. Parasitol.* **2010**, *172*, 135–138.
34. Alvar, J.; Cañavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine *Leishmania*sis. *Adv. Parasitol.* **2004**, *57*, 1–88.

35. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends Parasitol.* **2008**, *24*, 324–330.

36. Margaroni, M.; Agallou, M.; Athanasiou, E.; Kammona, O.; Kiparissides, C.; Gaitanaki, C.; Karagouni, E. Vaccination with Poly(D,L-Lactide-Co-Glycolide) Nanoparticles Loaded with Soluble *Leishmania* Antigens and Modified with a TNFα-Mimicking Peptide or Monophosphoryl Lipid Aconfers Protection against Experimental Visceral *Leishmania*sis. *Int. J. Nanomedicine* **2017**, *12*, 6169–6184.

37. Regina-Silva, S.; Feres, A.M.L.T.; França-Silva, J.C.; Dias, E.S.; Michalsky, É.M.; de Andrade, H.M.; Coelho, E.A.F.; Ribeiro, G.M.; Fernandes, A.P.; Machado-Coelho, G.L.L. Field Randomized Trial to Evaluate the Efficacy of the Leish-Tec® Vaccine against Canine Visceral *Leishmania*sis in an Endemic Area of Brazil. *Vaccine* **2016**, *34*, 2233–2239.

38. Fernández Cotrina, J.; Iniesta, V.; Monroy, I.; Baz, V.; Hugnet, C.; Marañon, F.; Fabra, M.; Gómez-Nieto, L.C.; Alonso, C. A Large-Scale Field Randomized Trial Demonstrates Safety and Efficacy of the Vaccine LetiFend® against Canine Leishmaniosis. *Vaccine* **2018**, *36*, 1972–1982.

39. Fernandes, A.P.; Costa, M.M.S.; Coelho, E.A.F.; Michalick, M.S.M.; de Freitas, E.; Melo, M.N.; Luiz Tafuri, W.; Resende, D. de M.; Hermont, V.; Abrantes, C. de F.; et al. Protective Immunity against Challenge with *Leishmania* (*Leishmania*) Chagasi in Beagle Dogs Vaccinated with Recombinant A2 Protein. *Vaccine* **2008**, *26*, 5888–5895.

40. Toepp, A.; Larson, M.; Wilson, G.; Grinnage-Pulley, T.; Bennett, C.; Leal-Lima, A.; Anderson, B.; Parrish, M.; Anderson, M.; Fowler, H.; et al. Randomized, Controlled, Double-Blinded Field Trial to Assess *Leishmania* Vaccine Effectiveness as Immunotherapy for Canine Leishmaniosis. *Vaccine* **2018**, *36*, 6433–6441.

41. Pitta, M.G.R.; Romano, A.; Cabantous, S.; Henri, S.; Hammad, A.; Kouriba, B.; Argiro, L.; El Kheir, M.; Bucheton, B.; Mary, C.; et al. IL-17 and IL-22 Are Associated with Protection against Human Kala Azar Caused by *Leishmania* Donovani. *J. Clin. Invest.* **2009**, *119*, 2379–2387.

42. Wu, W.; Huang, L.; Mendez, S. A Live *Leishmania* Major Vaccine Containing CpG Motifs Induces the de Novo Generation of Th17 Cells in C57BL/6 Mice. *Eur. J. Immunol.* **2010**, *40*, 2517–2527.

43. Bacallar, O.; Faria, D.; Nascimento, M.; Cardoso, T.M.; Gollob, K.J.; Dutra, W.O.; Scott, P.; Carvalho, E.M. Interleukin 17 Production among Patients with American Cutaneous *Leishmania*sis. *J. Infect. Dis.* **2009**, *200*, 75–78.

44. Boaventura, V.S.; Santos, C.S.; Cardoso, C.R.; De Andrade, J.; Dos Santos, W.L.C.; Clarêncio, J.; Silva, J.S.; Borges, V.M.; Barral-Netto, M.; Brodskyn, C.I.; et al. Human Mucosal *Leishmania*sis: Neutrophils Infiltrate Areas of Tissue Damage That Express High Levels of Th17-Related Cytokines. *Eur. J. Immunol.* **2010**, *40*, 2830–2836.

45. Nascimento, M.S.L.; Albuquerque, T.D.R.; Nascimento, A.F.S.; Caldas, I.S.; Do-Valle-Matta, M.A.; Souto, J.T.; Talvani, A.; Bahia, M.T.; Galvão, L.M.C.; Câmara, A.C.J.; et al. Impairment of Interleukin-17A Expression in Canine Visceral Leishmaniosis Is Correlated with Reduced Interferon-γ and Inducible Nitric Oxide Synthase Expression. *J. Comp. Pathol.* **2015**, *153*, 197–205.

46. Rodriguez-Cortes, A.; Martori, C.; Martinez-Florez, A.; Clop, A.; Amills, M.; Kubejko, J.; Llull, J.; Nadal, J.M.; Alberola, J. Canine *Leishmania*sis Progression Is Associated with Vitamin D Deficiency. *Sci. Rep.* **2017**, *7*, 3346.

47. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous Immune Mechanisms in Canine Leishmaniosis Due to *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2015**, *163*, 94–102.

Conclusions

1. Local meglumine antimoniate was a safe and clinical effective alternative for the treatment of papular dermatitis in dogs with *L. infantum* infection.
2. No dog treated with local meglumine antimoniate showed worsening clinical signs or relapse during the one-year follow-up.
3. PHMB (alone or in combination with TLR4a) showed a higher tendency towards resolution when compared to placebo, although results were considered non-significant.
4. PHMB (alone or in combination with TLR4a) proved to be a safe local treatment for dogs affected by papular dermatitis due to *L. infantum*.
5. Dogs diagnosed with papular dermatitis due to *L. infantum* presented a protective immune response at the time of diagnosis and during a year follow-up period.
6. Neutrophil activation, as assessed by NBT, was significantly higher in dogs with mild self-limiting disease (papular dermatitis), followed by healthy seropositive dogs, indicating an enhanced oxidative metabolism of neutrophils in these cases.
7. Dogs in more advanced stages of the disease did not exhibit significant differences in neutrophil activation compared to healthy seronegative dogs.
8. A positive association was found between neutrophil activation and the production of IFN-γ.
9. [redacted]
10. [redacted]
11. [redacted]

Addendum

INCIDENT SHEET FOR OWNER (treatment application, adverse effects, or others)

Dog ID ☐

Detection date (days after treatment)												
Erythema / crusts (indicate from 0 to 4)												
Edema (indicate from 0 to 4)												
Itching (indicate from 0 to 4)												
Pain (indicate 'yes' or 'no')												
Other skin lesions (mark with an X the day of visit)*												
Other clinical signs (mark with an X the day of visit)*												
Treatment application (indicate 'yes' or 'no')**												

* Indicate on the incident sheet of the corresponding visit (1, 2, 3, or 4)
** In the case that the treatment could NOT be applied, describe it in this incident sheet.

Day of visit: The treatment has not been applied for the following reason:

ADVERSE EFFECTS BY VETERINARIAN

Day of visit ...../...../..... **Dog ID**

ERYTHEMA / CRUSTS	**0**	**1**	**2**	**3**	**4**
EDEMA	**0**	**1**	**2**	**3**	**4**
ITCHING	**NO**				**SI**
PAIN	**NO**				**SI**

OTHER SKIN LESIONS OR OTHER CLINICAL SIGNS (SPECIFY):

Erythema and crusts	**Formation of edemas**	
Very slight erythema	very slight edema (barely noticeable)	1
Well-defined erythema	slight edema (edges of the area well defined by elevation)	2
Moderate to severe erythema	Moderate edema (elevation of approximately 1 mm)	3
Severe or crusts formation	Severe edema (elevation of more than 1 mm, extending beyond the exposure site)	4

PHOTO FOR ADVERSE EFFECT ASSESSMENT

WITHDRAWAL FROM THE STUDY AND REASON

Day of visit ...../……../…… **Dog ID**

WITHDRAWAL FROM THE STUDY	**YES**	**NO**
REASON		

VETERINARIAN

Signature: _____ **Date:** ____/____/____

Animal owner informed consent

In this study, two distinct informed consent forms were used. The first form was carefully reviewed and signed by the owners of dogs diagnosed with stage I leishmaniosis (papular dermatitis) (pag 1-2), while the second form was designated for the remaining dogs participating in the research (pag 3).

ANIMAL OWNER INFORMED CONSENT FOR DOGS WITH PAPULAR DERMATITIS

Study on *Leishmania infantum* infection in dogs

Department of Animal Medicine and Surgery
Faculty of Veterinary Medicine
Autonomous University of Barcelona

Objective and brief explanation of the study:

Leishmaniosis is an infectious disease that can affect both dogs and humans. In both cases, the infection is transmitted through the bite of a sandfly commonly found in the Mediterranean area. In dogs, *Leishmania* infection can be associated with various clinical signs, ranging from mild skin symptoms to systemic conditions. It is also known that some dogs can control the progression of the disease after infection and may develop very mild clinical signs, such as papular dermatitis, or even show no clinical signs of the disease at all. This characteristic is genetically determined. For example, Ibizan Hound dogs are more resistant to the disease than dogs of other breeds. The variability in clinical manifestations of canine leishmaniosis depends on different immune responses in the dog and can partially influence the treatment and its response.

The objective of this study is to assess the efficacy of a topical immunomodulatory or antiparasitic treatment for papular dermatitis caused by *Leishmania*.

Which patients are eligible to participate in this study?

Dogs with leishmaniosis, with papular dermatitis as the only clinical sign (stage I).

Voluntary participation:

Your dog's participation in this research is completely voluntary on your part.

What will happen if I decide to enroll my dog in this study?

The dogs will undergo some diagnostic tests to confirm that they are infected by *Leishmania*. Your dog will undergo a series of diagnostic tests, including blood, urine, lymph node, and skin tests. These procedures are not painful and can be performed without sedation or anesthesia. These tests are necessary for diagnosing

leishmaniosis and, in most cases, would need to be conducted even if you decide not to enroll your dog in the study.

Once in the study, your dog will be assigned to either the topical treatment group or the control group. Papular dermatitis is a sign of mild leishmaniosis that generally does not require treatment and tends to resolve on its own within a couple of months. Therefore, if your dog is assigned to the control group, it does not mean that it will affect their health or delay the resolution of the skin lesions.

It is essential to note that as the owner of the dog, you can withdraw your dog from the study at any time without any negative impact on your dog. If you observe any alterations in the treated area of the animal after withdrawing from the study, the animal will be attended to at the same center without any additional cost.

Consent Certificate

Animal Name: _____ **Sex:** _____
M(male)/F(female)

Age: _____ **Breed:** _____
(Weeks/Months/Years)

I hereby certify that I am the owner (or custodian) of the animal whose name is indicated above and that I am of legal age.

Furthermore,

I understand that the veterinarians of this institution are dedicated to researching the nature of canine leishmaniosis.

Veterinarian Dr. _____ has explained the study's characteristics to me, and I have carefully read the above information.

I have had the opportunity to ask questions and clarify points, and I have received specific answers to those questions.

I give my permission to publish the data obtained from this study for the benefit of the scientific community. I understand that my dog cannot be individually identified.

Therefore, I voluntarily consent to my dog's participation in this research.

Signed _____ Date _____

Owner or Custodian

Witnessed by: _____ Date _____

ANIMAL OWNER INFORMED CONSENT

Name of animal: ……. Identification (Chip): ……

- I, the undersigned ..... (name and surname) certify that I am the owner of the above-mentioned animal and that I am of legal age.

- I understand that I should not discuss aspects of the study “research project (register number 008/EPA-2383ESP)” with people outside the study.

- I have been duly informed by my veterinarian Dr. .....(Name and surname) about the objective of the study, the inclusion/exclusion/withdrawal criteria, the treatments to be followed, my obligations in the development of the study, possible adverse effects, waiting time and all relevant aspects that may affect my animal. I have had the opportunity to ask questions about the study and have received the answers.

- I agree to follow the indications received regarding my participation in the study. I have been informed of the possibility of withdrawing the animal at any time during the study.

- I declare that my animal is not on any type of medication at the time of inclusion and I have reported all medication received during the last 90 days.

- I understand that I must not administer any medication to the animal during the study except as indicated for the study.

- I agree to inform my veterinarian immediately if my animal shows signs of illness or if any adverse event occurs.

- I give my consent for the realization of the above-mentioned study promoted by Ecuphar Veterinaria S.L.U. in the animal of my property.

- I consent to the collection, processing, disclosure and transfer of my personal data for the study's administrative purposes or regulatory requirements.

- I give my permission to publish the data obtained from this study for the benefit of the scientific community. I understand that my dog cannot be individually identified.

- Therefore, I voluntarily consent to my dog's participation in this research.

Owner’s signature _____ Date: ___ / ___ / _____

Veterinarian’s signature _____ Date: ___ / ___ / _____

Supplementary material 2

Individual clinical record sheet for the assessment of clinical safety

Local Adverse Effects

Pruritus:			
Swelling:	0	1	2
Alopecia:	0	1	2
Skin hardening:	0	1	2
Erythema:	0	1	2
Local pain:	0	1	2
Others (ulcers, abscess, necrosis, regional lymphadenomegally …):			

Systemic Adverse Effects

Fever:	0	1	2
Depression:	0	1	2
Antalgic posture:	0	1	2
Diarrhea:	YES	NO	
Vomiting:	YES	NO	
Anorexia:	YES	NO	
Generalized lymphadenomegally:	YES	NO	
Others (anaphylaxis, seizures, death…):			

Examination of the injection site:

- Local reaction or pain on palpation (normal = 0; mild inflammation without pain = 1; moderate inflammation with pain =2).
- Presence of swelling, alopecia, skin hardening, erythema using a 0 to 2 scale (0= absence; 1= moderate presence; 2= severe presence).
- Presence of pruritus following the visual analogue scale.

Systemic reactions:

- Rectal temperature: normal (38 -39ºC) = 0; 39º - 39.5ºC = 1; >39.5º = 2.
- Pain or discomfort behaviour: normal = 0; hyporexia/anorexia = 1; antalgic posture = 2.
- Depression using a 0-2 scale (0= absence; 1= moderate presence; 2= severe presence).
- Presence or absence of vomiting, diarrhea and generalized lymphadenomegally.

During two hours after injection, the dogs will be observed by more than one person to detect possible anaphylactic reactions.

Corrective measures to prevent and manage possible adverse events after the vaccination:

- Total values of 0 are considered normal.
- With a total value of 1, frequency of monitorization is increased to 2 examinations per day, no additional treatment is applied.
- Total values of 2 during more than one day: meloxicam is administrated subcutaneously at 0.2 mg/kg (5mg/ml) SID at most 3 days.
- If an anaphylactic reaction is detected at the inoculation time or after, dexamethasone will be administrated intramuscularly at 0.1mg/kg (2mg/ml).
- If an anaphylactic shock is detected at the inoculation time or after, epinephrine will be administrated intramuscularly at 0.01mg/kg (1mg/ml) and dexamethasone will be administrated intramuscularly at 0.1mg/kg (2mg/ml).

Published papers

pathogens

MDPI

Article

Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis due to *Leishmania infantum* in Dogs

Icíar Martínez-Flórez [1], Maria Jose Guerrero [2], Annabel Dalmau [3], Maria Cabré [1,4], Maria Magdalena Alcover [5], Diana Berenguer [5], Liam Good [6], Roser Fisa [5], Cristina Riera [5], Laura Ordeix [1,4] and Laia Solano-Gallego [1,*]

1 Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain; imflorez.vet@gmail.com (I.M.-F.); mariacabregil@gmail.com (M.C.); laura.ordeix@uab.cat (L.O.)
2 Hospital Veterinario Alhaurín el Grande, 29120 Alhaurín el Grande, Spain; marjoxgr@gmail.com
3 AniCura Mediterrani Hospital Veterinari, 43204 Reus, Spain; annabeldalmaulopez@gmail.com
4 Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain
5 Departament de Biologia, Sanitat i Medi Ambient, Secció de Parasitologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, 08028 Barcelona, Spain; mmagdalenaalcoveramengual@ub.edu (M.M.A.); berenguer.diana@gmail.com (D.B.); rfisa@ub.edu (R.F.); mcriera@ub.edu (C.R.)
6 Department of Pathobiology and Population Sciences, The Royal Veterinary College, University of London, London NW1 0TU, UK; lgood@rvc.ac.uk
* Correspondence: laia.solano@uab.cat

check for updates

Citation: Martínez-Flórez, I.; Guerrero, M.J.; Dalmau, A.; Cabré, M.; Alcover, M.M.; Berenguer, D.; Good, L.; Fisa, R.; Riera, C.; Ordeix, L.; et al. Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis due to *Leishmania infantum* in Dogs. *Pathogens* **2023**, *12*, 821. https://doi.org/10.3390/pathogens12060821

Academic Editors: Nicola Carter, Maria Pereira and Teresa Letra Mateus

Received: 28 March 2023
Revised: 11 May 2023
Accepted: 8 June 2023
Published: 10 June 2023

Abstract: Papular dermatitis is a cutaneous manifestation of canine *Leishmania infantum* infection associated with mild disease. Although it is a typical presentation, nowadays, there is still no established treatment. This study evaluated the safety and clinical efficacy of local meglumine antimoniate, locally administered polyhexamethylene biguanide (PHMB) alone or PHMB in combination with a Toll-like receptor 4 agonist (TLR4a) for the treatment of papular dermatitis due to *L. infantum* and assessed parasitological and immunological markers in this disease. Twenty-eight dogs with papular dermatitis were divided randomly into four different groups; three of them were considered treatment groups: PHMB (n = 5), PHMB + TLR4a (n = 4), and meglumine antimoniate (n = 10)), and the remaining were considered the placebo group (n = 9), which was further subdivided into two sub-groups: diluent (n = 5) and TLR4a (n = 4). Dogs were treated locally every 12 h for four weeks. Compared to placebo, local administration of PHMB (alone or with TLR4a) showed a higher tendency towards resolution of papular dermatitis due to *L. infantum* infection at day 15 (χ^2 = 5.78; df = 2, p = 0.06) and day 30 (χ^2 = 4.; df = 2, p = 0.12), while local meglumine antimoniate administration demonstrated the fastest clinical resolution after 15 (χ^2 = 12.58; df = 2, p = 0.002) and 30 days post-treatment (χ^2 = 9.47; df = 2, p = 0.009). Meglumine antimoniate showed a higher tendency towards resolution at day 30 when compared with PHMB (alone or with TLR4a) (χ^2 = 4.74; df = 2, p = 0.09). In conclusion, the local administration of meglumine antimoniate appears to be safe and clinically efficient for the treatment of canine papular dermatitis due to *L. infantum* infection.

Keywords: canine; cutaneous lesions; immunotherapy; leishmaniosis; local treatment; toll-like agonist

1. Introduction

Canine leishmaniosis (CanL) is a vector-borne zoonotic disease with a worldwide distribution caused by the protozoan parasite *Leishmania infantum*. It is considered an endemic disease in the Mediterranean basin, Portugal, Latin America, and southern Asia [1]. Female phlebotomine sand flies are the biological vector, and dogs are considered the main reservoir [2].

Canine leishmaniosis is manifested by a broad spectrum of clinical signs and a wide range of disease severity that is classified into four stages: mild (stage I), moderate (stage II), severe (stage III), or very severe disease (stage IV) based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies [1]. Cutaneous manifestations are the most common clinical signs, and among them, papular dermatitis is considered a typical presentation in endemic areas and is associated with Leishvet stage I-mild leishmaniosis [1,3]. This cutaneous form is associated with an effective Th1 predominant parasite-specific cellular immunity and low humoral immune response, and thus, it is characterized by the absence of laboratory abnormalities and lack of systemic clinical signs. Self-healing of the skin lesions may occur at some point; however, it usually takes three to six months [4].

Clinical signs and outcomes of CanL depend on the interactions between the parasite and the host's innate and adaptive immune responses [5–7]. The adaptive response characterized by T-helper 1 (Th1) activation produces cytokines such as interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α), which help to control the infection. In contrast, the immune response mediated by Th2 induces the production of anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β) and is associated with disease progression [7,8]. Interleukin-17a (IL-17a) produced by several cells such as Th17, natural killer cells (NK), and macrophages, among others [9], plays a role in blocking parasite growth by the activation of inducible nitric oxide synthase (iNOS), among others [10,11]. Therefore, IL-17a is associated with resistance against *L. infantum* infection or disease in humans [10] and dogs [12], acting synergistically with IFN-γ.

Regarding the Th1 response, disease control requires a balanced interaction between a proinflammatory immune response mediated by T helper 1 type (Th1) CD4+ T cells, with the aim of controlling parasite replication, and a regulatory immune response mediated by T regulatory 1 cells, which are necessary to avoid a self-damaging overactivation of the immune system [13]. B cells also play a key role in the progression of CanL. They induce a humoral immune response and act as antigen-presenting cells modulating the activation of CD4+ T cells, which in turn activate B cells to produce antibodies [13]. As the disease progresses, the production of specific and non-specific IgG antibodies increases, reflected as hypergammaglobulinemia, and binds to *Leishmania* antigen creating immune complexes, which may contribute to disease progression [14–16].

One of the factors that regulate the activation of Th1 or Th2 immunity is pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which recognize molecules found in the promastigote that act as pathogen-associated molecular patterns (PAMPs) [17]. TLRs are located on the membrane or intracellular compartments of different types of cells, including T and B lymphocytes, macrophages, dendritic cells (DCs), and NK [18]. After binding their ligand, TLRs trigger the activation of the immune activity through diverse events such as the induction of inflammatory cytokines such as TNF-α and IFN-γ [19–22].

The innate immune response has an essential role in avoiding *Leishmania* survival. Cells such as neutrophils, macrophages, and DCs can kill the parasite via phagocytosis or facilitate their elimination by producing cytokines [7]. Neutrophils are activated following *L. infantum* inoculation and initiate several defense mechanisms, such as the production of reactive oxygen species (ROS) [23]. The nitroblue tetrazolium (NBT) reduction test is used to detect activated neutrophils in peripheral blood. NBT (soluble and colorless) is transformed into formazan (insoluble and blue-grey) in activated neutrophils, shown to be directly related to ROS production [24,25].

Treatment regarding Leishvet stage I has been scientifically neglected. Short-term treatment with one or two conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine and/or allopurinol) in dogs classified as Leishvet stage I has been described, as well as immune-potentiating treatments (domperidone or nucleotides plus- active hexose correlated compound (AHCC)) alone or in combination with the previously mentioned drugs [1,26]. Alternatively, monitoring without treatment can also be considered in some

cases. There is limited evidence for treatment outcomes for dogs in this stage, and therefore, the clinical efficacy of these treatment options remains unknown [27].

Similarly, there is no universally applicable treatment for human cutaneous leishmaniasis (CL) [28]. Local treatment for CL in humans has been recommended as an alternative treatment to systemic therapy by the World Health Organization and the Pan American Health Organization in patients with at least four lesions smaller than 4 cm in diameter, especially if the face and joints are not affected [29]. Local treatments are described as easy to use and with a lower risk, toxicity, and cost compared to traditional therapies, and they include intralesional antimonial injections, cryotherapy (with liquid nitrogen), thermotherapy (use of localized current field radiofrequency heat), and topical formulations such as paromomycin ointment [28,30–32]. However, local treatment has not been investigated in stage I-papular dermatitis due to canine *L. infantum* infection.

Polyhexamethylene biguanide (PHMB) is a synthetic cationic polymer with antimicrobial activity commonly used as a first-line treatment for locally infected wounds [33]. Furthermore, PHMB has been reported to have antileishmanial activity by inducing disruption of the parasite´s membrane integrity and causing chromosomal damage [34]. On the other hand, TLR4 activation induces a protective immune response against *Leishmania*, and will also regulate iNOS leading to the death of the parasite [22]. PHMB alone or in combination with a TLR4 agonist (TLR4a) has been reported to induce lower percentages of *Leishmania* infection in canine DH-82 cells in vitro [22].

The main aim of this randomized, controlled, double-blinded study was to evaluate the safety and clinical efficacy of locally administered PHMB alone or in combination with TLR4a and the local administration of meglumine antimoniate in the treatment of papular dermatitis due to *L. infantum* infection in dogs. The secondary objectives were to assess parasite-specific humoral and cellular immunity as well as parasitemia and to evaluate neutrophil function based on NBT at the time of diagnosis and during the follow-up period.

2. Materials and Methods

2.1. Dogs and Study Design

This study was designed as a multicentric randomized double-blinded controlled study with a follow-up of one year and involving five veterinary centers from Spain: Hospital Veterinario Alhaurín El Grande (Málaga), Hospital Mediterrani Veterinaris (Reus, Tarragona), Clínica veterinaria Paws Patas (Mula, Murcia), Hospital Veterinari Canis (Palma, Mallorca), and Fundació Hospital Clínic Veterinari (UAB, Bellaterra, Barcelona).

Dogs visited at the mentioned centers from 2019 to 2022 were considered for inclusion in this study. Inclusion criteria were a diagnosis of mild leishmaniosis (LeishVet stage I) characterized by the absence of laboratory abnormalities, negative or low antibody levels, and papular dermatitis as the only clinical sign at the time of diagnosis [1]. Withdrawal criteria were the development of systemic disease based on clinical signs and clinicopathological abnormalities, treatment with conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine, or allopurinol), or highly increased antibody levels. A signed informed consent was obtained from all dog owners. Ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (CEAAH 4526, November 2018) and by "Generalitat de Catalunya" (FUE-2018-00944112 i ID KSHYD6LVR, April 2019).

A complete physical examination, complete blood count (CBC), and biochemistry panel, including at least creatinine, urea, total proteins (TP), and alanine transaminase (ALT), were performed in all dogs to assess the clinical status. In some dogs, serum electrophoresis and urinalysis with urinary protein/creatinine ratio were also performed. Cutaneous lesions were photographed, borders around each lesion were measured to document size, and a cytology examination was performed from the lesions to diagnose *L. infantum* infection.

2.2. Treatment and Follow-Up

Five different products were manufactured as follows: PHMB alone (3 mg/mL PHMB in 30% ethanol), a combination of PHMB and TLR4a (1.5 µg/mL TLR4 agonist + 3 mg/mL PHMB in 30% ethanol), TLR4a alone (1.5 µg/mL TLR4 agonist in 30% ethanol), meglumine antimoniate (30% meglumine antimoniate + 70% pluronic F-127), and diluent (30% ethanol in nuclease-free water).

PHMB, PHMB + TLR4a, TLR4a, and diluent (30% ethanol in nuclease-free water) were formulated as liquid spray formulations. It was strongly advised to gently invert 20 times the sprayer vial before every application and to keep it upright when spraying.

Meglumine antimoniate was a lotion spray formulation and was stored in the refrigerator. It was recommended to remove the remaining product over the patient's skin from the last application before a new administration [35].

Dogs were randomly divided into four groups: three of them were considered treatment groups, PHMB (n = 5), PHMB + TLR4a (n = 4), and meglumine antimoniate (n = 10), and the remaining was considered the placebo group (n = 9), which was further subdivided into two sub-groups (diluent (n = 5) and TLR4a (n = 4)). TLR4a showed no statistical differences when compared to a diluent, and therefore, both groups were considered as placebo. Each group was treated locally over the papules every 12 h for four weeks, and a physical exam and lesion follow-up were performed on days 15, 30, 60, 90, 180, and 365. Clinical efficacy was defined as partial or total resolution of the lesions at days 15 and 30, respectively (Figure 1). All the procedures performed on each dog at the time of diagnosis and during follow-up are described at Figure 2.

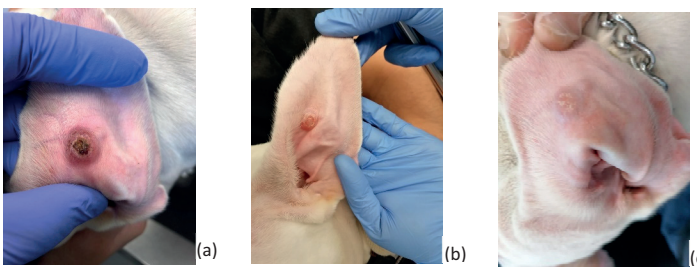


Figure 1. Clinical efficacy of local PHMB on papular dermatitis due to *L. infantum* at day 0 (**a**), day 15 (**b**) showing partial remission, and day 30 (**c**) full remission. Courtesy of Isaac Carrasco.

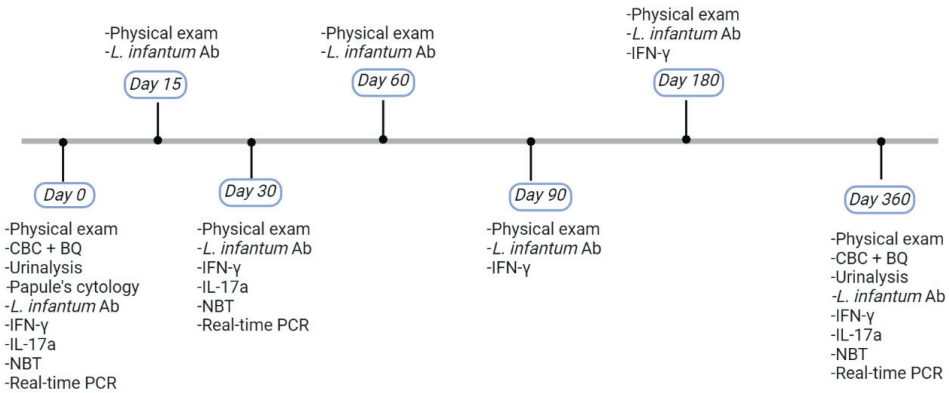


Figure 2. Timeline of the procedures performed on each dog at the time of diagnosis and during follow-up. Created with BioRender.com. CBC = complete blood count, BQ = biochemistry panel, Ab = antibody, IFN-γ = Interferon-gamma, IL-17a = Interleukin-17a, LSA = soluble *L. infantum* antigen, NBT = Nitroblue tetrazolium.

Persistent papules after sixty days from the beginning of the study or the appearance of new papules during the treatment were considered as a lack of response to treatment and relapse, respectively, and a rescue therapy (topical administration of 30% meglumine antimoniate + 70% pluronic F-127) was administered.

2.3. Safety Assessment

Drug safety assessment was performed based on a complete physical exam on days 15, 30, and 60 by veterinarians. Local side effects of the topical treatments at the application site, such as erythema and swelling, were recorded and graded (0–4) (Supplementary Material Table S1). Local pruritus and pain were also recorded. Systemic signs such as lymphadenomegaly or increased temperature, among others, were also registered. This information was recorded in the data collection form of both the veterinarian and the owner or caregiver of the dog (Supplementary Material Table S1). Furthermore, photographs of the application site were also reviewed by the first author to check for local reactions on days 15, 30, and 60.

2.4. ELISA for specific L. infantum Antibody Detection

Leishmania infantum antibody levels were measured by quantitative serology using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA) at the time of diagnosis and repeated on days 15, 30, 60, 90, 180, and 365 [5].

The in-house ELISA was performed on the sera of all dogs as previously described [36]. Briefly, the samples were diluted to 1:800 in phosphate buffer solution (PBS) with Tween 20 and 1% dry milk and incubated at 37° for 1 h. Then, the plates were washed three times with PBS-Tween and once with PBS and incubated with Protein A conjugated to horseradish peroxidase (Peroxidase Conjugate Protein A; Merck KGaA, Darmstadt, Germany) at 1:30,000 dilution for 1 h at 37 °C. After incubation, the plates were washed again, as described before. Then, o-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA, Darmstadt, Germany) was added to the plates, and the reaction was finally stopped with 5 M H2SO4. Absorbance values were read at 492 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd., Shenzhen, China), and the results were quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and set at 100 EU.

The cut-off of the serum in-house ELISA was already determined to be 35 EU using the ELISA results of 80 dogs from a non-endemic area, as previously described [37]. Cut-off was

established by the standard deviation (SD) method, consisting of multiplying the SD of the results by four and adding up the mean of the results obtained by the ELISA (mean + 4 SD). Serum was classified as high positive when the result was ≥300 EU, medium positive when the result was ≥150 EU and <300 EU, low positive when the result was ≥35 EU and <150 EU, and negative when the result was <35 EU [37]. All samples from each animal were analyzed on the same plate.

2.5. Cytokine Release Whole Blood Assay and Determination of Canine IFN-γ and IL-17a

IFN-γ concentration was measured at admission in all dogs and repeated at days 30, 90, 180, and 365, while IL-17a was performed at days 0 and 30.

A heparinized whole blood cytokine release assay was performed as described elsewhere [5]. Briefly, whole blood was separately incubated with three different conditions: (i) medium alone (unstimulated); (ii) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 μg/mL; and (iii) medium with the mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 μg/mL. Blood cultures were collected after five days at 37 °C in 5% of CO_2 and were centrifugated at 300 g for 10 min. Supernatants were collected and stored at −80 °C until tested. IFN-γ and IL-17a were determined in all samples by a commercial sandwich ELISA (DuoSet® ELISA by Development System R&DTM, Abingdon, UK) [5,6]. The standard curve for IFN-γ and IL-17a was calculated using a computer-generated four-parameter logistic curve fit with the program MyAssays (http://www.myassays.com/) [5].

Dogs were classified as IFN-γ producers when *L. infantum*-specific IFN-γ concentration was ≥110 pg/mL after subtracting the medium alone [38]. In the case of IL-17a, dogs were classified as producers when *L. infantum*-specific IL-17a concentration was ≥62.5 pg/mL after subtracting the medium alone [5].

2.6. Nitroblue Tetrazolium Reduction Test

Hematocrit capillary microtubes were filled with blood samples collected in EDTA tubes from all dogs and centrifugated at 2910 g for 5 min. Then, the buffy coat from each microtube was placed in an Eppendorf tube with the same volume of 0.1% NBT solution (1:1) (N6876, Sigma–Aldrich Co., St. Louis, MO, USA). The Eppendorf tube was mildly agitated and incubated for 15 min at 37 °C and then for another 15 min at room temperature. Two blood smears from each Eppendorf tube were obtained by placing 3 μL of NBT-stained samples on each slide. The slides were stained with Diff-Quick, and the NBT reduction rate was assessed by light microscopy after counting 300 neutrophils. The percentage was calculated as the number of activated neutrophils, defined by those containing blue-black formazan deposits, divided by the total number of neutrophils counted, multiplying the result by 100.

2.7. Blood DNA Extraction and Leishmania Real-Time PCR

Real-time PCR was carried out from blood samples at day 0, day 30, and 6 or 12 months.

DNA was isolated from blood samples collected in EDTA tubes using MagMax CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) using an automated system (KingFisher Flex Purification System, Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions for a simple workflow with whole blood samples. Briefly, 10 μL of proteinase K solution and 20 μL of magnetic beads were added to 100 μL of each sample. DNA was extracted by the robot after adding 700 μL of a mix of the binding and lysis solution in all the samples [36].

Leishmania real-time PCR was performed as previously described [39]. Each DNA sequence amplification for PCR was performed in triplicate. The mix reaction was prepared with 1x iTaq supermix with Rox (Bio-Rad), 0.2 μmoL of direct primer (5′-CTT TTC TGG TCC TCC GGG TAGG-3′), 0.2 μmoL of reverse primer (5′-CCA CCC GGC CCT ATT TTA CAC CAA -3′), 20 nmol of the labeled TaqMan probe (FAM- TTT TCG CAG AAC GCC CCT ACC CGC TAMRA), 0.5 μL of H20, and 2.5 μL of sample DNA. Positive and negative controls

were also included in each plate. A 10-fold dilution series of standard DNA from *L. infantum* promastigotes (CATB101) was used as a calibrator (serial dilution from 10^5 parasites/mL to 10^{-3} parasites/mL), allowing for the plotting of a standard curve. Cycling was performed using the QuantStudio™ 7 Pro (Thermo Fisher Scientific, Foster City, CA, USA) at 95 °C/55 °C for 45 cycles. The PCR was considered positive for *Leishmania* when the quantification cycle (Cq) was <40, and the amplification was detected in all the replicates.

2.8. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 8.0.1 for Windows software (GraphPad Software, San Diego, CA, USA). First, different normality tests were performed to know if the different variables (age, ELISA results, real-time PCR, LSA IFN-γ, ConA IFN-γ, LSA IL-17a, ConA IL-17a, and NBT rate) had a normal distribution at the time of diagnosis and follow-up. For each variable, the following tests were performed: Anderson–Darling, D'Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov. A p-value < 0.05 was considered statistically significant, meaning all variables did not follow a normal distribution except for the NBT rate. Therefore, non-parametric tests were used unless the comparison was between NBT rate groups, in which case, a parametric test was performed (paired t-test). A non-parametric Wilcoxon matched-pairs signed rank test was used for quantitative variables to compare two groups if the samples were paired. If samples were unpaired, a Mann–Whitney U-test was used. A Friedman test was used to compare more than two groups, and afterward, Dunn's multiple comparisons test was performed. The chi-square test or Fisher´s exact test was used to determine whether there was a significant association between two categorical variables. The Spearman Correlation Coefficient was used to evaluate differences in cytokine production of the dogs studied. Differences were considered significant, with a 5% significance level (p-value < 0.05).

3. Results

3.1. Dog Characteristics at the Time of Diagnosis

Twenty-eight dogs were included in this study (Figure 3) and were randomly divided into the aforementioned groups as follows: PHMB (n = 5), PHMB + TLR4a (n = 4), TLR4a-placebo (n = 4), meglumine antimoniate (n = 10), and diluent-placebo (n = 5). Unfortunately, complete blood samples and data were only obtained from 24, including 9 females and 15 males (37.5% and 62.5%, respectively).

Fifty-four percent of dogs were crossbred (n = 13); other represented breeds were Belgian Malinois (n = 4), German shepherd (n = 2), American bully (n = 1), Border collie (n = 1), Chihuahua (n = 1), Labrador retriever (n = 1), and Rottweiler (n = 1). Their ages ranged from 3 to 84 months, with a median of 6 months, with 91.7% being less than 12 months old and 45.8% less than 6 months old.

On physical examination, solitary (n = 1) or multiple (n = 23) erythematous non-pruritic papules were observed in non-haired skin areas (Table 1).

Table 1. Distribution of papules.

Site	Number of Lesions	%
Inner surface of the pinna	15	46.9
Abdomen	6	18.8
Eyelid	3	9.4
Lips	3	9.4
Foreskin	2	6.3
Elbow	1	3.1
Bridge of the nose	1	3.1
Nose	1	3.1

The onset of clinical lesions was mainly in autumn (70.8%), followed by winter (16.7%), spring (8.3%), and summer (4.2%) (Table 2).

Table 2. The onset of clinical lesions was analysed by season, sex, and median age.

	Total Number of Dogs	%	Median Age (Minimum-Maximum Months)
Autumn	17	70.8	6 (3–84)
Female	5	20.8	7 (3–84)
Male	12	50	6 (4–6)
Winter	4	16.7	6 (6–10)
Female	3	12.5	6 (6–10)
Male	1	4.2	5
Spring	2	8.3	3.5 (3–4)
Male	2	8.3	3.5 (3–4)
Summer	1	4.2	5
Female	1	4.2	5
Total	24	100	6

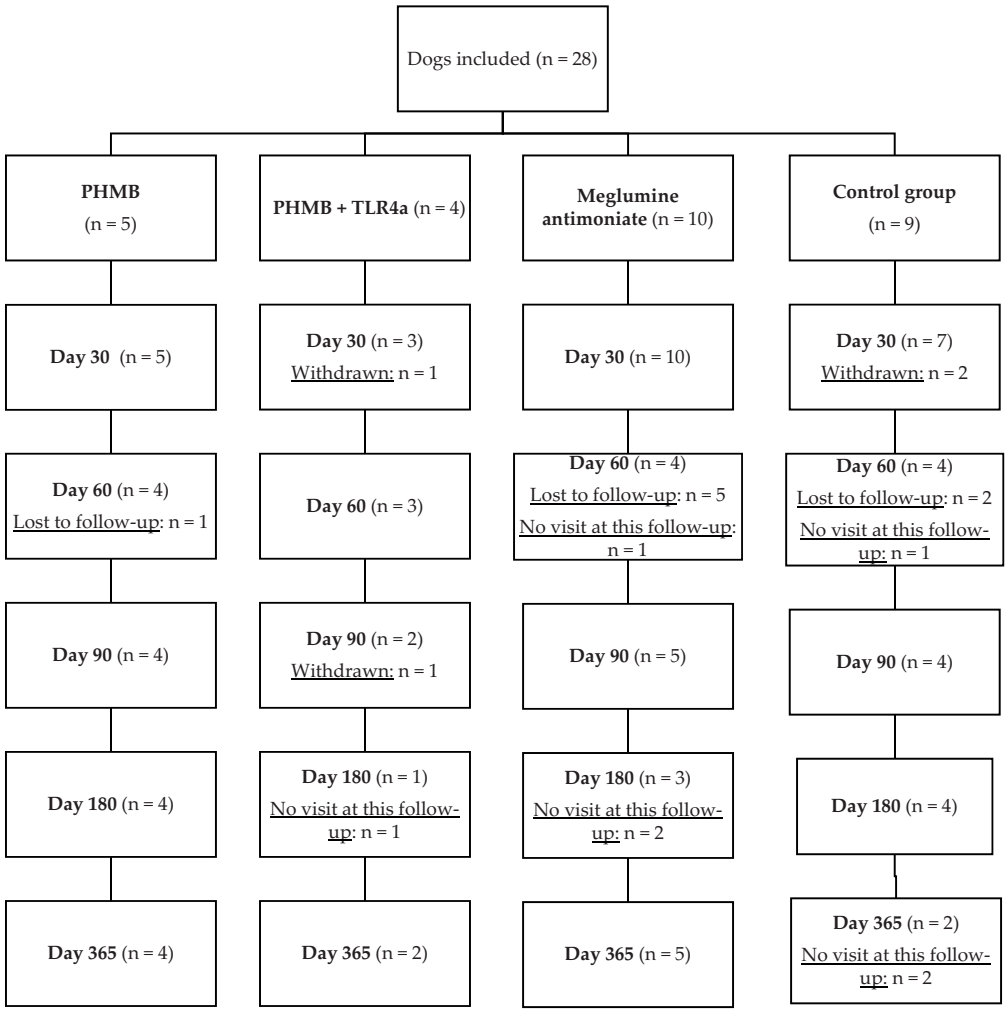


Figure 3. Flowchart displaying the number of dogs included at the time of diagnosis, treatment groups, as well as withdrawn dogs and lost to follow-up during the follow-up period. PHMB = polyhexamethylene biguanide, TLR4a = Toll-like receptor 4 agonist, Control group (n = 9): diluent (n = 5) + TLR4a (n = 4). Lost to follow-up = owners did not want to participate in the study anymore. No visit at this follow-up = owners did not show up at the Veterinary clinic that day. Withdrawn = dog was withdrawn of the study due to the development of systemic disease based on clinical signs and clinicopathological abnormalities, treatment with conventional anti-*Leishmania* drugs (meglumine antimoniate, miltefosine, or allopurinol), or highly increased antibody levels.

No other abnormalities were found on physical examination, except a mild lymphadenomegaly of regional lymph nodes in two dogs.

Most dogs showed no laboratory abnormalities except for one dog with a mild increase in TP and three dogs with a mild decrease in TP.

Clinical characteristics and laboratory tests according to different treatments at the time of diagnosis are displayed in Table 3.

Table 3. Clinical characteristics and laboratory tests at the time of diagnosis before starting local treatment.

Qualitative Characteristics		**PHMB + PHMB with TLR4a (n = 9)**	**Meglumine Antimoniate (n = 6/10) ***	**Control Group (n = 9)**	***p*-Value (Fisher's Exact Test)**
Breed	Crossbred	7 (78%)	3 (50%)	3 (33%)	0.162
	Purebred	2 (22%)	3 (50%)	6 (67%)	
Sex	Male	7 (78%)	2 (33%)	5 (56%)	0.227
	Female	2 (22%)	4 (67%)	4 (44%)	
ELISA at day 0	Low positive	3 (33%)	0 (0%)	3 (33%)	0.264
	Negative	6 (67%)	6 (100%)	6 (67%)	
Leishmania real-time PCR	Positive	5 (56%)	3 (50%)	5 (56%)	0.972
	Negative	4 (44%)	3 (50%)	4 (44%)	
IFN-γ	Producers	7 (78%)	3 (50%)	2 (22%)	0.1
	Non-prod.	2 (22%)	3 (50%)	7 (78%)	
IL-17a	Producers	8 (89%)	1 (17%)	1 (11%)	0.7
	Non-prod.	1 (11%)	5 (83%)	8 (89%)	
Quantitative Characteristics		**Median (min-max)**	**Median (min-max)**	**Median (min-max)**	***p*-Value (Kruskal-Wallis Test)**
Age (months)		6 (3–8)	5.5 (4–84)	6 (3–36)	0.875
IFN-γ (pg/mL)		768.6 (11.6–3997.5)	140.4 (0–762.4)	23.6 (0–309)	0.011 **
IL-17a (pg/mL)		151.1 (0–1933.3)	11.9 (0.148)	0 (0–71.5)	0.005 **
ELISA Units		26 (7.1–227.1)	5.7 (2.8–73.5)	22.3 (4.8–100.9)	0.11
Quantitative Characteristics		**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	***p*-Value (Ordinary One-Way ANOVA)**
Cq PCR		35.3 ± 1.9	35 ± 1.4	33.9 ± 2.7	0.58

PHMB = polyhexamethylene biguanide, TLR4a = Toll-like receptor 4 agonist, IFN-γ = Interferon-gamma, IL-17a = Interleukin-17a, Cq = Quantification cycle, Non-prod= non-producers. * Complete data were only obtained from 6 out of 10 dogs in this group. ** Significant difference was observed between PHMB + PHMB with TLR4a and control groups but not between PHMB + PHMB with TLR4 and meglumine antimoniate groups and between meglumine antimoniate and control groups.

3.2. Safety and Clinical Efficacy of Treatments

All treatments studied were considered safe. No side effects (local or systemic) were observed during the treatment period in any formulations studied.

Regarding the PHMB formulations, there were no statistically significant differences between the PHMB alone group and the PHMB with TLR4a at day 15 (chi-square: $\chi^2 = 0.9$; df = 2, $p = 0.64$) and day 30 (chi-square: $\chi^2 = 3.6$; df = 2, $p = 0.17$). On the other hand, although differences between local administration of PHMB alone or in combination with TLR4a when compared to the placebo administration were not statistically significant, and thus it is unclear if this would reflect on a large canine population, the results suggested a higher tendency towards resolution (both partial and total) at day 15 (chi-square: $\chi^2 = 5.78$; df = 2, $p = 0.06$) and day 30 post-treatment (chi-square: $\chi^2 = 4.$; df = 2, $p = 0.12$). Conversely, dogs treated with local administration of meglumine antimoniate had a statistically significant

fastest resolution compared with placebo treatment at 15 (chi-square: $\chi^2 = 12.58$; df = 2, $p = 0.002$) and 30 days post-treatment (chi-square: $\chi^2 = 9.47$; df = 2, $p = 0.009$). Although no statistically significant differences were observed between the administration of meglumine antimoniate and the administration of PHMB alone or in combination with TLR4a at 15 days post-treatment (chi-square: $\chi^2 = 2.57$; df = 2, $p = 0.27$), there was a higher tendency towards resolution at 30 days post-treatment (chi-square: $\chi^2 = 4.74$; df = 2, $p = 0.09$) when meglumine antimoniate was administered (Table 4).

Table 4. Clinical resolution of papules according to the different treatments (PHMB alone, PHMB in combination with TLR4a, and meglumine antimoniate).

Local Treatment (Number of Dogs)	Clinical Resolution (Number of Dogs/Total, %)					
	15 Days Post-Treatment			30 Days Post-Treatment		
	Without Resolution	Partial	Total	Without Resolution	Partial	Total
PHMB alone (n = 5)	1/5, 20%	3/5, 60%	1/5, 20%	1/5, 20%	2/5, 40%	2/5, 40%
PHMB+ TLR4a (n = 4)	1/4, 25%	3/4, 75%	0/4, 0%	0/4, 0%	4/4, 100%	0/4, 0%
Meglumine antimoniate (n = 10)	0/10, 0%	8/10, 80%	2/10, 20%	0/10, 0%	3/10, 30%	7/10, 70%
Control group (n = 9): diluent (n = 5) + TLR4a (n = 4)	7/9, 78%	2/9, 22%	0/9, 0%	5/9, 56%	3/9, 33%	1/9, 11%
Total (n = 28)	9	16	3	6	12	10

PHMB = polyhexamethylene biguanide, TLR4a = Toll-like receptor 4 agonist.

Regarding the dogs included in the control group (n = 9), some showed a total resolution of the papules at days 30 (n = 1/9), 90 (n = 2/9), and 180 (n = 1/9), two were excluded from the study at day 30 because of clinical worsening, and they were treated with meglumine antimoniate subcutaneously (2/9). The other three dogs (3/9) showed a partial resolution at day 60, but they were lost to follow-up (Figure 3).

3.3. Clinical Worsening (Withdrawal) and Lost to Follow-Up

Some dogs were lost to follow-up or withdrawn from the study for several reasons (Figure 3). In four (two from the control group and two from the PHMB + TLR4a group), other clinical signs such as loss of body weight, generalized lymphadenomegaly, and decreased appetite were observed during the follow-up, and *L. infantum*-specific antibody levels increased. They were treated with meglumine antimoniate subcutaneously and withdrawn from the study from that point.

Samples from several dogs could not be collected during the lockdown caused by SARS-CoV-2, but they were collected again in further follow-ups (Figure 3).

In one dog treated with PHMB, initially, clinical resolution of papules was observed, although new papules appeared during the treatment. This was considered a relapse, and a local rescue therapy (30% meglumine antimoniate + 70% pluronic F-127) was instituted. In this case, papules were all resolved on day 15 after the rescue therapy was started.

3.4. Leishmania Infantum-Specific Antibody Levels at the Time of Diagnosis and Follow-Up

The results of *L. infantum*-specific antibody levels at the time of diagnosis and during treatment follow-up are displayed in Table 5.

Table 5. Serological, molecular, IFN-γ and IL-17a concentrations and NBT results of dogs at diagnosis and follow-up.

	Number of Seropositive Dogs (%)	Median ELISA Units (min-max)	Number of LSA IFN-γ Producers (%)	Median LSA IFN-γ (pg/mL) (min-max)	Number of LSA IL-17a Producers (%)	Median LSA IL-17a (pg/mL) (min-max)	PCR Positives (%)	Mean Cq ± SD	Mean NBT Rate ± SD (%)
Day 0	6/24 (25%)	21.4 (2.8–227.1)	12/24 (50%)	127.9 (0–3998)	10/24 (42%)	23.2 (0–1933)	13 (54.2)	34.7 ± 2.1	18 ± 6%
Day 15	8/23 (34.8%)	17.8 (3.5–167.4)	-	-	-	-			-
Day 30	6/20 (30%)	13.8 (2.9–121.6)	9/20 (45%)	61.7 (0–3614)	11/20 (55%)	85.2 (0–630.3)	4 (20)	37.1 ± 0.5	19± 5%
Day 60	4/14 (28.6%)	13.3 (3.8–115.5)	-	-	-	-			-
Day 90	1/15 (6.7%)	12.4 (2.8–161.6)	9/12 (75%)	444.1 (4.6–6002.9)					
Day 180	0/13 (0%)	9.58 (3–26)	5/11 (45.5%)	41.6 (0–2660)					
Day 365	1/13 (8%)	16.3 (2.4–213.7)	5/12 (41.7%)	1250.2 (0–6947.7)			12 (92.3)	34.7 ± 1.2	

Cq = Quantification cycle, IFN-γ = Interferon-gamma, IL-17a = Interleukin-17a, LSA = soluble *L. infantum* antigen, NBT = Nitroblue tetrazolium, min = minimum, max = maximum.

At the time of diagnosis, all dogs presented negative or low antibody levels except one dog (n = 24, median: 21.4, ranging from 2.8 to 227.1 EU). At day 0, six dogs were positive against *L. infantum* antigen, five of which were low positive, and one was medium positive. The rest of the dogs (n = 18) were seronegative. At day 15 (n = 23, median: 17.8, ranging from 3.5 to 167.4 EU), seven dogs were low positive, one dog was medium positive, and the rest were seronegative. At day 30 (n = 20, median: 13.8, ranging from 2.9 to 121.6 EU), six dogs were low positive, and the rest were seronegative. At day 60 (n = 14, median: 13.3, ranging from 3.8 to 115.5 EU), four dogs were low positive, and the rest were seronegative. At day 90 (n = 15, median: 12.4, ranging from 2.8 to 161.6 EU), only one dog was medium positive, and the rest were seronegative. At day 180 (n = 13, median: 3, ranging from 2.8 to 26 EU), all dogs were seronegative. At day 365 (n = 13, median: 16.3, ranging from 2.4 to 213.7 EU), one dog was medium positive, and the rest were seronegative; this dog had not been tested at day 180 due to lack of compliance of the owner.

No significant differences were observed when comparing *L. infantum* antibody levels at day 0 with the follow-up at any time ($p = 0.98$). Regarding the follow-up, only in 10 dogs (41.7%) was it possible to complete it as planned (days 0, 15, 30, 60, 90, 180, and 365). At days 180 and 365, thirteen dogs out of a total of 24 (54.2%) were tested.

Twelve dogs remained seronegative, while only one dog remained seropositive during all the follow-ups. Interestingly, four dogs experimented with a seroreversion since the antibody levels changed from low positive to negative. In contrast, no dogs showed seroconversion during the follow-up period. One dog treated with PHMB + TLR4a changed from low positive to medium positive.

3.5. IFN-γ Concentration at the Time of Diagnosis and During Treatment Follow-Up

The results of IFN-γ concentration at the time of diagnosis and during treatment follow-up are displayed in Table 5.

At day 0, 12 of 24 dogs were considered IFN-γ producers. Supernatants from LSA-stimulated whole blood of IFN-γ producer dogs presented significantly higher concentrations of IFN-γ (median: 607.8, ranging from 171.9 to 3997.5 pg/mL) when compared with IFN-γ non-producers (median: 10.6, ranging from 0 to 84 pg/mL) ($p < 0.0001$) (Figure 4a). However, the concentration of IFN-γ of ConA stimulated blood from IFN-γ non-producers (median: 1287, ranging from 153.8 to 4254.2 pg/mL) did not show statistically significant differences ($p = 0.442$) with the IFN-γ producers' group (median: 1287.5, ranging from 459.3 to 5959.9 pg/mL).

At day 30, 9 of the 20 dogs analyzed were classified as IFN-γ producers. Supernatants from LSA-stimulated whole blood of IFN-γ producer dogs showed higher concentrations of IFN-γ (median: 668.9, ranging from 233.9 to 3614 pg/mL) when compared with the IFN-γ non-producers' group (median: 23.6, ranging from 0 to 62.7 pg/mL) ($p = 0.0017$). Regarding the concentration of IFN-γ of ConA stimulated blood from IFN-γ producers (median: 2503.7, ranging from 357.3 to 5073.2 pg/mL), there were no significant differences ($p = 0.14$) with the IFN-γ non-producers (median: 1295.6, ranging from 293.8 to 3576 pg/mL).

Concerning the rest of the follow-up, 9 dogs out of a total of 12 were considered IFN-γ producers at day 90, 5 out of 11 dogs were producers at day 180, while 5 dogs out of 12 remained IFN-γ producers at day 365.

Additionally, no statistically significant differences were observed when comparing IFN-γ concentrations in supernatants of blood stimulated with LSA from all dogs at day 0 and day 30 ($p = 0.35$) or any other time point during follow-up nor when comparing IFN-γ concentration after ConA stimulation at day 0 and day 30 ($p = 0.81$).

3.6. IL-17a Concentration at Time of Diagnosis and During Treatment Follow-Up

The results of IL-17a concentration at the time of diagnosis and during treatment follow-up are displayed in Table 5.

At diagnosis, 10 of 24 dogs were classified as IL-17a producers. Supernatants from LSA-stimulated whole blood of IL-17a producer dogs presented significantly higher concen-

trations of IL-17a (median: 149.5, ranging from 69.4 to 1933.3 pg/mL) when comparing with IL-17a non-producers (median: 0, ranging from 0 to 32.1 pg/mL) ($p < 0.0001$) (Figure 4b). However, concentrations of IL-17a of ConA stimulated blood from IL-17a non-producers (median: 6466.3, ranging from 721.3 to 23,050.2 pg/mL) did not show statistically significant differences ($p = 0.34$) with the IL-17a producers' group (median: 9228.7 ranging from 3363.5 to 18,143.3 pg/mL).

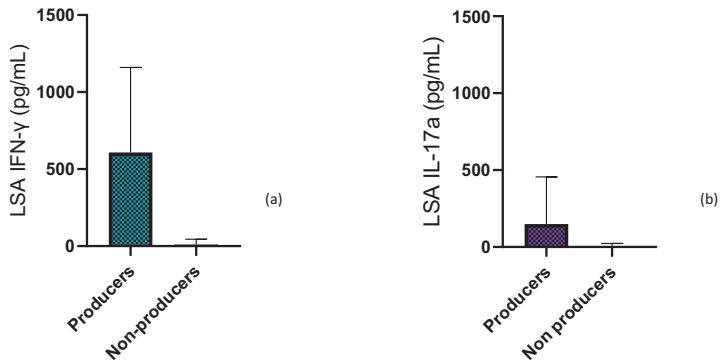


Figure 4. Median concentration with 95% CI (**a**) IFN-γ and (**b**) IL-17a concentration after LSA stimulation in cytokine producers and non-producers dogs at the time of diagnosis and prior to administration of local treatment.

On day 30, 11 dogs were considered IL-17a producers out of 20 dogs. IL-17a concentration after LSA stimulation of IL-17a producer dogs demonstrated higher concentrations of IL-17a (median: 155.7 ranging from 74 to 630.3 pg/mL) when comparing with the IL-17a non-producers' group (median: 2.8 ranging from 0 to 40 pg/mL) ($p < 0.0001$). IL-17a concentration after ConA stimulation was not statistically different ($p = 0.56$) between IL-17a producers (median: 14,088.6 ranging from 1082.1 to 41,390 pg/mL) and IL-17a non-producers (median: 12,425.3 ranging from 2637 to 31,150 pg/mL) dogs.

Furthermore, no statistically significant differences were observed when comparing IL-17a concentrations in supernatants of blood stimulated with LSA from all dogs at day 0 and day 30 ($p = 0.08$). However, significant differences were found when comparing IL-17a concentration after ConA stimulation at day 0 and day 30 ($p = 0.01$).

3.7. Correlation between Parameters Studied

Considering all dogs studied (n = 24), a correlation was not found between IFN-γ concentration after LSA stimulation and *L. infantum* specific antibody levels (Spearman r: 0.31, $p = 0.15$) neither between IL-17a after LSA stimulation and antibody levels (Spearman r: 0.25, $p = 0.24$). Moreover, there was no correlation between the age of the dogs and antibody levels (Spearman r: −0.28, $p = 0.18$).

No correlation was found between PCR Cq and *L. infantum* specific antibody levels (Spearman r: −0.07, $p = 0.8$) nor PCR Cq and IFN-γ concentration after LSA stimulation (Spearman r: 0.41, $p = 0.09$) nor between PCR Cq and IL-17a after LSA stimulation (Spearman r: 0.46, $p = 0.06$).

IFN-γ concentration after LSA stimulation was significantly positively correlated with IL-17a concentration after LSA stimulation (Spearman r: 0.59, $p < 0.0001$) (Figure 5).

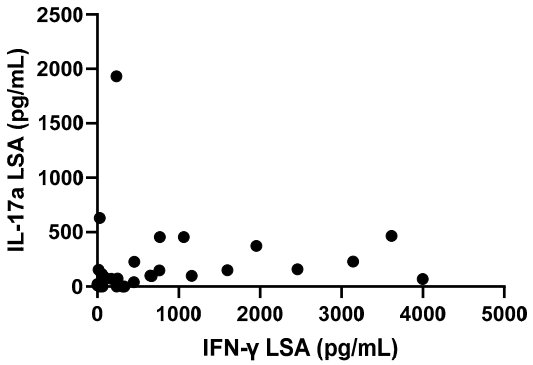


Figure 5. Correlation between IL-17a (pg/mL) and IFN-γ (pg/mL) after LSA stimulation in the total of dogs studied at the time of diagnosis and follow-up. Spearman r: 0.59, $p < 0.0001$.

Similarly, there was a significant correlation between IFN-γ concentration after Con A stimulation and IL-17a concentration after Con A stimulation (Spearman r: 0.54, $p = 0.0002$).

3.8. Nitroblue Tetrazolium Reduction Test

There were no statistically significant differences when comparing the NBT reduction rate at day 0 (mean ± SD: 18 ± 6%) with the NBT reduction rate at day 30 (mean ± SD: 19 ± 5%) ($p = 0.53$).

3.9. Blood PCR

The *Leishmania* real-time PCR results at the time of diagnosis and during the follow-up period are depicted in Table 5.

At the time of diagnosis, 13 out of a total of 24 (54.2%) dogs were PCR positive.

At day 30, only 4 dogs were PCR positive from a total of 20 (20%) dogs.

Interestingly, 2 dogs remained positive from day 0 to day 30. The other 2 negative dogs at day 0 were PCR positive at day 30. In contrast, 8 dogs that were PCR positive at day 0 were negative at day 30. Unfortunately, 4 dogs were lost to follow-up on day 30.

Regarding the last PCR performed from each dog, one was at day 60 and was positive, while it was negative at days 0 and 30.

On day 180, 3 dogs were tested, and all were PCR positive.

Finally, at day 365, 12 out of 13 dogs were PCR positive (92%). Two dogs remained always positive from day 0, only one remained negative from day 0, and the others changed depending on the time.

Regarding the proportion of positive dogs, significant differences were found when comparing day 0 to day 30 ($p = 0.03$), day 0 to day 365 ($p = 0.02$), and day 30 to day 365 ($p \leq 0.0001$). Statistically significant differences were observed when comparing PCR Cq results at day 0 (mean ± SD: 34.7 ± 2.1) and day 30 (mean ± SD: 37.1 ± 0.5) ($p = 0.04$), and when comparing PCR Cq results at day 30 and day 365 (mean ± SD: 34.7 ± 1.2) ($p = 0.0023$). However, no differences were observed when PCR Cq results at day 0 and day 365 were compared ($p = 0.93$).

4. Discussion

To the author's best knowledge, there are few studies evaluating the effectiveness of local treatment in canine cutaneous leishmaniosis (CCL), but no studies so far have evaluated local treatment of papular dermatitis due to *L. infantum* infection [40–42]. In the present randomized controlled study, 70% of dogs treated with meglumine antimoniate and 40% of dogs treated with PHMB alone showed a complete response after 30 days of

treatment, demonstrating a much faster response than the median of 98 ± 5 days observed in a previous observational study. However, it is difficult to compare these results as we only included dogs with stage I-mild disease-papular dermatitis, and in the previous study, all dogs had ulcerative lesions in which self-healing was not expected [1,40]. Moreover, no adverse effects were observed throughout the course of the study in any of the dogs. Therefore, we might conclude that all the studied substances were safe.

Although meglumine antimoniate is widely described as a systemic treatment for *L. infantum* infection both in humans and dogs [36], to the author's best knowledge, there is limited information regarding the efficacy of its topical use in dogs with either cutaneous leishmaniosis or papular dermatitis. Interestingly, a recent study demonstrated the efficacy of an intralesional meglumine antimoniate compared with a placebo for the treatment of CCL due to *L. braziliensis* [41]. Dogs with localized cutaneous ulcers were enrolled in this study and showed a higher and faster healing rate when they were treated with intralesional meglumine antimoniate [41]. In human medicine, meglumine antimoniate is commonly used as a local treatment through intralesional administration and is considered appropriate for the treatment of simple cutaneous lesions due to *L. panamensis* and *L. mexicana* [43]. Local application of meglumine antimoniate has also been tested in murine leishmaniosis models [44–47]. The main obstacle to the local administration of meglumine antimoniate is that it is considered a water-soluble molecule, and thus, it has limited interaction with lipophilic skin structures [29].

In murine models, different drug delivery systems have been evaluated. Moosavian Kalat et al. (2014) tested a liposome containing 22.5% meglumine antimoniate as a local treatment on infected BALB/c mice and observed a reduction in lesion size and a lower spleen parasite burden in treated mice compared to the control group [44]. The same group, in a further study (2019), tested a similar mixture by adding stearylamine, which improved liposome performance with good clinical improvement [45]. In another study published in 2013, meglumine antimoniate administered using a liposomal formulation showed a reduction in lesion size and amastigote counts, although it had no significant therapeutic difference compared to the control group [46]. Finally, Horoiwaone et al. (2020) tested the local application of meglumine antimoniate contained in maltodextrin polymeric colloidal nanocarriers in a murine leishmaniasis model, which showed similar efficacy to the intraperitoneal injection regarding parasite titer reduction and superior healing activity in terms of collagen area deposition [47]. Other delivery systems have also been tested for systemic administration of meglumine antimoniate, which could be considered for topical administration in future studies, including polymeric nanoformulations such as aqueous-core poly-L-lactide nanocapsules, which have shown a great antileishmanial activity on mice infected with *L. infantum* [48]. In addition, this new formulation promotes meglumine antimoniate uptake within the macrophages, increasing its efficacy and decreasing its cellular-negative outcome [48].

In the present study, the administration of meglumine antimoniate with pluronic F-127 as a delivery system was tested, which is a non-ionic detergent used to facilitate the solubilization of water-insoluble materials in physiological media. A significant improvement was observed in our patients when compared to the control group, and no adverse effects were detected during the length of administration nor the posterior follow-ups, which could mean that pluronic F-127 is a safe and adequate delivery system and that locally administered meglumine antimoniate could be considered as a viable treatment option in dogs with localized papular dermatitis due to *L. infantum.* This new local formulation of meglumine antimoniate has shown to be safe for its use in humans, and it has been previously tested in vitro in three cell lines, ex vivo using human skin samples, and in vivo testing on human volunteers [35].

Regarding PHMB, to the author's best knowledge, there are no previous in vivo studies reporting its use as a topical formulation for the treatment of papules due to *L. infantum* infection, neither in dogs nor in humans. On the other hand, it has been described in previous publications as a topical antimicrobial for locally infected wounds

in dogs [49,50], and there is a study about the safety of its use in combination with other drugs as an ear flush in dogs [51]. In humans, PHMB has been used as a first-line treatment for infected wounds [52,53] and as a treatment for *Acanthamoeba* keratitis [54].

As mentioned previously, in the present study, dogs treated with topical PHMB showed a higher tendency toward resolution compared with the control group, although the results were not statistically significant. PHMB has been shown to have antileishmanial properties, such as disruption of the parasite´s membrane integrity and chromosomal damage, in previous in vitro studies [22,34]. We also evaluated its combination with TLR4a, as according to a previous study, TLR4 activation leads to the parasite's death by inducing a protective immune response against *Leishmania* and regulating iNOS [22]. According to our results, no statistically significant differences were observed between the PHMB alone group and the PHMB combined with TLR4a. This lack of difference could be due to a lack of effectiveness regarding the TLR4a stimulation, insufficient dosage, or due to complications such as lack of absorption of the TLR4a. However, a small number of dogs were included in these groups, and thus a significant difference could have been observed if more dogs were included.

To the author's best knowledge, there are only two published studies in which a follow-up over dogs with papular dermatitis was performed [4,55]. In one of them, 15 out of 17 dogs were treated with subcutaneous meglumine antimoniate for 25–30 days, showing complete resolution of lesions by day 25 in all dogs [4]. The remaining dog received no treatment and was only revisited four months later, showing only partial remission [4]. In the other study, 3 out of 8 dogs were treated twice a day with meglumine antimoniate subcutaneously (50 mg/kg SC BID) and allopurinol orally (10 mg/kg PO BID), showing total resolution by 10 days of treatment [55]. The other four dogs received no treatment, showing improvement only after 3 to 5 months [55]. Similarly, in the present study, all dogs treated with topical meglumine antimoniate showed improvement one month after starting treatment, although only 70% had a complete resolution of lesions. However, taking into account the mean of administration (much less painful and stressful for the dog and the owner) and the lower risk of adverse effects, we can consider topical treatment as an advantageous alternative.

As mentioned previously, self-healing of the skin lesions may occur between three and six months in previous studies [4,55]. This fact is similar to the results of the present study, in which some dogs included in the control group showed a total resolution of the papules at days 90 (22%) and 180 (11%) or partial resolution at day 60 (11%), although one showed total resolution by day 30 (11%).

The immune response was also evaluated in this study. IFN-γ is one of the cytokines expressed by a Th1 response (considered an effective immune response). In the present study, 50% (12/24) of dogs at day 0 and 45% (9/20) at day 30 were considered IFN- γ producers. These findings are similar to previous studies using IFN-γ release whole blood assay in dogs with stage I and papular dermatitis. In one study [5], dogs in stage I (25.7%) and IIa (48.5%) showed a higher production of IFN-γ than other dogs classified as stage IIb (8.5%), III (14.2%), and IV (2.8%). In another study, 15 of 19 dogs in stage I (79%) were IFN-γ producers, whereas only 6 out of 15 dogs (40%) were in stage II-III [6]. Interestingly, in this study, the dogs that were followed up at different time points presented a production of parasite-specific IFN-γ that ranged from 41 to 75% of the dogs studied.

The results of IFN-γ concentration showed a positive correlation with IL-17a concentration after LSA and Con A stimulation. This correlation was expected regarding previous studies where it was demonstrated that IL-17a played a synergic role with IFN-γ by blocking parasite growth after checking IFN-γ and IL-17a levels in infected mice [10]. In another study, mRNA expression for iNOS and IFN- γ was positively correlated with IL-17a gene transcription in dogs [11]. However, another study concluded that SNPs located in analogous regions of canine IL-17a gene promoter did not show an association with *Leishmania* spp. resistance [56]. Further studies on the role played by IL-17a in *L. infantum* infection are required to determine if it might be used as a prognostic marker.

In addition, it is important to highlight that, similarly to previous studies, the majority of these dogs were seronegative or presented very low positive antibody levels throughout the study period, and four dogs even showed seroreversion [6]. Therefore, even if dogs were only treated with local treatment or no treatment, most of them did not have relapses or worsening of the infection. Regarding this, only one dog treated with PHMB alone showed relapse of papular dermatitis, another dog treated with PHMB + TLR4a demonstrated an increase in serology levels through treatment, and four dogs were withdrawn from the study due to worsening of clinical signs, two associated to the control group (2/4) and two to PHMB + TLR4a group (2/4). Conversely, no dogs treated with local meglumine antimoniate showed worsening or relapse of clinical signs throughout the study period, nor an increase in antibody levels, and the dog that had relapsed while PHMB was being administrated, did show complete resolution after changing to local meglumine antimoniate, demonstrating, in these cases, a good efficacy not only in the treatment of clinical signs but also to avoid relapses or worsening of the disease.

According to previous papers, in this study, there was no association between sex or age and NBT reduction rate [24,25]. In a previous study, the NBT reduction rate of 40 healthy dogs (4.57 ± 1.72%), 20 dogs in Stage-I (34 ± 10.05%), and 20 dogs in Stage-IV (3.7 ± 2.03%) was compared, showing that NBT reduction rate was significantly higher in dogs in Stage-I [24]. In another study, the NBT reduction rate was compared between ten healthy dogs treated with 0.5 mg/kg oral (PO) of domperidone once a day (SID) for 30 days and ten non-treated healthy dogs [25]. In this previous study, the results from the non-treated group showed similar means to the first study (5.9 ± 1.6%) [25]. According to these previous papers, our results of NBT reduction rate at day 0 (mean ± SD: 18 ± 6%) and day 30 (mean ± SD: 19 ± 5%) were closer to the Stage-I results [24,25].

Concerning real-time PCR, the results of the present study are in agreement with previous studies where dogs with mild leishmaniosis showed a lower proportion of positive cases than dogs in more advanced stages [5,6,57]. It is well known that sick dogs with high antibody levels also show high parasitemia levels [57]. It is worth noticing that a difference was observed between the proportion of PCR-positive dogs at day 0 (54.2%) and day 30 (20%) ($p = 0.03$), and when comparing dogs at day 30 and day 365 (92.3%) ($p = 0.0001$), showing a significantly lower result at day 30 and a significantly higher result at day 365. This could be interpreted as a signal of infection recrudescence. However, it was not associated with clinical or analytical worsening, and the parasitemia (PCR Cq) was the same at day 365 (34.7 ± 1.2) and at day 0 (34.7 ± 2.1), indicating a possible clinical cure with infection persistence or progression. Nevertheless, it should be taken into account that in this study, dogs were changing between PCR positive or negative depending on the day of the follow-up. Intermittent or transitory parasitemia has been commonly observed in previous studies in dogs with *Leishmania* infection and is the most likely explanation for the variability of the results throughout the follow-up [58]. Moreover, the number of dogs that were available for PCR testing on day 365 was much lower than initially, and this could affect the results. It should also be pointed out that PCR performed on DNA extracted from whole blood samples is a less sensitive technique than PCR performed on bone marrow, lymph node, spleen, or skin, which could be regarded as a limitation of this study [59,60]. Invasive sampling for PCR was considered not ethical in this scenario.

Regarding the onset of clinical signs, 87.5% of the papules appeared in autumn and winter, as previously described [4,61]. This period belongs to the end of the sandfly season, which could mean a delay between *Leishmania* inoculation and the development of papular dermatitis. This might be explained by a period of parasite amplification, as it was described in mice [62] and dogs [63]. The majority of dogs in this study were young, 91.7% were below 12 months of age, and 45.8% were below 6 months of age, as was observed in previous studies [4,61]. This could mean that papular dermatitis might be more common at the time of the first contact of the parasite with the host's immune system. In the same way, the distribution of papular lesions was similar to these previous studies, 46.9% were located on the inner surface of the pinna, and 18.8% were on the abdomen [4,61].

5. Conclusions

In conclusion, the results of this study showed that dogs with papular dermatitis treated with local meglumine antimoniate healed faster than dogs treated with a placebo. Furthermore, no dog treated with topical meglumine antimoniate showed worsening clinical signs or relapse during the one-year follow-up. On the other hand, PHMB (alone or in combination with TLR4a) showed a higher tendency towards resolution when compared to placebo, although results were considered non-significant. Moreover, no adverse effects were observed in any of the drugs throughout the study in any of the dogs. Therefore, we might conclude that local meglumine antimoniate is a safe and effective alternative for the treatment of papular dermatitis in dogs with *L. infantum* infection. The present study also showed that most dogs presented a protective immune response at the time of diagnosis and during a year follow-up period without clinical failure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens12060821/s1, Table S1: Incident sheet for owner (treatment application, adverse effects, or others).

Author Contributions: Conceptualization, L.S.-G.; formal analysis, I.M.-F.; investigation, I.M.-F., M.J.G., A.D., M.C., L.O. and M.M.A.; resources; L.S.-G., M.M.A., D.B., L.G., R.F. and C.R.; data curation, I.M.-F.; writing—original draft preparation, I.M.-F.; writing—review and editing, L.S.-G. and L.O.; visualization, L.S.-G.; supervision, L.S.-G.; project administration, L.S.-G.; funding acquisition, L.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish ministry grant, Ministerio de Economía y competitividad, and Fondo Europeo de Desarrollo Regional (FEDER, EU) (AGL2015-68477).

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona (CEAAH 4526, November 2018) and by Generalitat de Catalunya (FUE-2018-00944112 i ID KSHYD6LVR, April 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting reported results can be obtained by emailing the corresponding author.

Acknowledgments: Authors would like to acknowledge Isaac Carrasco Rivero and Amalia Melendreras Montesinos for their contribution to clinical cases, Lourdes Alarcón Sánchez for her priceless technical help and support, Marta Baxarias Canals for her considerable assistance with the study, and Carles Blasi Brugué for his valuable work reviewing the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet guidelines for the practical management of canine leishmaniosis. *Parasites Vectors* **2011**, *4*, 1–16. [CrossRef]
2. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine leishmaniosis—New concepts and insights on an expanding zoonosis: Part one. *Trends Parasitol.* **2008**, *24*, 324–330. [CrossRef]
3. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological findings and detection of Toll-like receptor 2 in cutaneous lesions of canine leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163. [CrossRef] [PubMed]
4. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular dermatitis due to *Leishmania infantum* infection in seventeen dogs: Diagnostic features, extent of the infection and treatment outcome. *Parasites Vectors* **2014**, *7*, 1–11. [CrossRef]
5. Solano-Gallego, L.; Montserrrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-specific production of IFN-γ and IL-10 in stimulated blood from dogs with clinical leishmaniosis. *Parasites Vectors* **2016**, *9*, 317. [CrossRef] [PubMed]
6. Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P.; Solano-Gallego, L. Parasite specific antibody levels, interferon-γ and TLR2 and TLR4 transcripts in blood from dogs with different clinical stages of leishmaniosis. *Vet. Sci.* **2018**, *5*, 31. [CrossRef]
7. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on adaptive and innate immunity in canine leishmaniosis. *Parasitology* **2017**, *144*, 95–115. [CrossRef] [PubMed]
8. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2015**, *163*, 94–102. [CrossRef]

9. Hernandez-Bures, A.; Gillen, J.; Apostolidis, K.; Saridomichelakis, M.; Di Loria, A.; Santoro, D. Evaluation of the cutaneous inflammatory cells in dogs with leishmaniosis and in dogs without the disease that were naturally infected by *Leishmania infantum* (syn. *L. chagasi*). *Vet. Dermatol.* **2021**, *32*, 99-e19. [CrossRef]
10. Nascimento, M.S.L.; Carregaro, V.; Lima-Júnior, D.S.; Costa, D.L.; Ryffel, B.; Duthie, M.S.; de Jesus, A.; de Almeida, R.P.; da Silva, J.S. Interleukin 17A acts synergistically with interferon γ to promote protection against *Leishmania infantum* infection. *J. Infect. Dis.* **2015**, *211*, 1015–1026. [CrossRef]
11. Nascimento, M.S.L.; Albuquerque, T.D.R.; Nascimento, A.F.S. Impairment of Interleukin-17A Expression in Canine Visceral Leishmaniosis is Correlated with Reduced Interferon-γ and Inducible Nitric Oxide Synthase Expression. *J. Comp. Pathol.* **2015**, *153*, 197–205. [CrossRef] [PubMed]
12. Rebech, G.T.; Venturin, G.L.; Siqueira Ito, L.T.; Bragato, J.P.; de Carvalho Fonseca, B.S.; Melo, L.M.; Costa, S.F.; de Rezende Eugênio, F.; Dos Santos, P.S.P.; de Lima, V.M.F. PD-1 regulates leishmanicidal activity and IL-17 in dogs with leishmaniasis. *Vet. Immunol. Immunopathol.* **2020**, *219*, 109970. [CrossRef]
13. Toepp, A.J.; Petersen, C.A. The balancing act: Immunology of leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25. [CrossRef]
14. Esch, K.J.; Schaut, R.G.; Lamb, I.M.; Clay, G.; Lima, L.M.; Nascimento, R.P.; Whitley, E.M.; Jeronimo, S.M.; Sutterwala, F.S.; Haynes, J.S.; et al. Activation of Autophagy and Nucleotide-Binding Domain Leucine-Rich Repeat Containing-Like Receptor Family, Pyrin Domain Containing 3 Inflammasome during *Leishmania infantum* Associated Glomerulonephritis. *Am. J. Pathol.* **2015**, *185*, 2105–2117. [CrossRef]
15. Gibson-Corley, K.N.; Hostetter, J.M.; Hostetter, S.J.; Mullin, K.; Ramer-Tait, A.E.; Boggiatto, P.M.; Petersen, C.A. Disseminated *Leishmania infantum* infection in two sibling foxhounds due to possible vertical transmission. *Can. Vet. J.* **2008**, *49*, 1005–1008.
16. Gibson-corley, K.N.; Boggiatto, P.M.; Mukbel, R.M.; Petersen, C.A.; Jones, D.E. A deficiency in the B cell response of C57BL / 6 mice correlates with loss of macrophage-mediated killing of *Leishmania amazonensis*. *Int. J. Parasitol.* **2010**, *40*, 157–161. [CrossRef]
17. Pereira-Fonseca, D.C.M.; Oliveira-Rovai, F.M.; Rodas, L.A.C.; Beloti, C.A.C.; Torrecilha, R.B.P.; Ito, P.K.R.K.; Avanço, S.V.; Cipriano, R.S.; Utsunomiya, Y.T.; Hiramoto, R.M.; et al. Dog skin parasite load, TLR-2, IL-10 and TNF-α expression and infectiousness. *Parasite Immunol.* **2017**, *39*, e12493. [CrossRef]
18. Carpenter, S.; O'Neill, L.A.J. How important are Toll-like receptors for antimicrobial responses? *Cell Microbiol.* **2007**, *9*, 1891–1901. [CrossRef]
19. Kumar, H.; Kawai, T.; Akira, S. Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* **2011**, *30*, 16–34. [CrossRef] [PubMed]
20. Kawai, T.; Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **2011**, *34*, 637–650. [CrossRef] [PubMed]
21. Ordeix, L.; Montserrat-Sangrà, S.; Martínez-Orellana, P.; Baxarias, M.; Solano-Gallego, L. Toll-like receptors 2, 4 and 7, interferon-gamma and interleukin 10, and programmed death ligand 1 transcripts in skin from dogs of different clinical stages of leishmaniosis. *Parasites Vectors* **2019**, *12*, 575. [CrossRef]
22. Martínez-Orellana, P.; Baxarias, M.; Good, L.; Solano-Gallego, L. The effects of polyhexamethylene biguanide (PHMB) and TLR agonists alone or as polyplex nanoparticles against *Leishmania infantum* promastigotes and amastigotes. *Vet. Sci.* **2020**, *7*, 179. [CrossRef] [PubMed]
23. Winterbourn, C.C.; Kettle, A.J.; Hampton, M.B. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **2016**, *85*, 765–792. [CrossRef]
24. Gómez-Ochoa, P.; Lara, A.; Couto, G.; Marcen, J.M.; Peris, A.; Gascón, M.; Castillo, J.A. The nitroblue tetrazolium reduction test in canine leishmaniosis. *Vet. Parasitol.* **2010**, *172*, 135–138. [CrossRef]
25. Gómez-Ochoa, P.; Sabate, D.; Homedes, J.; Ferrer, L. Use of the nitroblue tetrazolium reduction test for the evaluation of Domperidone effects on the neutrophilic function of healthy dogs. *Vet. Immunol. Immunopathol.* **2012**, *146*, 97–99. [CrossRef] [PubMed]
26. Dea-Ayuela, M.A.; Segarra, S.; Serrano, D.R.; Bolás-Fernández, F. Nucleotides and AHCC Enhance Th1 Responses in Vitro in *Leishmania*-Stimulated/Infected Murine Cells. *Molecules* **2020**, *25*, 3918. [CrossRef]
27. Ordeix, L.; Rodríguez, A.; Martínez-Orellana Pamela Montserrat Sangrà, S.; Solano-Gallego, L. Clinical follow up of a series of dogs with papular dermatitis due to *Leishmania infantum*. In Proceedings of the 29th Annual Congress of the ESVD-ECVD, Lausanne, Switzerland, 14 September 2017.
28. Aronson, N.; Herwaldt, B.L.; Libman, M.; Pearson, R.; Lopez-Velez, R.; Weina, P.; Carvalho, E.; Ephros, M.; Jeronimo, S.; Magill, A. Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin. Infect. Dis.* **2016**, *63*, 1539–1557. [CrossRef]
29. Garza-Tovar, T.F.; Sacriste-Hernández, M.I.; Juárez-Durán, E.R.; Arenas, R. An overview of the treatment of cutaneous leishmaniasis. *Fac. Rev.* **2020**, *9*, 28. [CrossRef]
30. Burza, S.; Croft, S.L.; Boelaert, M. Leishmaniasis. *Lancet* **2018**, *392*, 951–970. [CrossRef]
31. Nassif, P.W.; França, T.; Mello, P.D.E.; Navasconi, T.R.; Mota, C.A.; Demarchi, I.G.; Aristides, S.M.A.; Lonardoni, M.V.C.; Teixeira, J.J.V.; Silveira, T.G.V. Safety and efficacy of current alternatives in the topical treatment of cutaneous leishmaniasis: A systematic review. *Parasitology* **2017**, *144*, 995–1004. [CrossRef] [PubMed]

32. Uribe-Restrepo, A.F.; Prieto, M.D.; Cossio, A.; Desai, M.M.; Del Mar Castro, M. Eligibility for Local Therapies in Adolescents and Adults with Cutaneous Leishmaniasis from Southwestern Colombia: A Cross-Sectional Study. *Am. J. Trop. Med. Hyg.* **2019**, *100*, 306–310. [CrossRef]
33. Mulder, G.; Cavorsi, J.; Lee, D. Polyhexamethylene Biguanide (PHMB): An Addendum to Current Topical Antimicrobials. *Wounds* **2007**, *19*, 173–182.
34. Firdessa, R.; Good, L.; Amstalden, M.C.; Chindera, K.; Kamaruzzaman, N.F.; Schultheis, M.; Röger, B.; Hecht, N.; Oelschlaeger, T.A.; Meinel, L.; et al. Pathogen- and Host-Directed Antileishmanial Effects Mediated by Polyhexanide (PHMB). *PLoS Negl. Trop. Dis.* **2015**, *9*, e0004041. [CrossRef]
35. Berenguer, D.; Sosa, L.; Alcover, M.; Sessa, M.; Halbaut, L.; Guillén, C.; Fisa, R.; Calpena-Campmany, A.C.; Riera, C. Development and characterization of a semi-solid dosage form of meglumine antimoniate for topical treatment of cutaneous leishmaniasis. *Pharmaceutics* **2019**, *11*, 613. [CrossRef]
36. Solano-Gallego, L.; Di Filippo, L.; Ordeix, L.; Planellas, M.; Roura, X.; Altet, L.; Martínez-Orellana, P.; Montserrat, S. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. *Parasites Vectors* **2016**, *9*, 1–9. [CrossRef]
37. Solano-Gallego, L.; Villanueva-Saz, S.; Carbonell, M.; Trotta, M.; Furlanello, T.; Natale, A. Serological diagnosis of canine leishmaniosis: Comparison of three commercial ELISA tests (Leiscan®, ID Screen® and *Leishmania* 96®), a rapid test (Speed Leish K®) and an in-house IFAT. *Parasites Vectors* **2014**, *7*, 1–10. [CrossRef] [PubMed]
38. Martínez-Orellana, P.; González, N.; Baldassarre, A.; Álvarez-Fernández, A.; Ordeix, L.; Paradies, P.; Soto, M.; Solano-Gallego, L. Humoral Responses and Ex Vivo IFN-γ Production after Canine Whole Blood Stimulation with *Leishmania infantum* Antigen or KMP11 Recombinant Protein. *Vet. Sci.* **2022**, *9*, 116. [CrossRef] [PubMed]
39. Martín-Ezquerra, G.; Fisa, R.; Riera, C.; Rocamora, V.; Fernández-Casado, A.; Barranco, C.; Serra, T.; Baró, T.; Pujol, R. Role of *Leishmania* spp. infestation in nondiagnostic cutaneous granulomatous lesions: Report of a series of patients from a Western Mediterranean area. *Br. J. Dermatol.* **2009**, *161*, 320–325. [CrossRef] [PubMed]
40. Piragauta, S.P.; Higuita-Castro, J.L.; Arbeláez, N.; Restrepo, A.M.; Archbold, R.; Quiñones, W.; Torres, F.; Echeverri, F.; Escobar, G.; Vélez, I.D.; et al. Utility of the combination of hederagenin glucoside saponins and chromane hydrazone in the topical treatment of canine cutaneous leishmaniasis. An observational study. *Parasitol. Res.* **2022**, *121*, 1419–1428. [CrossRef]
41. Lago, J.; Fraga, D.; Guimarães, L.H.; Lago, T.; Santos, Y.; Lago, E.; Werneck, G.L.; Bacellar, O.; Carvalho, E.M. Efficacy of intralesional meglumine antimoniate in the treatment of canine tegumentary leishmaniasis: A Randomized controlled trial. *PLoS Negl. Trop. Dis.* **2023**, *17*, e0011064.
42. Barbosa Santos, E.G.O.; Marzochi, M.C.A.; Conceição, N.F.; Brito, C.M.M.; Barroso, J.A.; Pacheco, R.S. N-methylglucamine antimonate (SbV+): Intralesional canine tegumentary leishmaniasis therapy. *Parasite* **1998**, *5*, 175–180. [CrossRef] [PubMed]
43. Aronson, N.E.; Joya, C.A. Cutaneous Leishmaniasis: Updates in Diagnosis and Management. *Infect. Dis. Clin. N Am.* **2019**, *33*, 101–117. [CrossRef]
44. Moosavian Kalat, S.A.; Khamesipour, A.; Bavarsad, N.; Fallah, M.; Khashyarmanesha, Z.; Feizi, E.; Neghabi, K.; Abbasi, A.; Jaafari, M. Use of topical liposomes containing meglumine antimoniate (Glucantime) for the treatment of *L. major* lesion in BALB/c mice. *Exp. Parasitol.* **2014**, *143*, 5–10. [CrossRef]
45. Moosavian, S.A.; Fallah, M.; Jaafari, M.R. The activity of encapsulated meglumine antimoniate in stearylamine-bearing liposomes against cutaneous leishmaniasis in BALB/c mice. *Exp. Parasitol.* **2019**, *200*, 30–35. [CrossRef]
46. Momeni, A.; Rasoolian, M.; Momeni, A.; Navaei, A.; Emami, S.; Shaker, Z.; Mohebali, M.; Khoshdel, A. Development of liposomes loaded with anti-leishmanial drugs for the treatment of cutaneous leishmaniasis. *J. Liposome Res.* **2013**, *23*, 134–144. [CrossRef]
47. Aragão Horoiwa, T.; Cortez, M.; Sauter, I.P.; Migotto, A.; Bandeira, C.L.; Cerize, N.N.P.; de Oliveira, A.M. Sugar-based colloidal nanocarriers for topical meglumine antimoniate application to cutaneous leishmaniasis treatment: Ex vivo cutaneous retention and in vivo evaluation. *Eur. J. Pharm. Sci.* **2020**, *147*, 105295. [CrossRef] [PubMed]
48. Cosco, D.; Bruno, F.; Castelli, G.; Puleio, R.; Bonacci, S.; Procopio, A.; Britti, D.; Fresta, M.; Vitale, F.; Paolino, D. Meglumine antimoniate-loaded aqueous-core PLA nanocapsules: Old drug, new formulation against *Leishmania*-related diseases. *Macromol. Biosci.* **2021**, *21*, e2100046. [CrossRef] [PubMed]
49. Nolff, M.C.; Winter, S.; Reese, S.; Meyer-Lindenberg, A. Comparison of polyhexanide, cold atmospheric plasma and saline in the treatment of canine bite wounds. *J. Small Anim. Pract.* **2019**, *60*, 348–355. [CrossRef]
50. Llorens, E.; Calderón, S.; Del Valle, L.J.; Puiggalí, J. Polybiguanide (PHMB) loaded in PLA scaffolds displaying high hydrophobic, biocompatibility and antibacterial properties. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2015**, *50*, 74–84. [CrossRef]
51. Mills, P.C.; Ahlstrom, L.; Wilson, W.J. Ototoxicity and tolerance assessment of a TrisEDTA and polyhexamethylene biguanide ear flush formulation in dogs. *J. Vet. Pharmacol. Ther.* **2005**, *28*, 391–397. [CrossRef]
52. Barrigah-Benissan, K.; Ory, J.; Sotto, A.; Salipante, F.; Lavigne, J.-P.; Loubet, P. Antiseptic Agents for Chronic Wounds: A Systematic Review. *Antibiotics* **2022**, *11*, 350. [CrossRef]
53. Worsley, A.; Vassileva, K.; Tsui, J.; Song, W.; Good, L. Polyhexamethylene Biguanide_Polyurethane Blend Nanofibrous Membranes for Wound Infection Control. *Polymers* **2019**, *11*, 915. [CrossRef]

54. Bagga, B.; Sharma, S.; Gour, R.P.S.; Mohamed, A.; Joseph, J.; MRathi, V.; Garg, P. A randomized masked pilot clinical trial to compare the efficacy of topical 1% voriconazole ophthalmic solution as monotherapy with combination therapy of topical 0.02% polyhexamethylene biguanide and 0.02% chlorhexidine in the treatment of *Acanthamoeba* keratitis. *Eye* **2021**, *35*, 1326–1333. [PubMed]
55. Bottero, E.; Poggi, M.; Viglione, M. Lesioni papulari indotte da *Leishmania* spp. in 8 cani giovani. *Veterinaria* **2006**, *20*, 33–36.
56. Gonçalves-de-albuquerque, C.; Gomes, L.; Sousa-paula LCDe Gaud, K.; Sales, S.; Boegel, A. Exploring IL-17 gene promoter polymorphisms in canine leishmaniasis. *Acta Trop.* **2022**, *232*, 106452. [CrossRef]
57. Rodríguez-Cortés, A.; Ojeda, A.; López-Fuertes, L.; Timón, M.; Altet, L.; Solano-Gallego, L.; Sánchez-Robert, E.; Francino, O.; Alberola, J. A long term experimental study of canine visceral leishmaniasis. *Int. J. Parasitol.* **2007**, *37*, 683–693. [CrossRef]
58. Pietro SDi Crinò, C.; Falcone, A.; Crupi, R.; Francaviglia, F.; Vitale, F.; Giudice, E. Parasitemia and its daily variation in canine leishmaniasis. *Parasitol. Res.* **2020**, *119*, 3541–3548. [CrossRef] [PubMed]
59. Solano-Gallego, L.; Rodriguez-Cortes, A.; Trotta, M.; Zampieron, C.; Razia, L.; Furlanello, T.; Caldin, M.; Roura, X.; Alberola, J. Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniosis. *Vet. Parasitol.* **2007**, *147*, 315–319. [CrossRef]
60. Solano-Gallego, L.; Koutinas, A.; Miró, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet. Parasitol.* **2009**, *165*, 1–18. [CrossRef] [PubMed]
61. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular dermatitis due to *Leishmania* spp. infection in dogs with parasite-specific cellular immune responses. *Vet. Dermatol.* **2005**, *16*, 187–191. [CrossRef]
62. Belkaid, Y.; Mendez, S.; Lira, R.; Kadambi, N.; Milon, G.; Sacks, D. A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* **2000**, *165*, 969–977. [CrossRef] [PubMed]
63. Aslan, H.; Oliveira, F.; Meneses, C.; Castrovinci, P.; Gomes, R.; Teixeira, C.; Derenge, C.A.; Orandle, M.; Gradoni, L.; Oliva, G.; et al. New insights into the transmissibility of *Leishmania infantum* from dogs to sand flies: Experimental vector-transmission reveals persistent parasite depots at bite sites. *J. Infect. Dis.* **2016**, *213*, 1752–1761. [CrossRef] [PubMed]

veterinary sciences

MDPI

Article

Exploring the Relationship between Neutrophil Activation and Different States of Canine *L. infantum* Infection: Nitroblue Tetrazolium Test and IFN-γ

Carles Blasi-Brugué †, Icíar Martínez-Flórez †, Marta Baxarias, Joan del Rio-Velasco and Laia Solano-Gallego *

Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; carles.blasi@uab.cat (C.B.-B.); imflorez.vet@gmail.com (I.M.-F.); martabaxcan@gmail.com (M.B.); rio.joan@gmail.com (J.d.R.-V.)

* Correspondence: laia.solano@uab.cat

† These authors contributed equally to this work.

Simple Summary: This study aimed to understand the role of neutrophils in canine leishmaniosis (CanL) by assessing neutrophil activation and its relationship with different states of *Leishmania infantum* infection and antibody and IFN-γ production. The results showed that sick dogs in stage I-mild disease had significantly higher neutrophil activation compared to healthy seronegative and seropositive dogs and sick dogs in advanced stages (II, III–IV). Healthy seropositive dogs exhibited higher neutrophil activation compared to all other groups except sick dogs in stage I. Dogs in advanced disease stages (II, III–IV) did not show significant differences in neutrophil activation compared to healthy seronegative dogs. Furthermore, dogs in stage I had significantly higher IFN-γ concentrations compared to healthy seronegative and sick dogs in advanced disease stages. Dogs in stage II showed higher IFN-γ concentrations compared to healthy seronegative dogs, while no significant differences were observed in dogs in stage III–IV. Healthy seropositive dogs had elevated IFN-γ concentrations compared to healthy seronegative dogs and dogs in stage III–IV. These findings indicate that neutrophil activation is predominant in dogs with mild disease and healthy seropositive dogs with an association with potent IFN-γ production.

Abstract: This study aimed to investigate the role of neutrophils in canine leishmaniosis by assessing neutrophil activation and its relationship with different states of *L. infantum* infection and antibody and IFN-γ production. Dogs were categorized into five groups: healthy-seronegative (n = 25), healthy-seropositive (n = 21), LeishVet-stage I (n = 25), Leishvet-stage II (n = 41), and LeishVet-stage III–IV (n = 16). Results of the nitroblue tetrazolium reduction test (NBT) showed significantly higher neutrophil activation in stage I (median:17.17, range: [7.33–31.50]%) compared to in healthy-seronegative (4.10 [1.20–18.00]%), healthy-seropositive (7.65 [3.98–21.74]%), stage II (6.50 [1.50–28.70]%), and stage III–IV (7.50 [3.00–16.75]%) groups ($p < 0.0001$). Healthy-seropositive dogs also displayed higher values than all groups except stage I. Stages II and III–IV did not show significant differences compared to healthy-seronegative. Regarding IFN-γ, stage I dogs had higher concentrations (median:127.90, range: [0–3998.00] pg/mL) than healthy-seronegative (0 [0–109.50] pg/mL) ($p = 0.0002$), stage II (9.00 [0–5086.00] pg/mL) ($p = 0.045$), and stage III–IV (3.50 [80.00–548.80] pg/mL) ($p = 0.02$) dogs. Stage II dogs showed increased IFN-γ compared to healthy-seronegative dogs ($p = 0.015$), while stage III–IV dogs had no significant differences compared to healthy-seronegative dogs ($p = 0.12$). Healthy-seropositive dogs had elevated IFN-γ concentrations compared to healthy-seronegative dogs ($p = 0.001$) and dogs in stage III–IV ($p = 0.03$). In conclusion, neutrophil activation was higher in dogs with mild disease and healthy-seropositive dogs, and a relationship between neutrophil activation and the production of IFN-γ was found.

Keywords: NBT; dog; immunity; interferon-gamma; neutrophil; leishmaniosis; oxidative burst; oxidative metabolism

Citation: Blasi-Brugué, C.; Martínez-Flórez, I.; Baxarias, M.; del Rio-Velasco, J.; Solano-Gallego, L. Exploring the Relationship between Neutrophil Activation and Different States of Canine *L. infantum* Infection: Nitroblue Tetrazolium Test and IFN-γ. *Vet. Sci.* **2023**, *10*, 572. https://doi.org/10.3390/vetsci10090572

Academic Editors: Anastasia Diakou and Donato Traversa

Received: 19 July 2023
Revised: 4 September 2023
Accepted: 11 September 2023
Published: 13 September 2023

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic vector-borne disease caused by the protozoan parasite *Leishmania infantum* and primarily transmitted through the bite of female Phlebotomine sand flies [1]. It has a worldwide distribution and is considered endemic in regions, such as the Mediterranean basin, Latin America, and southern Asia [1]. Dogs serve as the main reservoir for this infection. *Leishmania infantum* is an obligate intracellular parasite that infects and survives within various myeloid lineage cells, including monocytes, macrophages, dendritic cells, and neutrophils. Its presence in the body has unique implications for the immune system during the course of the infection [2].

Clinical manifestations of CanL can vary widely, ranging from subclinical infection to severe systemic disease [1]. The severity of the disease is classified into four stages: mild disease-LeishVet stage I, characterized by mild clinical signs like papular dermatitis, exhibiting no noteworthy clinicopathological abnormalities, and displaying antibody levels that range from negative to low; moderate disease-LeishVet stage II, characterized by diffuse clinical manifestations, like extensive skin lesions, widespread lymph node enlargement or weight loss, clinicopathological abnormalities without a rise in creatinine concentrations, and antibody levels varying from weakly positive to strongly positive; severe disease-LeishVet stage III, defined by extensive clinical manifestations along with clinical signs linked to the deposition of immune complexes (like uveitis or glomerulonephritis), clinicopathological abnormalities, including either IRIS Stage 1 proteinuric or IRIS Stage 2 chronic kidney disease (CKD), accompanied by antibody levels ranging from moderate to high positivity; and very severe disease-LeishVet stage IV, determined by diffuse clinical signs, clinicopathological abnormalities, and advanced CKD stage 3 or 4, accompanied by antibody levels ranging from moderate to high positivity [1]. The interplay between the host's innate and adaptive immune responses in response to the parasite infection plays a crucial role in determining the clinical signs and overall outcome of the disease [3,4]. Dogs with a predominant cellular immune response are associated with disease resistance or subclinical forms, while dogs exhibiting a predominantly humoral response are associated with clinical illness [2,5]. Adaptive T-cell immunity has been widely investigated to play a crucial role in disease development and resistance. The T helper 1 (Th1)-mediated immune response, characterized by the secretion of cytokines, such as interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α), is associated with immune protection [4,6]. Conversely, the Th2-mediated immune response leads to the production of cytokines, like interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β), which are linked to a predominantly humoral immune response and disease progression [4,6]. A balanced response between proinflammatory Th1 CD4+ T cells that inhibit parasite replication and an immune regulatory response mediated by T regulatory 1 (Tr1) cells is crucial for disease control [2]. The former stimulates macrophage activity through IFN-γ production, promoting parasite elimination [7]. However, prolonged exposure to antigens can lead to T cell exhaustion, resulting in reduced T cell proliferation and IFN-γ production. This, in turn, allows for parasite survival and expansion within macrophages [7].

In recent years, growing evidence has highlighted the significant role of the innate immune response in controlling *Leishmania* infection. Among the components of the innate immune system, neutrophils have emerged as key players, particularly in the early stages of *Leishmania* infection [8]. While macrophages are considered the primary host target cells for the replication of *L. infantum*, neutrophils also play a distinct role in the control or progression of leishmaniosis, thanks to their ability to kill parasites and produce interleukins [9,10]. As the first line of defense, neutrophils are the initial cells that migrate to the site of infection and internalize the *Leishmania* promastigote [11–14]. Upon phagocytosis, neutrophils activate their defense mechanisms, including the production of reactive oxygen species (ROS), which is a crucial antileishmanial mechanism produced in phagocytes (oxidative burst) [15–18]. The phagocyte's NADPH oxidase generates the superoxide anion (O_2^-), which serves as a precursor to hydrogen peroxide and other ROS [15–17,19–21].

Subsequently, the neutrophil releases these ROS within the phagosomes containing *L. infantum* amastigotes, effectively killing the parasite [19]. In addition to the oxidative burst, neutrophils that ingest *Leishmania* amastigotes initiate further defensive strategies. These include the secretion of TNF-α and IFN-γ, which aid in stimulating macrophage activation and recruitment to the infection site [22,23] and the release of neutrophil extracellular traps, which also potentially play a significant role in combatting protozoal infections, such as *Leishmania* [24].

However, *Leishmania* amastigotes have the ability to deactivate the oxidative activity of polymorphonuclear leukocytes, allowing them to survive within neutrophils and exploit them as a means to hide from the immune system while silently infecting the final host cell, the macrophage [10,22,25]. Alternatively, if the oxidative burst is successful and limits parasite growth within cells, the infection can be controlled [26,27]. Therefore, the ability or predisposition of neutrophils to activate the oxidative burst can have a significant impact on disease progression or containment.

Interferons (IFNs) are a family of proteins that exhibit strong antiviral and antibacterial effects and play crucial roles in regulating effector cells within both innate and adaptive immune responses [28–31]. Among these, IFN-γ stands out for having the most diverse and potent effects [32]. While much of the research on IFN-γ has focused on its effects on adaptive immunity, recent studies have revealed its ability to modulate myeloid cell activity, including neutrophils, in diverse ways. These include enhancing NADPH oxidase activity and the oxidative burst, promoting nitric oxide (NO) production, increasing the expression of MHCII proteins, altering cytokine and chemokine production, inhibiting chemotaxis, and suppressing apoptosis [33]. Although these effects have been predominantly observed in vitro based on mature neutrophils, studies suggest that more substantial changes occur when IFN-γ interacts with maturing myeloid cells [34]. Thus, IFN-γ could potentially have an impact on the capacity of neutrophils to eliminate phagocyted *Leishmania* amastigotes and promastigotes by stimulating the cellular immune response and activating phagosomes to induce an oxidative burst. Therefore, a positive relationship within IFN-γ and the oxidative metabolism of neutrophils could be suspected.

The nitroblue tetrazolium reduction test (NBT) is a diagnostic assay that utilizes the ability of neutrophils and macrophages to generate free radicals (oxidative burst) during the process of phagocytosis [35,36]. This test relies on the reduction of nitroblue tetrazolium, a colorless compound, to formazan, a blue compound, by the NADPH oxidase present in activated neutrophils within the phagocytic vacuole. As a result, the cytoplasm of the cells undergoes a color change, indicating the production of ROS [36]. The NBT reaction provides an assessment of the ROS-generating activity in the cytoplasm of cells and has been shown to correlate strongly with the levels of ROS produced during the oxidative burst [37,38]. By employing conventional light microscopy, the rate of NBT can be determined by calculating the percentage of neutrophils with formazan present in their cytoplasm, reflecting the activation status of neutrophils and monocytes in the peripheral blood [35]. The primary objective of this study was to assess the variations in peripheral blood neutrophil ROS generation in dogs with different stages of *L. infantum* infection by means of the NBT. Additionally, we aimed to investigate the potential association between neutrophil ROS generation and IFN-γ concentrations, as well as antibody levels, in dogs with different states of *L. infantum* infection.

2. Materials and Methods

All dogs included in this study belonged to volunteer owners who had granted permission to a physical examination and blood collection from their dogs through a signed consent form.

2.1. Dogs and Clinical Data

Dogs of different breeds from an endemic area were selected to enter the study. A complete blood count, serum biochemistry profile (at least containing creatinine, alanine

aminotransferase, and albumin), urinalysis, serum electrophoresis, and ELISA serology were performed for all dogs, as well as cytology of the lesions in dogs in stage I.

A total of 128 dogs were enrolled in this study and divided into five groups according to the LeishVet staging system [1]: Group 1 included healthy seronegative dogs with no clinical signs or hematological or biochemical abnormalities (n = 25); Group 2 included *Leishmania*-seropositive but clinically healthy dogs with no clinical signs, hematological and serum and urinary biochemical parameters within normal limits, and presenting low to medium antibody levels (n = 21); Group 3 included dogs with mild disease-LeishVet stage I presenting papular dermatitis as the sole clinical sign [39], the identification of intralesional amastigotes via cytology, hematological, serum, and urinary biochemical parameters within normal limits (including creatinine < 1.4 mg/dL), and presenting negative to low antibody levels (n = 25); Group 4 included dogs with moderate disease-LeishVet stage II, including the characteristic clinical signs (diffuse clinical manifestations, like extensive skin lesions, widespread lymph node enlargement, or weight loss among others), hematological and serum biochemical abnormalities with normal creatinine values (<1.4 mg/dL), and medium-to-high antibody levels (n = 41); Group 5 included dogs with severe disease-LeishVet stage III and very severe disease-Leishvet stage IV, including the characteristic clinical signs, hematological, biochemical abnormalities (including creatinine values of 1.4–2.8 mg/dL or UPC > 1 for stage III and creatinine values > 2.8 for stage IV or UPC > 5), and presenting positive antibody levels (n = 16) [1]. The urine protein creatinine ratio was assessed in 12/25 dogs in stage I and in all dogs in stages II, III, and IV. *Leishmania infantum*-specific antibody levels were assessed in all dogs using an end point ELISA. As for IFN-γ, this test was conducted on most of the dogs, although technical limitations prevented its performance in all cases. IFN-γ measurements were obtained from 10 out of 25 healthy seronegative dogs, 15 out of 21 healthy seropositive dogs, 24 out of 25 stage I dogs, 34 out of 41 stage II dogs, and 13 out of 16 stage III/IV dogs. A follow-up of up to one year was available for all dogs with papular dermatitis without conventional anti-*Leishmania* systemic treatment instituted. Some dogs in this group received local treatment whit either topical meglumine antimoniate as a lotion spray (n = 6/25), polyhexamethylene biguanide alone or in combination with Toll like receptor 4 agonist as a spray (n = 9/25), or placebo (n = 9/25), and one received no treatment at all [39].

2.2. Blood Collection

The blood was collected via jugular or cephalic venipuncture and placed in EDTA tubes for the hematologic analysis and the NBT test, into heparin tubes to perform cytokine release whole blood assay, and into serum tubes for the antileishmanial antibody quantification.

2.3. Antileishmanial Antibody Quantification via ELISA

Antibody levels against *L. infantum* were assessed using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA), as outlined in previous studies [3,40]. Dog sera were diluted to a ratio of 1:800 in a phosphate buffer solution (PBS) containing Tween 20 and 1% dry milk. This mixture was then incubated in plates previously coated overnight with sonicated promastigotes of *L. infantum* (MHOM/MON-1/LEM-75) obtained from an infected dog at a concentration of 20 µg/mL, and the incubation took place at 37 °C for 1 h [41]. Following this, the plates underwent a series of washes using PBS-Tween and PBS. Next, Protein A conjugated to horseradish peroxidase (Peroxidase Conjugate Protein A; Merck KGaA, Darmstadt, Germany) was added to the plates at a dilution of 1:30,000 and incubated for 1 h at 37 °C. After another round of washing, the plates were treated with o-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA, Darmstadt, Germany). The reaction was terminated using 5 M H_2SO_4. Absorbance readings were taken at 492 nm using a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd., Shenzhen, China). The quantification of results was performed in terms of ELISA units (EUs), normalized against positive canine serum utilized as a calibrator and set at 100 EU.

The threshold was established at 35 EU [39]. Serum samples were categorized as negative when their value fell below 35 EU. If the result ranged from 35 EU up to but not exceeding 150 EU, it was considered low positive. Similarly, if the result fell between 150 EU and 300 EU, it was categorized as medium positive. Finally, if the value equaled or surpassed 300 EU, it was classified as high positive.

For a more in-depth examination of samples identified as medium or high positive, an ELISA involving a two-fold serial dilution was conducted. This process was initiated at a dilution of 1:800 and continued with an additional 7 to 11 dilutions. Quantification was predicated on arbitrary units (EU) in relation to a calibrator set at 100 EU, which corresponded to an optical density (OD) value of 1 at the 1:800 dilution. The mean values of dilutions displaying an OD approximate to one were chosen for calculating the EU. This computation was executed using the following formula: (sample OD/calibrator OD) $\times$ 100 $\times$ dilution factor [40].

2.4. Nitroblue Tetrazolium Reduction Test

For each EDTA tube, blood was drawn and filled into three hematocrit capillary microtubes (Servoprax, Wiesel, Germany). These microtubes were then subjected to centrifugation at 2910$\times$ *g* for 5 min (Fugevet+ GDC005, Nahita International Ltd., London, UK) to isolate the buffy coat. The obtained buffy coat was transferred to an Eppendorf tube, where it was mixed with an equal volume of 0.1% NBT solution (1:1) (N6876, Sigma-Aldrich Co., St. Louis, MO, USA). The mixture was gently agitated and then placed in a heater at 37.5 °C for a duration of 15 min (INB 200, Memmert GmbH + Co. KG, Schwabach, Germany), followed by an additional 15 min at room temperature. Subsequently, two blood smears were prepared from each Eppendorf tube, with 3 µL of NBT-stained blood being placed on each glass slide. These slides were further stained using the Diff-Quik method. To assess the NBT rate, a total of 300 neutrophils displaying a clear morphology were counted on each slide using standard light microscopy. Neutrophils that were aggregated or damaged were excluded from the count. The NBT rate was calculated by determining the percentage of activated neutrophils, characterized by the presence of blue-black formazan deposits, among the total counted neutrophils [42].

Dogs were considered to have an increased NBT rate when the NBT value exceeded the cut-off. This cut-off was calculated by considering the NBT values of the 25 healthy seronegative dogs as the control group. A high sensitivity was desired for this cut-off. The standard deviation was added to the mean value of this group, resulting in a cut-off of 10.80%. With this cut-off, considering the healthy seronegative dogs as negative and the dogs with papular dermatitis as positive, the sensitivity was 92% and the specificity 84%.

2.5. Cytokine Release Whole Blood Assay and Determination of Canine IFN-γ

The heparinized cytokine release whole blood assay was conducted following established procedures [3]. To outline the process, the assay involved incubating whole blood separately under three distinct conditions: (i) medium alone (unstimulated); (ii) medium containing *L. infantum* soluble antigen (LSA) at a concentration of 10 µg/mL [43]; and (iii) medium supplemented with the mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 µg/mL. LSA was obtained through a process involving triple cycles of freezing and thawing of cultured *L. infantum* promastigotes (MHOM/MON-1/LEM-75) suspended at a concentration of 1 $\times$ 10^9 cells/mL in PBS. Subsequently, the supernatant was harvested following centrifugation (8.000$\times$ *g*, 20 min, 4 °C), adhering closely to the methodology outlined in Carrillo et al., 2015, albeit with minor adjustments [44]. Following five days of incubation at 37 °C in an environment containing 5% CO_2, supernatants were obtained through centrifugation at 300$\times$ *g* for a duration of 10 min. These supernatants were then collected and stored at −80 °C until the testing phase. The measurement of IFN-γ was carried out for all samples utilizing a commercially available sandwich ELISA (Du-oSet® ELISA by Development System R&DTM, Abingdon, UK) using a canine IFN-γ antibody. A standard curve for canine IFN-γ was established

through a computer-generated four-parameter logistic curve-fit using the MyAssays program (http://www.myassays.com/) [3]. Dogs were categorized as IFN-γ producers if the concentration of *L. infantum*-specific IFN-γ exceeded or was equal to 110 pg/mL after accounting for the medium-alone baseline [43].

2.6. Statistical Analysis

The statistical analysis of data was performed using GraphPad Prism 8.0.1 for Windows software (GraphPad Software, San Diego, CA, USA).

Several normality tests (Anderson–Darling, D'Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov) were performed to assess the normality of the different variables (NBT rate, serological results, and IFN-γ concentration). No variables followed a normal distribution except for the NBT rate in stage I and stage III–IV considering a p-value < 0.05 as statistically significant. Therefore, non-parametric tests were used to assess the data, except for the unpaired t-test (parametric test) performed between the parameters with a normal distribution. A non-parametric Mann–Whitney U-test was used for quantitative variables to compare two groups.

A Kruskal–Wallis test and a Dunn's multiple comparisons test were used to compare more than two groups when data did not follow a normal distribution. If data followed a normal distribution, an ordinary one-way ANOVA and a Tukey's multiple comparisons test were performed. The Chi-square test or Fisher's exact test was performed to determine if an association between categorical variables existed. The Spearman (non-normal distribution) or Pearson (normal distribution) correlation coefficient was used to evaluate if there were correlations among different parameters studied. Results were considered statistically significant at a p-value < 0.05.

3. Results

3.1. Dog Clinical Characteristics and Performed Tests

Dog clinical characteristics and results of performed tests are summarized and displayed in Tables 1 and 2.

Table 1. Distribution of qualitative characteristics including breed, sex, positive/negative NBT, positive/negative serology, and productive/non-productive status of IFN-γ among dogs categorized within the five states of infection studied.

Qualitative Characteristics		Healthy Seronegative (*n* = 25)	Healthy Seropositive (*n* = 21)	Stage I (*n* = 25)	Stage II (*n* = 41)	Stage III–IV (*n* = 16)	*p*-Value (Chi-Square, *df*)
Breed (*n* = 128)	**Crossbred (*n* = 54)**	9 (36.00%)	11 (52.38%)	14 (56.00%)	16 (39.02%)	4 (25.00%)	0.25 (5.30, 4)
	Purebred (*n* = 74)	16 (64.00%)	10 (47.62%)	11 (44.00%)	25 (60.98%)	12 (75.00%)	
Sex (*n* = 128)	**Male (*n* = 74)**	12 (48.00%)	10 (47.62%)	16 (64.00%)	23 (56.10%)	13 (81.25%)	0.2 (5.90, 4)
	Female (*n* = 54)	13 (52.00%)	11 (52.38%)	9 (36.00%)	18 (43.90%)	3 (18.75%)	
NBT (*n* = 128)	**Increased (*n* = 46)**	4 (16.00%)	5 (23.81%)	23 (92.00%)	10 (24.39%)	3 (18.75%)	<0.0001 * (44.60, 4)
	WNL (*n* = 82)	21 (84.00%)	16 (76.19%)	2 (8.00%)	31 (75.61%)	13 (81.25%)	
Serology (*n* = 128)	**Positive (*n* = 84)**	0 (0.00%)	21 (100.00%)	6 (24.00%)	41 (100.00%)	16 (100.00%)	<0.0001 * (107.80, 4)
	Negative (*n* = 44)	25 (100.00%)	0 (0.00%)	19 (76.00%)	0 (0.00%)	0 (0.00%)	
IFN-γ (*n* = 97)	**Producers (*n* = 34)**	0 (0.00%)	9 (60.00%)	12 (50.00%)	9 (26.47%)	4 (30.77%)	0.01 * (12.54, 4)
	Non-prod. (*n* = 63)	10 (100.00%)	6 (40.00%)	12 (50.00%)	25 (73.53%)	9 (69.23%)	

NBT = nitroblue tetrazolium reduction test, WNL = within normal limits, IFN-γ = interferon-gamma, Non-prod. = non-producers, ELISA = Enzyme-Linked ImmunoSorbent Assay. * Significant differences were observed between groups.

Table 2. Median, minimum, and maximum values for quantitative traits, like age, NBT, serology, and IFN-γ within each group.

Quantitative Characteristics Median (Min–Max)	Healthy Seronegative (*n* = 25)	Healthy Seropositive (*n* = 21)	Stage I (*n* = 25)	Stage II (*n* = 41)	Stage III–IV (*n* = 16)	*p*-Value (Kruskal-Wallis Test)
Age (years)	1.30 (1.00–1.60)	5.00 (1.00–13.00)	0.50 (0.25–7.00)	4.00 (1.00–13.00)	7.50 (1.00–11.00)	<0.0001 *
NBT (%)	4.13 (1.15–17.96)	7.65 (3.98–21.74)	17.17 (7.33–31.50)	6.50 (1.50–28.70)	7.50 (3.00–16.75)	<0.0001 *
IFN-γ (pg/mL)	0 (0–109.50)	204.10 (0–4763.00)	127.90 (0–3998.00)	9.00 (0–5086.00)	3.48 (0–548.80)	0.001 *
Serology (ELISA units)	5.50 (0.30–28.49)	133.90 (75.79–956.30)	21.42 (2.76–227.10)	1894.00 (57.16–10,293.00)	1857.00 (96.79–11,114.00)	<0.0001 *

NBT = nitroblue tetrazolium reduction test, IFN-γ = interferon-gamma, ELISA = Enzyme-Linked ImmunoSorbent Assay. * Significant differences were observed between groups.

Forty-two percent of dogs were crossbred (*n* = 54), and the other most represented breeds were Beagle (*n* = 10), German shepherd (*n* = 8), and Greyhound (*n* = 5).

3.2. Age-Stratified Analysis of NBT Rate in Dogs with Papular Dermatitis (Stage I): Insights from Younger and Older Canine Cohorts

Dogs within the stage I group were further subdivided between dogs younger than one year of age (*n* = 22) and dogs older than one year of age (*n* = 3) for a further statistical analysis. No statistical differences were found when comparing the NBT rate of dogs in both groups ($p = 0.674$).

Additionally, the statistical study comparing the stage I group with the other groups was repeated but only taking in consideration the dogs that were older than 1 year of age, showing similar results to the previous study performed. Stage I (older than 1 year of age) had a significantly higher NBT rate than dogs in the healthy seronegative group ($p = 0.02$), the healthy seropositive group ($p = 0.011$), the stage II group ($p = 0.002$), and the stage III–IV group ($p < 0.0001$).

3.3. Follow-Up of Dogs with Papular Dermatitis (Stage I)

Over the following months after diagnosis, the majority of the dogs exhibited a notable improvement or the resolution of their cutaneous lesions, and the antibody levels demonstrated a declining trend. However, among the 25 dogs studied, three exhibited a deterioration in their cutaneous lesions, which progressed to the point of ulceration in two cases. Consequently, a systemic conventional anti-*Leishmania* treatment approach was initiated to address their condition.

For the purpose of conducting a statistical analysis, the three dogs that experienced a worsening of their clinical signs were categorized as belonging to group PD-B, and the remaining 22 dogs that displayed an improvement or the resolution of their clinical signs without the need for systemic treatment were designated as members of group PD-A. Detailed results pertaining to NBT, IFN-γ, and serology, both for these delineated groups and for each individual dog within group PD-B, can be found in Table 3.

Upon conducting a comparative analysis of the NBT, IFN-γ, and serology results between the two groups, no statistically significant differences were found ($p = 0.074$, $p = 0.176$, and $p = 0.271$, respectively). However, it is worth noting that the sample size for group PD-B was limited to only three dogs, which could have influenced the robustness of the statistical outcomes.

Interestingly, despite the limitations in sample size, a significant correlation was established between the number of dogs surpassing the NBT cut-off value and the achievement of a clinical improvement without the requirement for systemic treatment. This observation indicated that dogs in group PD-B (those experiencing worsening clinical signs) were more prone to exhibit NBT results within the normal range, unlike dogs in group PD-A (demonstrating an improvement without systemic treatment). However, no corresponding link was identified between the various groups and the status of being an IFN-γ producer or having a positive serology.

Table 3. NBT and IFN-γ results for dogs that presented with papular dermatitis and divided into two groups: Group PD-A (clinical improvement without systemic treatment) and Group PD-B (clinical worsening and need for systemic treatment).

	NBT (%)	NBT Status (Cut-Off = 10.8)	IFN-γ	IFN-γ Status	Serology	Serological Status	Clinical Evolution
Group PD-A (*n* = 22)	Median: 17.59 (min: 9.67, max: 31.50)	22/22 Over cutoff	Median: 171.90 (min: 0, max: 3998.00)	10/10 Producers	Median: 18.21 (min: 2.76, max: 227.10)	5/22 positive	Partial or total improvement
Group PD-B (*n* = 3)	Median: 8.80 (min: 7.33, max: 20.83)	2/3 WNL	Median: 7.03 (min: 0, max: 234.20)	1/3 Producer	Median: 27.41 (min: 22.34, max: 79.90)	1/3 positive	-
Dog PD-B.1	8.80	WNL	7.03	Non-producer	79.90	Positive	Worsening of cutaneous clinical signs: diffuse worsening.
Dog PD-B.2	7.33	WNL	234.24	Producer	27.41	Negative	Worsening of cutaneous clinical signs: ulcerative lesion at the nose bridge.
Dog PD-B.3	20.83	Over cutoff	0	Non-producer	22.34	Negative	Worsening of cutaneous clinical signs: ulcerative lesion at the level of the right lower eyelid.

Group PD-A = dogs diagnosed with papular dermatitis that showed an improvement in clinical signs without systemic treatment, Group PD-B = dogs diagnosed with papular dermatitis that showed the worsening of clinical signs and in which systemic treatment was considered necessary, NBT = nitroblue tetrazolium reduction test, WNL = within normal limits, IFN-γ = interferon-gamma.

3.4. Total Leucocyte Concentration and Differential Leukocyte Concentration

The results from the total leucocyte concentration and the differential leukocyte concentration analysis within the different groups studied are displayed in Table 4. Upon scrutinizing the outcomes across different groups, no statistically significant differences were identified in terms of total leukocyte, neutrophil, and eosinophil concentrations. However, significant differences were observed in relation to the lymphocyte concentration. Specifically, healthy seronegative dogs exhibited notably higher lymphocyte concentrations in comparison to the healthy seropositive dogs ($p = 0.028$), as well as to dogs in stage II ($p = 0.048$) and stage III–IV ($p = 0.001$). Interestingly, no substantial variance was found when comparing the lymphocyte concentration between healthy seronegative dogs and dogs in the stage I group. Furthermore, regarding the lymphocyte concentration within the stage I group, significantly higher values were also recorded in contrast to healthy seropositive dogs ($p = 0.022$), dogs in stage II ($p = 0.033$), and dogs in stage III–IV ($p = 0.001$). Notably, no significant differences were evident when comparing the stage II group with the stage III–IV group.

Regarding the monocyte concentration, statistically significant differences were observed between healthy seropositive dogs and those in stage I ($p = 0.004$), as well as between dogs in stage II and those in stage I ($p = 0.04$). In both cases, the stage I group exhibited markedly higher values.

When exploring potential correlations between the NBT rate and both neutrophil and lymphocyte concentrations, a comprehensive analysis encompassing all of the dogs collectively revealed no significant correlations ($p = 0.491$ and $p = 0.199$, respectively). This pattern persisted when conducting separate evaluations within distinct groups, which included healthy seronegative ($p = 0.682$ and $p = 0.811$, respectively), healthy seropositive ($p = 0.766$ and $p = 0.144$, respectively), stage I ($p = 0.187$ and $p = 0.507$, respectively), stage II ($p = 0.408$ and $p = 0.494$, respectively), and stage III–IV ($p = 0.625$ and $p = 0.525$, respectively) groups.

Table 4. Median (min–max) results for total leukocyte concentrations and differential leukocyte concentrations in each group.

Median (Min–Max)	Healthy Seronegative	Healthy Seropositive	Stage I	Stage II	Stage III–IV	*p*-Value
Leucocytes (cell/µL)	11,330 (6390–15,020)	9710 (3360–16,920)	11,180 (6200–20,540)	10,470 (3990–19,430)	9650 (3420–13,630)	0.115
Neutrophils (cell/µL)	6675 (3706–9913)	5889 (2755–11,844)	6939 (3300–17,860)	7171 (3032–14,378)	6482 (2633–10,223)	0.936
Lymphocytes (cell/µL)	3006 (1968–3930)	1711 (235–3849)	3590 (900–5800)	1921 (518–4696)	1472 (616–2385)	<0.0001 *
Eosinophils (cell/µL)	409 (300–934)	514 (63–2369)	500 (20–1100)	479 (0–2176)	386 (0–1204)	0.541
Monocytes (cell/µL)	668 (313–1502)	413 (34–1157)	750 (100–1310)	517 (129–1269)	560 (171–1204)	0.003 *

* Significant differences were observed between groups.

Regarding the monocyte concentration, a significant negative correlation with the NBT was observed exclusively within the stage I group ($p = 0.005$). In contrast, no such correlations emerged when examining the healthy seronegative ($p = 0.785$), healthy seropositive ($p = 0.128$), stage II ($p = 0.451$), and stage III–IV ($p = 0.73$) groups separately. Similarly, no overall correlation was identified when considering all dogs collectively ($p = 0.159$).

3.5. Nitroblue Tetrazolium Reduction Test

The results of NBT are depicted in Tables 1 and 2 and Figure 1. A significantly lower median NBT rate was observed in the healthy seronegative group (median: 4.13% range: [1.15–17.96]%) when comparing this to the to the healthy seropositive group (7.65% [3.98–21.74]%) ($p = 0.006$) and stage I group (17.17% [7.33–31.50]%) ($p < 0.0001$). However, no significant differences were found between the healthy seronegative group and stage II (6.50% [1.50–28.70]%) ($p = 0.08$) or stage III–IV group (7.50% [3.00–16.75]%) ($p = 0.09$).

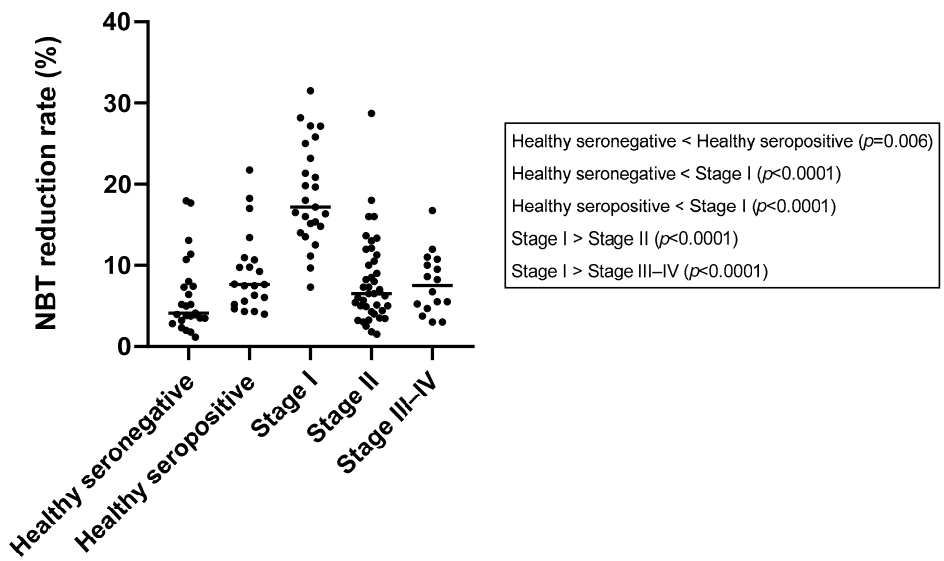


Figure 1. NBT reduction rate results (median, minimum, and maximum values) in different *L. infantum* states of infection.

Regarding healthy seropositive dogs, a significantly lower median NBT rate was also observed when comparing this group to stage I ($p < 0.0001$). However, no significant differences were found with the other groups: stage II ($p = 0.23$) and stage III–IV ($p = 0.53$). A significantly higher median NBT rate was found when comparing stage I to both stage II ($p < 0.0001$) and stage III–IV ($p < 0.0001$). Conversely, there were no significant differences between stage II and stage III–IV ($p = 0.8$).

Regarding the number of dogs over the cut-off value for NBT, there was a significantly higher proportion of dogs that surpassed the cut-off in the stage I group (92.00%) when compared to seronegative (16.00%) ($p < 0.0001$), healthy seropositive (23.8%) ($p < 0.0001$), stage II (24.40%) ($p < 0.0001$), and stage III–IV dogs (18.75%) ($p < 0.0001$). No additional significant differences were found when comparing the other groups (Table 1).

3.6. Leishmania Infantum-Specific Antibody Levels

All dogs in the healthy seronegative group were considered negative (median: 5.50, range: [0.30–28.49] EU). Among the healthy seropositive group (median: 133.90, range: [75.79–956.30] EU), 76.19% of dogs were classified as low positive, 14.29% as medium positive, and 9.52% exhibited high antibody levels. In the stage I group (median: 21.42, range: [2.76–227.10] EU), all dogs had negative (76.00%) or low (20.00%) antibody levels, except for one dog (4.00%), which was considered medium positive.

For the stage II group (median: 1894, range: [57.16–10,293.00] EU), 21.95% of dogs were classified as low positive, 34.14% as medium positive, and 43.90% as high positive. In the stage III–IV group (median: 1857, range: [96.79–11,114.00] EU), 37.50% of dogs were classified as medium positive, another 37.50% as high positive, and 25.00% fell into the low positive category.

Statistically significant lower values were found when comparing the healthy seronegative group to the healthy seropositive group ($p < 0.0001$), stage I ($p = 0.0003$), stage II ($p < 0.0001$), and stage III–IV ($p < 0.0001$) groups. The healthy seropositive group showed significantly higher values when compared to stage I ($p < 0.0001$) and significantly lower values when compared to stage II ($p < 0.0001$) and stage III–IV ($p = 0.0001$) groups. Significantly lower values were also observed when comparing stage I to stage II ($p < 0.0001$) and stage III–IV ($p < 0.0001$) groups. No statistically significant differences were observed between the stage II and stage III–IV groups ($p = 0.93$).

3.7. IFN-γ Concentration

The results of the IFN-γ concentration are depicted in Tables 1 and 2 and Figure 2. In the healthy seronegative group, all tested dogs (10/10) were classified as IFN-γ-non-producers. Among the healthy seropositive group, 9 out of 15 dogs (60.00%) were considered as IFN-γ-producers. In the stage I group, 50.00% of dogs (12/24) were classified as IFN-γ-producers. In stage II, a total of 9 out of 34 dogs (26.47%) were classified as IFN-γ-producers, while the majority, 73.53%, were non-producers. Similarly, in stage III–IV, only 4 out of 13 dogs (30.77%) were identified as IFN-γ-producers, with 69.23% classified as non-producers.

Supernatants from LSA-stimulated whole blood of healthy seronegative dogs (median: 0, range: [0–109.50] pg/mL) showed significantly lower concentrations of IFN-γ compared to healthy seropositive dogs (median: 204.10, range: [0–4763.00] pg/mL) ($p = 0.001$), dogs in stage I (median: 127.90 pg/mL, range: [0–3998.00] pg/mL) ($p = 0.0002$), and dogs in stage II (median: 9.00 pg/mL, range: [0–5086.00] pg/mL) ($p = 0.015$). However, no differences were observed when comparing IFN-γ concentration between healthy seronegative dogs and dogs in stage III–IV (median: 3.48 pg/mL, range [0–548.80] pg/mL) ($p = 0.12$).

Regarding healthy seropositive dogs, no significant differences were found when comparing their supernatants from LSA-stimulated whole blood results to dogs in stage I ($p = 0.6$) and stage II ($p = 0.06$). However, statistically significant higher values were observed in healthy seropositive dogs when comparing them to dogs in stage III–IV ($p = 0.03$).

Concentrations of IFN-γ from dogs in stage I were significantly higher than concentrations of IFN-γ from dogs in stage II ($p = 0.045$) and stage III–IV ($p = 0.02$). No statistically significant differences were found between IFN-γ concentrations from dogs in stage II and dogs in stage III–IV ($p = 0.5$).

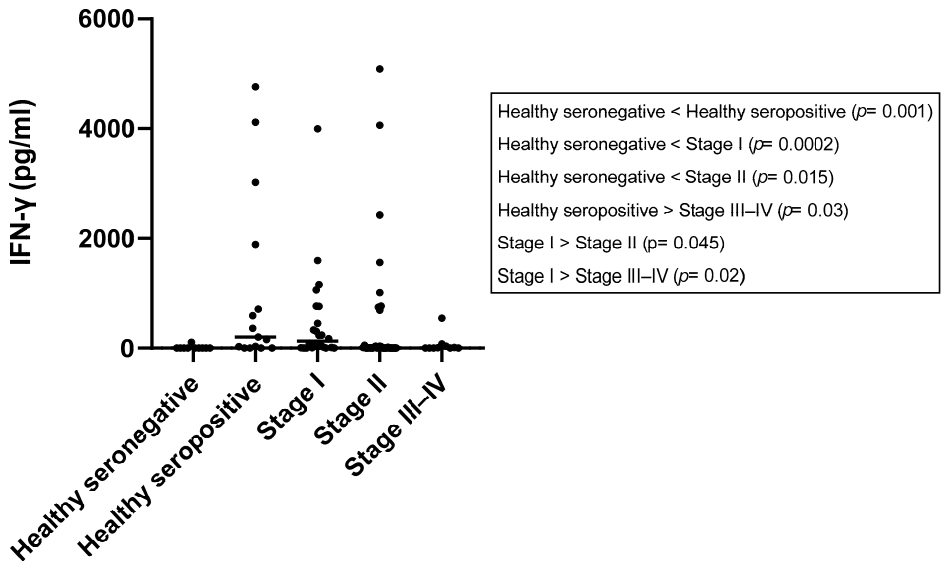


Figure 2. IFN-γ concentrations (median, minimum, and maximum values) after stimulation with LSA in different *L. infantum* states of infection. IFN-γ = interferon-gamma. LSA = *L. infantum* soluble antigen.

3.8. Correlation between Parameters Studied

No significant correlation was observed between the concentration of IFN-γ after LSA stimulation and *L. infantum*-specific antibody levels ($p = 0.107$) when considering all the groups. Similarly, there was no correlation between the NBT rate and antibody levels ($p = 0.088$). Additionally, no correlation was found between the NBT rate and the concentration of IFN-γ after LSA stimulation ($p = 0.087$).

When examining the correlation within specific groups, no significant correlation was observed between the concentration of IFN-γ and *L. infantum*-specific antibody levels in the healthy seronegative group ($p = 0.2$) and in the stage I group ($p = 0.146$). Conversely, a significant negative correlation was observed between IFN-γ and *L. infantum*-specific antibody levels when evaluating the results in the healthy seropositive ($p = 0.028$), the stage II ($p = 0.001$), and stage III–IV groups ($p = 0.002$).

No significant correlation was found between the NBT results and *L. infantum*-specific antibody levels when comparing different groups, including healthy seronegative dogs ($p = 0.778$), the healthy seropositive group ($p = 0.938$), stage I ($p = 0.663$), stage II ($p = 0.9$), and stage III–IV ($p = 0.164$).

Furthermore, no correlation was observed when comparing the NBT results and the concentration of IFN-γ after LSA stimulation within specific groups, including healthy seronegative dogs ($p = 0.8$), healthy seropositive group ($p = 0.609$), stage I ($p = 0.94$), stage II ($p = 0.13$), and stage III–IV ($p = 0.16$).

In terms of qualitative values, a significant positive association was found between IFN-γ-producers and increased NBT results when evaluating all dogs ($p = 0.009$) (Figure 3).

Additionally, there was a negative association between the proportion of the increased NBT rate and ELISA seropositivity when evaluating all dogs (p = 0.035) (Figure 4). A negative association was also found between the IFN-γ-producer status and ELISA positivity (p = 0.04). These associations were not found when evaluating the dogs separately by groups.

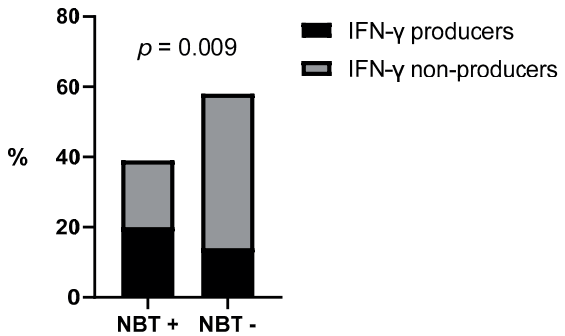


Figure 3. NBT association with IFN-y-producer status. NBT + = increased nitroblue tetrazolium reduction test, NBT − = nitroblue tetrazolium reduction test within normal limits, IFN-y = interferon-gamma.

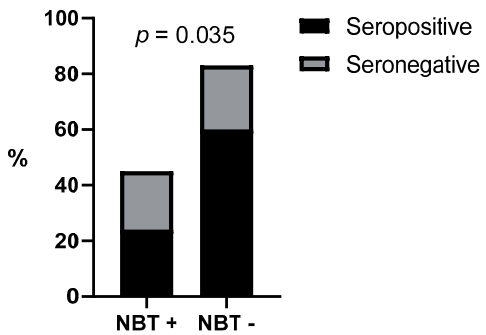


Figure 4. NBT association with the serological status. NBT + = increased nitroblue tetrazolium reduction test, NBT − = nitroblue tetrazolium reduction test within normal limits, ELISA = Enzyme-Linked ImmunoSorbent Assay.

4. Discussion

Neutrophils are suspected to be a key factor for disease development in CanL; however, their role remains poorly understood [2,8]. The objective of this study was to assess neutrophil activation and its relationship with different clinical manifestations ranging from subclinical infection to advanced disease and antibody production and to evaluate if the ability of IFN-γ to stimulate neutrophils correlates with the activation of its oxidative metabolism through NBT measurements. This is the first study to comprehensively evaluate the NBT across all states of this infection, including healthy seropositive and seronegative dogs, and to establish a relationship between antibody levels and the IFN-γ production status.

Regarding the assessment of oxidative metabolism in different clinical stages of leishmaniosis, the present findings revealed that dogs in the mild disease LeishVet stage I

exhibited significantly higher NBT values compared to healthy seronegative and seropositive dogs, as well as to dogs in the moderate and severe disease stages. This indicates elevated oxidative metabolism in neutrophils specifically in dogs with mild, self-limiting disease, whereas this increase was not observed in dogs with moderate-to-very severe disease [37]. Additionally, although the healthy seropositive group showed a significantly lower NBT rate than the stage I group, it still displayed significantly higher values compared to all other groups. These results provide support for the hypothesis that neutrophil activation is closely linked to an effective immune response against leishmaniosis. This hypothesis is further supported by the observed association between the lack of an NBT rate increase and the deterioration of clinical signs during the follow-up of dogs with papular dermatitis. It is important to note that among the 25 dogs, only three exhibited worsening clinical signs necessitating systemic treatment, aligning with existing research portraying papular dermatitis as a predominantly self-resolving condition linked to a robust immune response [39,45–47]. Remarkably, two of these three dogs did not display an elevated NBT rate, while all dogs manifesting with self-limiting disease (22 out of 25) demonstrated NBT values surpassing the established threshold.

The diminished response observed in the healthy seropositive group, when compared to dogs with mild self-limiting disease (papular dermatitis), could potentially be attributed to different states of infection, resulting in varying levels of immune stimulation or activation. Thus, enhanced oxidative metabolism in neutrophils may serve as an indicator of an effective immune response against *Leishmania* infection, further supporting the significance of neutrophils in disease management.

However, it is important to consider that differences in neutrophil activity within the stage I group could potentially arise from variations in age between these dogs and those belonging to the other groups. Notably, dogs with papular dermatitis are markedly younger than their counterparts, boasting a median age of 6 months, with ages ranging from 3 months to 7 years. This is in line with previous studies, as papular dermatitis is consistently diagnosed in dogs under one year of age [39,48,49]. This has been theorized to be attributed to the condition's propensity to manifest during the initial encounter between the parasite and the host's immune system, making it more prevalent in young dogs, particularly in areas where the parasite is endemic [39]. To mitigate the potential bias introduced by age, the statistical analysis was re-executed, focusing exclusively on dogs older than 12 months. This refined analysis yielded outcomes similar to those obtained when considering the entire dog population, despite the limited population of older dogs with papular dermatitis (three cases). Additionally, no significant differences were observed when comparing dogs aged 12 months and above with their younger counterparts within the stage I group. Consequently, the authors consider it unlikely for age to act as a confounding factor in neutrophil activation levels. An alternative hypothesis could be considered, suggesting that the neutrophilic response observed in patients at stage I could be linked to an early phase of the disease rather than a profile of resistance. However, this proposition seems less plausible given that, in the current study, every dog included within the stage I category exclusively displayed papular dermatitis as the solitary clinical manifestation. This particular clinical presentation is commonly associated with a robust and protective immune response. Notably, these dogs spontaneously resolve cutaneous lesions, as evidenced by long-term follow-up, which also indicates an absence of clinical relapse or the emergence of systemic clinical signs, and thus, they are more likely to be representative of an effective immune response and not just an early phase of the disease [39,45–47,49]. To further support this, the long-term follow-up in the current study showed that only three animals out of 25 were considered to get worse without systemic treatment, and the other 22 dogs resolved cutaneous lesions and remained healthy. Additionally, as mentioned previously, an association between the lack of an NBT rate increase and the deterioration of clinical signs was found.

In contrast to stage I and healthy seropositive dogs, dogs in stages II and III–IV did not exhibit significant differences compared to healthy seronegative dogs. This observation

could indicate a correlation between the absence of neutrophil activation and the progression of clinical disease, mirroring the findings obtained while assessing dogs in stage I who exhibited a deterioration in clinical signs and reflecting a non-effective immune phenotype, including immunity exhaustion [7]. Previous studies have demonstrated an increased rate of neutrophil apoptosis in severe clinical stages of CanL, which may contribute to the reduction in the NBT rate [50]. While an initial increase in oxidative metabolism by neutrophils might be viewed as a protective response, if *Leishmania* infection is not controlled, prolonged neutrophil activation can lead to the generation of oxidative stress and the depletion of antioxidant defenses and ultimately trigger neutrophil apoptosis [51]. In addition, in dogs with LeishVet stage III–IV, chronic kidney disease may also contribute to an increased apoptotic rate and reduced neutrophil superoxide production [52,53]. Regardless of the underlying cause, this negatively impacts neutrophil oxidative metabolism, which can significantly compromise the organism's primary defense mechanisms. This impairment ultimately results in a reduced ability to control CanL and likely increases the vulnerability to co-infections [50,52,54,55].

When assessing the total leukocyte and leukocyte differential concentrations, despite observing no distinctions in leukocyte, neutrophil, and eosinophil concentrations across the groups, some differences were found when evaluating the lymphocytes and monocytes. Specifically, healthy seropositive dogs and those with moderate-to-advanced leishmaniosis (stage II and stage III–IV) displayed significantly diminished lymphocyte concentrations compared to both healthy seronegative dogs and those in stage I. This partly concurs with earlier research linking clinical systemic CanL to a reduced lymphocyte concentration, consistent with the present observations in stage II and III-V cohorts [56,57]. This could be due to multifactorial mechanisms, and although the most likely explanation is stress-induced changes in the leukogram, other causes, like lymphocyte entrapment or hindered hematopoiesis attributed to bone marrow parasitism, could also contribute to lymphopenia [57]. It is also worth noting that dogs in the stage I group exhibited lymphocyte concentrations comparable to those of healthy seronegative dogs. This observation suggests the absence of a stress leukogram in dogs with mild self-healing disease. Furthermore, dogs in stage I showed a significant increase in the monocyte concentration compared to seropositive dogs and those in stage II. While elevated monocyte concentrations in infected dogs have been documented previously, particularly in dogs with positive splenic cultures for *L. infantum* [58], our study indicated a distinct pattern: solely dogs in stage I exhibited significantly elevated levels, particularly when contrasted against other infection statuses (healthy seropositive and stage II). This could be explained by the increased mobilization of monocytes linked to an effective cellular immune response. Additionally, a correlation between the NBT rate and leukocyte differential was explored, and while none was found when evaluating the neutrophil and lymphocyte concentrations, an inverse correlation surfaced when evaluating the monocyte concentration, though this was only observed within the stage I group. This negative correlation could be explained by the increased recruitment of monocytes to the infection site induced by activated neutrophils, consequently depleting the circulating monocyte pool [22,23].

Previous research has provided evidence for the crucial role of neutrophils in the early stages of *L. infantum* infection [8,11,59]. These cells form an essential part of the initial nonspecific immune response by internalizing the promastigote and acting as a "trojan horse" to infect definitive host cells, such as macrophages [8,11,59]. However, this process could only occur if the parasite is able to evade or suppress the parasite-killing activity of polymorphonuclear cells, which has been observed to happen primarily through inhibiting or evading its oxidative damage capacity [10,59,60]. The findings of the current study support this hypothesis, as dogs with mild self-healing disease and seropositive healthy dogs exhibited a higher rate of NBT in their circulating white blood cells. This indicates increased neutrophil activity and enhanced parasite-killing capacity through the oxidative burst mechanism. This could explain why the infection was confined to the site of inoculation (papular dermatitis) and systemic disease did not develop [39].

Previous studies on neutrophil oxidative metabolism in CanL have reported conflicting results. Some studies suggested a decrease in oxidative metabolism in infected dogs, while others found increased superoxide production and oxidative metabolism [9,36,61,62]. However, these studies had limitations, such as small sample sizes and the lack of disease severity assessments.

Our study findings align with the conclusions drawn in two more recent publications, further demonstrating that neutrophil oxidative metabolism is dependent on the disease stage [36,50]. In line with the study by Gómez-Ochoa et al., our results indicate that dogs with mild disease-LeishVet stage I exhibited significantly higher neutrophil reactivity compared to both healthy dogs and dogs presenting with severe disease [36]. However, in contrast to the findings of Almeida et al., which reported a significantly increased NBT rate in stage II dogs when compared to healthy and stage IV dogs, our study revealed that both stage II and stages III–IV groups exhibited reduced oxidative metabolism, with no significant differences observed between them or the healthy seronegative group [50]. It is important to note that our study included a much larger population, with 41 dogs in stage II (compared to 15 in the previous study), which reduces the risk of population bias. Additionally, we employed a modified NBT technique that mitigates potential biases by evaluating a minimum of 300 cells per sample, whereas previous studies evaluated a minimum of 100 cells per sample, thus enhancing the reliability of our results in this specific aspect [36,50,62]. Furthermore, our study is the first to perform a direct comparison between dogs in stage I and stage II, revealing a significantly higher proportion of activated neutrophils in dogs with mild stage I disease, as well as to evaluate and compare healthy seropositive dogs with both healthy seronegative and diseased dogs. Interestingly, we found a mild yet significant increase in the NBT rate among healthy seropositive dogs when compared to the healthy seronegative group. Finally, this is also the first study to assess the clinical progression of dogs diagnosed with papular dermatitis and compare it to the NBT rate at diagnosis, showing an association of clinical worsening with a lack of neutrophil activation. These findings highlight the distinct neutrophil activation patterns in different states of *L. infantum* infection in dogs and provide valuable insights into the immune responses in CanL.

Gómez-Ochoa et al. proposed a hypothesis suggesting that the variations in neutrophil oxidative metabolism and its augmentation during the early stages of *L. infantum* infection may be associated with the heightened production of chemotactic agents, such as cytokines, in healthy infected dogs, thereby promoting resistance against infection [36]. IFN-γ is a potent immunoregulatory cytokine that not only modulates the adaptive immune system but also plays a role in enhancing neutrophil functions [29,32]. In agreement with this, when evaluating our results, IFN-γ was found to be increased in dogs with mild self-limiting clinical leishmaniosis.

The production of *Leishmania*-specific IFN-γ in stimulated blood, being this a cytokine associated with a robust Th1 immune response, has been previously correlated with a resistant phenotype or a less-severe clinical presentation [2–4,63–66].

Previous studies demonstrated that different clinical stages of leishmaniosis in dogs were associated with different cytokine profiles in stimulated blood [3,66]. They reported that the IFN-γ concentration was significantly higher in dogs staged I and IIa when compared to more severe clinical stages, although results were not compared to healthy dogs [3]. Our study yielded similar findings, as dogs in stage I exhibited significantly higher concentrations of IFN-γ compared to healthy dogs, dogs in the moderate stage I,I, and dogs in the severe stage III–IV disease categories. Notably, dogs in stage II displayed a mild yet significant increase in IFN-γ concentrations compared to healthy seronegative dogs, whereas no significant differences were observed in dogs in stage III–IV when compared to healthy seronegative dogs. Additionally, the majority of dogs in stage II and stage III–IV were classified as IFN-γ-non-producers. Thus, in line with previous studies, dogs in more advanced clinical stages showed lower levels of IFN-γ, which could reflect a less effective immune response, away from a Th1 phenotype [7,67]. It has been previously reported that,

as disease progresses, T cells develop certain unresponsiveness to *L. infantum* antigens, showing an impairment in CD4+ T cell proliferation and IFN-γ production, called T-cell exhaustion [7,67].

Interestingly, in our study, healthy seropositive dogs displayed elevated IFN-γ concentrations in comparison to both healthy seronegative dogs and dogs in stage III–IV, the latter of which aligns with a recent study performed in seropositive dogs [40]. However, the previous study did not compare the results to healthy seronegative dogs [40]. This observation could indicate the presence of an effective Th1 response that effectively maintains the infection in a subclinical state.

In a recent study performed on humans, the administration of IFN-γ was found to have notable effects on circulating neutrophils, leading to significant changes in gene expression, protein expression, and overall function. These findings indicate an enhancement in neutrophil activity [32]. The results of that study further suggested that IFN-γ not only stimulates NADPH oxidase activity, thereby promoting the oxidative burst, but also impacts Fc receptor-mediated ingestion, pathogen recognition by innate immune receptors, antigen presentation (including MHCI and MHCII), the activation of GBPs (guanylate-binding proteins), the upregulation of NO production, and the augmentation of neutrophil release into circulation [32]. Therefore, it is plausible that IFN-γ has a direct correlation with NBT by stimulating the oxidative burst of white blood cells, which in turn may be associated with an effective immune response through neutrophil reactivity.

In our study, we discovered a significant association between IFN-γ production and an increased NBT rate. Moreover, both factors exhibited a substantial increase in dogs with subclinical or controlled infection (healthy seropositive), as well as dogs with mild self-limiting disease (stage I). These findings lend support to the notion that IFN-γ plays a role in promoting the oxidative metabolism of neutrophils, thereby contributing to an effective immune response [32]. This suggests that the modulation of neutrophil function through IFN-γ-mediated pathways could serve as one of the mechanisms by which the immune system achieves an efficient defense against the disease [32]. Furthermore, the reduction in IFN-γ associated with T-cell exhaustion could also contribute to the lack of an increase in the oxidative metabolism of neutrophils observed in advanced stages [7,32]. The findings of this investigation also revealed an inverse relationship between IFN-γ and levels of *L. infantum*-specific antibody levels across the healthy seropositive, stage II, and stage III–IV groups. Additionally, an association was found between the IFN-γ-producer status and negative ELISA results. This is in agreement with a previous article by Solano-Gallego et al. published in 2016 [3]. These negative correlations and associations could be explained by IFN-γ's role as a marker for an effective Th1 response, primarily characterized as cellular rather than humoral. Conversely, heightened antibody levels align with a robust humoral immune response, linked to other cytokines, such as IL-4, IL-10, and TGF-β [4,6,68]. Overall, these findings contribute to a better understanding of the complex immune response in CanL and emphasize the significance of neutrophil activation and IFN-γ production in disease management. Further research is needed to explore other underlying mechanisms and potential therapeutic targets for enhancing the immune response against *Leishmania* infection.

One limitation of this study was the lack of a sufficient number of dogs in stage IV, preventing a separate evaluation of this group. As a result, dogs in stage IV had to be combined with those in stage III in a sole group. However, the classification of dogs into healthy, mild disease, moderate disease, and severe disease categories still holds relevance. Another limitation is inherent to the NBT technique, which relies on a subjective measurement through light microscopy to assess activated neutrophils. To mitigate potential operator bias, a rigorous approach was adopted: a minimum of 300 cells was evaluated per sample, and at least 50% of the samples were independently reviewed by two different investigators.

5. Conclusions

In conclusion, neutrophil activation, as assessed using NBT, is significantly higher in dogs with mild self-limiting disease, followed by healthy seropositive dogs, indicating enhanced oxidative metabolism in neutrophils in these cases. Additionally, dogs in stage I exhibiting an NBT rate within normal limits were found to be linked to clinical deterioration when contrasted with dogs displaying an elevated NBT rate at the time of diagnosis. On the other hand, dogs in more advanced stages of the disease did not exhibit significant differences in neutrophil activation compared to healthy seronegative dogs. Furthermore, the study demonstrates a relationship between neutrophil activation and the production of IFN-γ, a key cytokine associated with an effective immune response against *Leishmania* infection. This study provides valuable insights into the role of neutrophil activation and its relationship with subclinical states and degrees of disease severity and suggests that neutrophils and oxidative metabolism could play a crucial role in the effective immune response against leishmaniosis.

Author Contributions: Conceptualization, C.B.-B., I.M.-F. and L.S.-G.; investigation, C.B.-B., I.M.-F., J.d.R.-V. and M.B.; formal analysis, I.M.-F.; writing—original draft preparation, C.B.-B. and I.M.-F.; writing—review and editing, M.B. and L.S.-G.; supervision, L.S.-G.; funding acquisition, L.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the Spanish ministry grant, Ministerio de Economía y competitividad, and Fondo Europeo de Desarrollo Regional (FEDER, EU) (AGL2015-68477). This research was also partially funded by Ecuphar veterinaria SLU with funding number CF617624.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Universitat Autònoma de Barcelona (for dogs in group 1, ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (CEAAH 4526, November 2018) and by "Generalitat de Catalunya" (FUE-2018-00944112 i ID KSHYD6LVR, April 2019). For the 10 dogs purchased at Isoquimen S.L., ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (OH-CEEA100 i CEEA 4747, July 2019) and by "Generalitat de Catalunya" (10649, June 2021). For the rest of the dogs, study authorization was obtained from the Spanish authority Agencia Española de Medicamentos y Productos Sanitarios (AEMPS), with the authorization number 008/EPA-2383ESP.

Informed Consent Statement: All dogs included in this study belonged to volunteer owners who had granted permission to a physical examination and blood collection from their dogs through a signed consent form.

Data Availability Statement: Data of the study are available from the corresponding author upon reasonable request.

Acknowledgments: Authors would like to acknowledge Lourdes Alarcón Sánchez and Tamara Rivero Balsera for their priceless technical help and support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasites Vectors* **2011**, *4*, 86. [CrossRef] [PubMed]
2. Toepp, A.J.; Petersen, C.A. The Balancing Act: Immunology of Leishmaniosis. *Res. Vet. Sci.* **2020**, *130*, 19–25. [CrossRef] [PubMed]
3. Solano-Gallego, L.; Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-Specific Production of IFN-γ and IL-10 in Stimulated Blood from Dogs with Clinical Leishmaniosis. *Parasites Vectors* **2016**, *9*, 317. [CrossRef] [PubMed]
4. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, *144*, 95–115. [CrossRef]
5. Alvar, J.; Cañavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine Leishmaniasis. *Adv. Parasitol.* **2004**, *57*, 1–88. [CrossRef]
6. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous Immune Mechanisms in Canine Leishmaniosis Due to *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2015**, *163*, 94–102. [CrossRef]

7. Boggiatto, P.M.; Ramer-Tait, A.E.; Metz, K.; Kramer, E.E.; Gibson-corley, K.; Mullin, K.; Hostetter, J.M.; Gallup, J.M.; Jones, D.E.; Petersen, C.A. Immunologic Indicators of Clinical Progression during Canine *Leishmania infantum* Infection. *Clin. Vaccine Immunol.* **2010**, *17*, 267–273. [CrossRef]
8. Venuprasad, K.; Chattopadhyay, S.; Saha, B. CD28 Signaling in Neutrophil Induces T-Cell Chemotactic Factor(s) Modulating T-Cell Response. *Hum. Immunol.* **2003**, *64*, 38–43. [CrossRef]
9. Brandonisio, O.; Panunzio, M.; Faliero, S.M.; Ceci, L.; Fasanella, A.; Puccini, V. Evaluation of Polymorphonuclear Cell and Monocyte Functions in *Leishmania infantum*-Infected Dogs. *Vet. Immunol. Immunopathol.* **1996**, *53*, 95–103. [CrossRef]
10. Panaro, M.A.; Puccini, V.; Faliero, S.M.; Marzio, R.; Marangi, A.; Lisi, S.; Brandonisio, O. *Leishmania donovani* lipophosphoglycan (LPG) inhibits respiratory burst and chemotaxis of dog phagocytes. *New Microbiol.* **1996**, *19*, 107–112.
11. van Zandbergen, G.; Klinger, M.; Mueller, A.; Dannenberg, S.; Gebert, A.; Solbach, W.; Laskay, T. Cutting Edge: Neutrophil Granulocyte Serves as a Vector for *Leishmania* Entry into Macrophages. *J. Immunol.* **2004**, *173*, 6521–6525. [CrossRef]
12. Mantovani, A.; Cassatella, M.A.; Costantini, C.; Jaillon, S. Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity. *Nat. Rev. Immunol.* **2011**, *11*, 519–531. [CrossRef]
13. Nauseef, W.M.; Borregaard, N. Neutrophils at Work. *Nat. Immunol.* **2014**, *15*, 602–611. [CrossRef]
14. Mócsai, A. Diverse Novel Functions of Neutrophils in Immunity, Inflammation, and Beyond. *J. Exp. Med.* **2013**, *210*, 1289–1299. [CrossRef]
15. Roos, D.; Van Bruggen, R.; Meischl, C. Oxidative Killing of Microbes by Neutrophils. *Microbes Infect.* **2003**, *5*, 1307–1315. [CrossRef] [PubMed]
16. Nauseef, W.M. How Human Neutrophils Kill and Degrade Microbes: An Integrated View. *Immunol. Rev.* **2007**, *219*, 88–102. [CrossRef] [PubMed]
17. Hampton, M.B.; Kettle, A.J.; Winterbourn, C.C. The undefined Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. *Blood* **1998**, *92*, 3007–3017. [CrossRef] [PubMed]
18. Underhill, D.M.; Ozinsky, A. Phagocytosis of Microbes: Complexity in Action. *Annu. Rev. Immunol.* **2002**, *20*, 825–852. [CrossRef] [PubMed]
19. Winterbourn, C.; Kettle, A.; Hampton, M. Reactive Oxygen Species and Neutrophil Function. *Annu. Rev. Biochem.* **2016**, *85*, 765–792. [CrossRef] [PubMed]
20. Babior, B.M. NADPH Oxidase: An Update. *Blood* **1999**, *93*, 1464–1476. [CrossRef]
21. Babior, B.M. The Respiratory Burst Oxidase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2006**, *65*, 49–95. [CrossRef]
22. Wardini, A.B.; Pinto-da-Silva, L.H.; Nadaes, N.R.; Nascimento, M.T.; Roatt, B.M.; Reis, A.B.; Viana, K.F.; Giunchetti, R.C.; Saraiva, E.M. Neutrophil Properties in Healthy and *Leishmania infantum*-Naturally Infected Dogs. *Sci. Rep.* **2019**, *9*, 6247. [CrossRef] [PubMed]
23. Carlsen, E.D.; Liang, Y.; Shelite, T.R.; Walker, D.H.; Melby, P.C.; Soong, L. Permissive and Protective Roles for Neutrophils in Leishmaniasis. *Clin. Exp. Immunol.* **2015**, *182*, 109–118. [CrossRef] [PubMed]
24. Guimaraes-Costa, A.B.; Nascimento, M.T.; Froment, G.S.; Soares, R.P.; Morgado, F.N.; Conceicao-Silva, F.; Saraiva, E. *Leishmania amazonensis* Promastigotes Induce and Are Killed by Neutrophil Extracellular Traps. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 6748–6753. [CrossRef]
25. Pereira, M.; Valério-Bolas, A.; Santos-Mateus, D.; Alexandre-Pires, G.; Santos, M.; Rodrigues, A.; Rocha, H.; Santos, A.; Martins, C.; Tomas, A.; et al. Canine Neutrophils Activate Effector Mechanisms in Response to *Leishmania infantum*. *Vet. Parasitol.* **2017**, *248*, 10–20. [CrossRef]
26. Kaye, P.; Scott, P. Leishmaniasis: Complexity at the Host-Pathogen Interface. *Nat. Rev. Microbiol.* **2011**, *9*, 604–615. [CrossRef]
27. Terrazas, C.; Oghumu, S.; Varikuti, S.; Martinez-Saucedo, D.; Beverley, S.M.; Satoskar, A.R. Uncovering *Leishmania*-Macrophage Interplay Using Imaging Flow Cytometry. *J. Immunol. Methods* **2015**, *423*, 93–98. [CrossRef]
28. Pollard, K.M.; Cauvi, D.M.; Toomey, C.B.; Morris, K.V.; Kono, D.H. Interferon-γ and Systemic Autoimmunity. *Discov. Med.* **2013**, *16*, 123.
29. Schoenborn, J.R.; Wilson, C.B. Regulation of Interferon-Gamma during Innate and Adaptive Immune Responses. *Adv. Immunol.* **2007**, *96*, 41–101. [CrossRef]
30. Hertzog, P.; Forster, S.; Samarajiwa, S. Systems Biology of Interferon Responses. *J. Interferon Cytokine Res.* **2011**, *31*, 5–11. [CrossRef]
31. Baccala, R.; Kono, D.H.; Theofilopoulos, A.N. Interferons as Pathogenic Effectors in Autoimmunity. *Immunol. Rev.* **2005**, *204*, 9–26. [CrossRef] [PubMed]
32. Ambruso, D.R.; Briones, N.J.; Baroffio, A.F.; Murphy, J.R.; Tran, A.D.; Gowan, K.; Sanford, B.; Ellison, M.; Jones, K.L. In Vivo Interferon-Gamma Induced Changes in Gene Expression Dramatically Alter Neutrophil Phenotype. *PLoS ONE* **2022**, *17*, e0263370. [CrossRef] [PubMed]
33. Ellis, T.N.; Beaman, B.L. Interferon-Gamma Activation of Polymorphonuclear Neutrophil Function. *Immunology* **2004**, *112*, 2–12. [CrossRef] [PubMed]
34. Ellison, M.A.; Gearheart, C.M.; Porter, C.C.; Ambruso, D.R. IFN-γ Alters the Expression of Diverse Immunity Related Genes in a Cell Culture Model Designed to Represent Maturing Neutrophils. *PLoS ONE* **2017**, *12*, e0185956. [CrossRef] [PubMed]
35. Gómez-Ochoa, P.; Sabate, D.; Homedes, J.; Ferrer, L. Use of the Nitroblue Tetrazolium Reduction Test for the Evaluation of Domperidone Effects on the Neutrophilic Function of Healthy Dogs. *Vet. Immunol. Immunopathol.* **2012**, *146*, 97–99. [CrossRef]

36. Gómez-Ochoa, P.; Lara, A.; Couto, G.; Marcen, J.M.; Peris, A.; Gascón, M.; Castillo, J.A. The Nitroblue Tetrazolium Reduction Test in Canine Leishmaniosis. *Vet. Parasitol.* **2010**, *172*, 135–138. [CrossRef]
37. Esfandiari, N.; Sharma, R.K.; Saleh, R.A.; Thomas, A.J.; Agarwal, A. Utility of the Nitroblue Tetrazolium Reduction Test for Assessment of Reactive Oxygen Species Production by Seminal Leukocytes and Spermatozoa. *J. Androl.* **2003**, *24*, 862–870. [CrossRef]
38. Muniz-Junqueira, M.I.; de Paula-Coelho, V.N. Meglumine Antimonate Directly Increases Phagocytosis, Superoxide Anion and TNF-Alpha Production, but Only via TNF-Alpha It Indirectly Increases Nitric Oxide Production by Phagocytes of Healthy Individuals, in Vitro. *Int. Immunopharmacol.* **2008**, *8*, 1633–1638. [CrossRef]
39. Martínez-Flórez, I.; Guerrero, M.J.; Dalmau, A.; Cabré, M.; Alcover, M.M.; Berenguer, D.; Good, L.; Fisa, R.; Riera, C.; Ordeix, L.; et al. Effect of Local Administration of Meglumine Antimoniate and Polyhexamethylene Biguanide Alone or in Combination with a Toll-like Receptor 4 Agonist for the Treatment of Papular Dermatitis Due to *Leishmania infantum* in Dogs. *Pathogens* **2023**, *12*, 821. [CrossRef]
40. Baxarias, M.; Jornet-Rius, O.; Donato, G.; Mateu, C.; Alcover, M.M.; Pennisi, M.G.; Solano-Gallego, L. Signalment, Immunological and Parasitological Status and Clinicopathological Findings of *Leishmania*-Seropositive Apparently Healthy Dogs. *Animals* **2023**, *13*, 1649. [CrossRef]
41. Riera, C.; Valladares, J.E.; Gállego, M.; Aisa, M.J.; Castillejo, S.; Fisa, R.; Ribas, N.; Carrió, J.; Alberola, J.; Arboix, M. Serological and Parasitological Follow-Up in Dogs Experimentally Infected with *Leishmania infantum* and Treated with Meglumine Antimoniate. *Vet. Parasitol.* **1999**, *84*, 33–47. [CrossRef] [PubMed]
42. Baxarias, M.; Solano-Gallego, L. Effect of Storage on Nitro Blue Tetrazolium Reduction Test in Dog Blood Samples. *Vet. Clin. Pathol.* **2023**. *online ahead of print*. [CrossRef] [PubMed]
43. Martínez-Orellana, P.; González, N.; Baldassarre, A.; Álvarez-Fernández, A.; Ordeix, L.; Paradies, P.; Soto, M.; Solano-Gallego, L. Humoral Responses and Ex Vivo IFN-γ Production after Canine Whole Blood Stimulation with *Leishmania infantum* Antigen or KMP11 Recombinant Protein. *Vet. Sci.* **2022**, *9*, 116. [CrossRef]
44. Carrillo, E.; Carrasco-Antón, N.; López-Medrano, F.; Salto, E.; Fernández, L.; San Martín, J.V.; Alvar, J.; Aguado, J.M.; Moreno, J. Cytokine Release Assays as Tests for Exposure to *Leishmania*, and for Confirming Cure from Leishmaniasis, in Solid Organ Transplant Recipients. *PLoS Negl. Trop. Dis.* **2015**, *9*, e0004179. [CrossRef] [PubMed]
45. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular Dermatitis Due to *Leishmania infantum* Infection in Seventeen Dogs: Diagnostic Features, Extent of the Infection and Treatment Outcome. *Parasites Vectors* **2014**, *7*, 120. [CrossRef]
46. Ordeix, L.; Rodríguez, A.; Martínez-Orellana, P.; Montserrat-Sangrà, S.; Solano-Gallego, L. Clinical follow up of a series of dogs with papular dermatitis due to *Leishmania infantum*. In Proceedings of the 29th Annual Congress of the ESVD-ECVD, Lausanne, Switzerland, 7–9 September 2017.
47. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular Dermatitis Due to *Leishmania* Spp. Infection in Dogs with Parasite-Specific Cellular Immune Responses. *Vet. Dermatol.* **2005**, *16*, 187–191. [CrossRef] [PubMed]
48. Bottero, E.; Poggi, M.; Viglione, M. Lesioni Papulari Indotte Da *Leishmania* spp. in 8 Cani Giovani. *Veterinaria* **2006**, *20*, 33–36.
49. Noli, C.; Cornegliani, L. Leishmaniosi Bottoniforme. Descrizione Di Cinque Casi Italiani e Confronto Con La Letteratura. *Quad. Dermatol.* **2006**, *1*, 23–26.
50. Almeida, B.F.M.; Narciso, L.G.; Bosco, A.M.; Pereira, P.P.; Braga, E.T.; Avanço, S.V.; Marcondes, M.; Ciarlini, P.C. Neutrophil Dysfunction Varies with the Stage of Canine Visceral Leishmaniosis. *Vet. Parasitol.* **2013**, *196*, 6–12. [CrossRef]
51. Anwar, S.; Whyte, M.K.B. Neutrophil Apoptosis in Infectious Disease. *Exp. Lung Res.* **2007**, *33*, 519–528. [CrossRef]
52. Silva, A.C.R.A.; de Almeida, B.F.M.; Soeiro, C.S.; Ferreira, W.L.; de Lima, V.M.F.; Ciarlini, P.C. Oxidative Stress, Superoxide Production, and Apoptosis of Neutrophils in Dogs with Chronic Kidney Disease. *Can. J. Vet. Res.* **2013**, *77*, 136. [PubMed]
53. Barbosa, T.S.; Mori, C.K.; Ciarlini, P.C. Efeito Inibidor Do Soro Urêmico Sobre o Metabolismo Oxidativo Dos Neutrófilos de Cães. *Arq. Bras. Med. Veterinária Zootec.* **2010**, *62*, 1352–1358. [CrossRef]
54. Kannan, K.; Jain, S.K. Oxidative Stress and Apoptosis. *Pathophysiology* **2000**, *7*, 153–163. [CrossRef]
55. Kennedy, A.D.; Deleo, F.R. Neutrophil Apoptosis and the Resolution of Infection. *Immunol. Res.* **2009**, *43*, 25–61. [CrossRef] [PubMed]
56. Abbehusen, M.M.C.; Almeida, D.A.V.; Solcà, S.M.; Da Silva Pereira, L.; Costa, D.J.; Gil-Santana, L.; Bozza, P.T.; Fraga, D.B.M.; Veras, P.S.T.; Dos-Santos, W.L.C.; et al. Clinical and Immunopathological Findings during Long Term Follow-up in *Leishmania infantum* Experimentally Infected Dogs. *Sci. Rep.* **2017**, *7*, 15914. [CrossRef]
57. Reis, A.B.; Martins-Filho, O.A.; Teixeira-Carvalho, A.; Carvalho, M.G.; Mayrink, W.; França-Silva, J.C.; Giunchetti, R.C.; Genaro, O.; Corrêa-Oliveira, R. Parasite Density and Impaired Biochemical/Hematological Status Are Associated with Severe Clinical Aspects of Canine Visceral Leishmaniasis. *Res. Vet. Sci.* **2006**, *81*, 68–75. [CrossRef] [PubMed]
58. Almeida, V.; Lima, I.; Fraga, D.; Carrillo, E.; Moreno, J.; Dos-Santos, W.L.C. Hematological Changes in Dogs with Visceral Leishmaniasis Are Associated with Increased Ifn-γ and Tnf Gene Expression Levels in the Bone Marrow. *Microorganisms* **2021**, *9*, 1618. [CrossRef]
59. Gueirard, P.; Laplante, A.; Rondeau, C.; Milon, G.; Desjardins, M. Trafficking of *Leishmania donovani* Promastigotes in Non-Lytic Compartments in Neutrophils Enables the Subsequent Transfer of Parasites to Macrophages. *Cell. Microbiol.* **2008**, *10*, 100–111. [CrossRef]

60. Brandonisio, O.; Panaro, M.A.; Marzio, R.; Marangi, A.; Faliero, S.M.; Jirillo, E. Impairment of the Human Phagocyte Oxidative Responses Caused by *Leishmania* Lipophosphoglycan (LPG): In Vitro Studies. *FEMS Immunol. Med. Microbiol.* **1994**, *8*, 57–62. [CrossRef]
61. Vuotto, M.L.; De Luna, R.; Ielpo, M.T.L.; De Sole, P.; Moscatiello, V.; Simeone, I.; Gradoni, L.; Mancino, D. Chemiluminescence Activity in Whole Blood Phagocytes of Dogs Naturally Infected with *Leishmania infantum*. *Luminescence* **2000**, *15*, 251–255. [CrossRef]
62. Ciarlini, P.C.; Valadares, T.C.; Ikeda-Garcia, F.A.; Marcondes, M.; De Lima, V.M.F. Leucograma E Metabolismo Oxidativo dos Neutrófilos de Cães com Leishmaniose Visceral Antes e Após O Tratamento com Antimoniato de Meglumina e Alopurinol. *Ciência Anim. Bras.* **2010**, *11*, 369–375. [CrossRef]
63. Santos-Gomes, G.M.; Rosa, R.; Leandro, C.; Cortes, S.; Romão, P.; Silveira, H. Cytokine Expression during the Outcome of Canine Experimental Infection by *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2002**, *88*, 21–30. [CrossRef]
64. do Nascimento, P.R.P.; Martins, D.R.A.; Monteiro, G.R.G.; Queiroz, P.V.; Freire-Neto, F.P.; Queiroz, J.W.; Morais Lima, Á.L.; Jeronimo, S.M.B. Association of Pro-Inflammatory Cytokines and Iron Regulatory Protein 2 (IRP2) with *Leishmania* Burden in Canine Visceral Leishmaniasis. *PLoS ONE* **2013**, *8*, e73873. [CrossRef] [PubMed]
65. Aslan, H.; Oliveira, F.; Meneses, C.; Castrovinci, P.; Gomes, R.; Teixeira, C.; Derenge, C.A.; Orandle, M.; Gradoni, L.; Oliva, G.; et al. New Insights Into the Transmissibility of *Leishmania infantum* from Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J. Infect. Dis.* **2016**, *213*, 1752–1761. [CrossRef] [PubMed]
66. Martínez-Orellana, P.; Marí-Martorell, D.; Montserrat-Sangrà, S.; Ordeix, L.; Baneth, G.; Solano-Gallego, L. *Leishmania infantum*-Specific IFN-γ Production in Stimulated Blood from Dogs with Clinical Leishmaniosis at Diagnosis and during Treatment. *Vet. Parasitol.* **2017**, *248*, 39–47. [CrossRef]
67. Esch, K.J.; Juelsgaard, R.; Martinez, P.A.; Jones, D.E.; Petersen, C.A. PD-1-Mediated T Cell Exhaustion during Visceral Leishmaniasis Impairs Phagocyte Function. *J. Immunol.* **2013**, *191*, 5542. [CrossRef]
68. Scott, P.; Riley, E.M. Acquired Immunity to Intracellular Protozoa. *Immune Response Infect.* **2014**, *24*, 301–311. [CrossRef]