Universitat Autònoma de Barcelona

“The spectrum of cutaneous manifestations in canine leishmaniosis: insights into diagnosis and immune responses

Laura Ordeix i Esteve

THE SPECTRUM OF CUTANEOUS MANIFESTATIONS IN CANINE LEISHMANIOSIS: INSIGHTS INTO DIAGNOSIS AND IMMUNE RESPONSES

Tesi Doctoral

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INFORMA:

Que el treball de tesi doctoral titulat

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del que és autora la llicenciada en veterinària

Laura Ordeix i Esteve

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 què així consti, signo el present informe a Bellaterra, 21 de setembre de 2018.

Dra. Laia Maria Solano Gallego
Directora

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Doctoranda

A la meva mare li deia…

"Mama, jo vull ser metge d'animals"

A la meva filla li dic …

"Maria, jo vull ser doctora"

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LIST OF ABBREVIATIONS

ADCL	Anergic Diffuse Cutaneous Leishmaniasis
CanL	Canine Leishmaniosis
CD	Cluster of Differentiation
cDNA	Complement Deoxyribonucleic Acid
CL	Cutaneous Leishmaniasis
ConA	Concanavalin A
DAMPs	Damage-Associated Molecular Patterns
DCL	Disseminated Cutaneous Leishmaniasis
DCs	Dendritic Cells
ddCt	Delta-Delta-Cycle threshold
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assay
GWA	Genome-Wide Association
HE	Haematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HPF	High Power Field
IFAT	Indirect Fluorescent Antibody Test
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IRF-5	interferon regulatory factor-5
LPG	Lipophosphoglycan
LR	Leishmaniasis Recidivans
LSA	*L. infantum* Soluble Antigen
LST	Leishmanin Skin Test
ML	Mucosal Leishmaniasis

mRNA	Messenger Ribonucleic Acid
NK	Natural Killer
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
P-MAPA	Protein aggregate Magnesium–Ammonium Phospholinoleate–palmitoleate Anhydride
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
PD-L1	Programmed Death Ligand 1
PKDL	Post-Kala-azar Dermal Leishmaniasis
PRR	Pattern Recognition Receptor
qPCR	quantitative PCR
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT	Room Temperature
SD	Standard Deviation
TGF-β	Transforming Growth Factor betta
Th	T helper
TLRs	Toll Like Receptors
TNF-α	Tumor Necrosis Factor alpha
UK	United Kingdom
UPC	Urinary Protein/Creatinine
USA	United States of America
VL	Visceral Leishmaniasis
WBA	Whole Blood Assay
WHO	World Health Organization

SUMMARY

Canine leishmaniosis (CanL) is a protozoan infectious disease caused by *Leishmania infantum* in the Mediterranean basin among other geographical areas. This infection can manifest as chronic subclinical infection, self-limiting disease or severe illness. In addition, a wide range of clinical disease has been documented in dogs. As in human beings, the canine immunological response might dictate the wide range of clinical manifestations associated with this infection. Effective T helper 1 specific immune response is associated with control of parasite proliferation and disease progression. However, the role of innate immune response in instructing the polarization of the T helper response should not be ignored. Cutaneous manifestations are among the most common clinical signs of CanL and a wide range of dermatological problems is encountered. In an endemic area, papular dermatitis is one typical manifestation of CanL and is considered a mild manifestation of the disease associated with favorable prognosis (stage I leishmaniosis) while other common cutaneous clinical signs such as exfoliative or ulcerative dermatitis appear to be associated with moderate to severe disease.

The hypothesis of this doctoral thesis was that dogs with papular dermatitis as the sole clinical manifestation were dogs with a distinctive innate and adaptive immune response able to control parasite infection and disease progression in comparison with dogs with more severe cutaneous manifestations. It was also hypothesized that leishmanin skin test (LST) positive reaction in resistant dogs was histoimmunologically similar to papular dermatitis. The last hypothesis of this doctoral thesis was that papular dermatitis may be more common in breeds known to be resistant to *Leishmania* infection. Therefore, the general objectives of this doctoral thesis were to compare clinical, pathological, parasitological and immunological parameters of dogs with papular dermatitis as the sole clinical manifestation *versus* dogs with more severe cutaneous manifestations; to evaluate the histoimmunological features of positive LST reactions of resistant dogs such as Ibizan hounds; and to evaluate the prevalence of papular dermatitis in dogs living in endemic areas.

The specific objectives were: 1) to evaluate and compare clinical, pathological, parasitological and systemic immunological parameters in sick dogs with different severities of CanL with cutaneous manifestations including papular, exfoliative and ulcerative dermatitis (chapters 3, 4 and 6); 2) to determine, the prevalence of papular dermatitis in resistant Ibizan hound in comparison with other dog breeds (chapter 5); 3) to assess and compare the expression of several immune genes in the clinically-lesioned and normal-looking skin of dogs with different severities of CanL with cutaneous manifestations including papular, exfoliative and ulcerative dermatitis

(chapter 6); 4) to investigate the histological features and the expression of several immune genes in positive LST reactions of Ibizan hound dogs (chapters 7 and 8).

Descriptive studies presented in the present doctoral thesis corroborated those clinical aspects of papular dermatitis previously reported in the literature. Papular dermatitis presented homogeneous clinicopathological pattern being the presence of papules, with the "volcanic" appearance, on sparsely haired skin in young dogs the most distinctive feature. Characteristically, dogs solely afflicted with this condition did not present clinicopathological abnormalities indicative of disease severity opposite with findings encountered in dogs with more severe cutaneous manifestations (chapters 4, 5 and 7).

In this doctoral thesis, new diagnostic results on canine papular dermatitis when compared with exfoliative-ulcerative dermatitis were found. Cytological examination with *Leishmania*-specific quantitative polymerase chain reaction (qPCR) on stained smears permitted the confirmation of the infectious nature of the papules in the majority of cases suggesting this combination technique as a promising diagnostic tool (chapter 5). Moreover, the histopathological studies showed that papular lesions were characterized by a nodular to diffuse pyogranulomatous dermatitis and granuloma formation with low parasite load demonstrated by means of *Leishmania*-specific qPCR, whereas a perivascular to interstitial lymphoplasmacytic dermatitis characterized lesioned skin of more severe afflicted dogs presenting exfoliative or ulcerative dermatitis. Moreover, normal-looking skin of dogs with papular dermatitis was less frequently inflamed and presented a lower parasite density than normal-looking skin of more severe sick dogs (chapters 3 and 4).

Chapters 4, 5 and 6 supported the adaptive immunological findings described previously in the literature for papular dermatitis and stage I leishmaniosis. In fact, a low or absent humoral immune response was documented in dogs with papular dermatitis while high antibody levels were found in dogs with exfoliative-ulcerative dermatitis. In addition, dogs with papular dermatitis showed a predominant cell-mediated immune response characterized by positive LST positive reactions. However, this doctoral thesis described for the first time, a high blood parasite specific IFN-γ production in dogs with papular dermatitis while an absent or reduced cell mediated immunity was found in dogs with more severe cutaneous manifestations (chapters 5 and 6). In addition, a significant slight agreement between LST, IFN-γ production and papular dermatitis was originally documented (chapter 5).

Papular dermatitis was more frequently diagnosed in young Ibizan hounds than in dogs belonging to other breeds in chapter 5.

In chapter 6, a study evaluating the expression of several immune genes in clinically-lesioned and paired normal-looking skin of dogs with papular dermatitis and more severe diseased dogs was described. Interestingly, this study demonstrated a distinct pattern of immune genes' expression in the skin of dogs with papular dermatitis and stage I leishmaniosis compared with more severe sick dogs. Papules were characterized by higher TLR2, TLR4, IL-10 and IFN-γ transcripts whereas TLR7 was downregulated and PD-L1 transcript was similar in comparison with more severe cutaneous lesions. On the other hand, normal-looking skin from dogs with papular dermatitis presented lower expression of TLR2, TLR7, IL-10, IFN-γ and PD-L1 than more severe sick dogs whereas TLR4 transcript was similar among both groups. Although the nodular to diffuse pattern with granuloma formation was not observed in LST positive reactions, the analysis of the expression of the same immune genes showed a similar pattern of expression as found in papules (chapters 7 and 8).

In conclusion, this doctoral thesis demonstrated that dogs with papular dermatitis and stage I leishmaniosis presented a distinctive innate and adaptive immune response as well as clinicopathological and parasitological features when compared with more severe sick dogs that protects them against parasite proliferation and disease progression. In addition, a similar local immune response between papular lesions and LST positive reactions from resistant dogs was demonstrated, suggesting LST positive reactions as a surrogate of cutaneous lesions in dogs with a protective immunity. Furthermore, a higher frequency of papular dermatitis was encountered in young Ibizan hounds than in dogs of other breeds, suggesting that the immunologic background of resistant dogs predisposes them to the development of papular dermatitis as the sole clinical sign of *Leishmania* infection.

RESUM

La leishmaniosis canina és una malaltia infecciosa que en la conca mediterrània, entre d'altres àrees geogràfiques, està causada per un protozou anomenat *Leishmania infantum*. Aquesta infecció es pot manifestar com una infecció subclínica crònica, una malaltia auto limitant o una malaltia greu. A més a més, s'ha documentat una àmplia gamma de malalties clíniques associades a la leishmaniosis canina. Com succeeix en els éssers humans, és la resposta immunològica la que dirigeix l'aparició d'un ampli ventall de manifestacions clíniques associat a aquest infecció en el gos. Una resposta immunitària cel·lular de tipus 1 específica i eficaç s'associa amb el control de la proliferació del paràsit i progressió de la malaltia. No obstant això, no s'ha d'ignorar el paper de la resposta immunitària innata en la polarització de la resposta adaptativa. Les manifestacions cutànies són el signe clínic més habitual de la leishmaniosis canina i es poden diagnosticar una àmplia gamma de problemes dermatològics. En una regió endèmica, la dermatitis papular és considera una manifestació típica de la leishmaniosis canina. Aquest problema dermatològic és tipificat com una manifestació lleu de la malaltia associat a un pronòstic favorable (estadi I de la leishmaniosis), mentre que altres signes clínics cutanis freqüents, com una dermatitis descamativa o ulcerativa, semblen ser associats a una malaltia de moderada a greu.

La hipòtesi d'aquesta tesi doctoral va ser que els gossos amb dermatitis papular com a única manifestació clínica eren gossos amb una resposta immunitària innata i adaptativa particular en comparació amb gossos amb manifestacions cutànies més greus. Aquesta resposta immunitària conferiria, doncs, la capacitat per controlar la proliferació parasitària i la progressió de la malaltia. També es va formular la hipòtesi que les reaccions positives obtingudes en la prova de la leishmanina en gossos resistents fos similar des del punt de vista histològic i immunològic a la dermatitis papular. La última hipòtesi d'aquesta tesi doctoral va ser que la dermatitis papular pogués ser més comú en races conegudes per ser resistents a la infecció per *Leishmania*. Per tant, els objectius generals d'aquesta tesi doctoral van ser comparar els paràmetres clínics, patològics, parasitològics i immunològics observats en gossos amb dermatitis papular com a única manifestació clínica amb aquells observats en gossos amb una manifestació cutània més greu; avaluar els trets histològics i immunològics de les reaccions positives a la leishmanina obtingudes en gossos resistents, com per exemple en el ca eivissenc; i en fi, avaluar la prevalença de la dermatitis papular en gossos que viuen en àrees endèmiques.

Els objectius específics foren: 1) avaluar i comparar paràmetres clínics, patològics, parasitològics i immunològics sistèmics en gossos malalts amb diferents graus de severitat de la leishmaniosis canina amb manifestacions cutànies incloent dermatitis papular, dermatitis

descamativa i ulcerativa (capítols 3, 4 i 6); 2) determinar la prevalença de la dermatitis papular en el ca eivissenc en comparació amb altres races de gossos (capítol 5); 3) avaluar i comparar l'expressió de diversos gens immunitaris en la pell clínicament lesionada i d'aspecte normal de gossos amb diferents graus de severitat de la leishmaniosis canina amb manifestacions cutànies incloent dermatitis papular, dermatitis descamativa i ulcerativa (capítol 6); 4) investigar la histologia i l'expressió de diversos gens immunitaris en reaccions positives al test de la leishmanina en el ca eivissenc (capítols 7 i 8).

Els estudis descriptius presentats en la tesi doctoral corroboraven aquells aspectes clínics de la dermatitis papular senyalats anteriorment en la literatura científica. La dermatitis papular presentava un patró clinicopatològic homogeni, sent la presència de pàpules, amb la típica aparença de "volcà", en la pell pobre en pèl de gossos joves, el tret més distintiu. Característicament, i contrari amb les troballes en gossos amb malaltia cutània més greu, els gossos afligits únicament amb aquesta condició no presentaven anomalies clinicopatològiques hemàtiques indicatives de la gravetat de la malaltia (capítols 4, 5 i 7).

En aquesta tesi doctoral, es varen descriure aspectes sobre el diagnòstic directe de la dermatitis papular nous. Es va poder confirmar la naturalesa infecciosa de les pàpules a través de l'anàlisi quantitatiu de la reacció en cadena de la polimerasa específica per *Leishmania* (qPCR) realitzat en frotis tenyits utilitzats anteriorment pel seu examen citològic, suggerint aquesta tècnica combinada com una prometedora eina diagnòstica (capítol 5). Els estudis histopatològics varen demostrar que les pàpules es caracteritzaven per una dermatitis de nodular a difusa piogranulomatosa amb formació de granulomes amb una càrrega parasitaria baixa demostrada mitjançant *Leishmania*-qPCR. Les lesions en gossos amb malaltia més greu, en canvi, es caracteritzaven per una dermatitis de perivascular a intersticial limfoplasmacítica. D'altra banda, la pell d'aspecte normal de gossos amb dermatitis papular es va mostrar menys freqüentment inflamada i amb una densitat parasitaria més baixa comparada amb la pell d'aspecte normal de gossos amb malaltia més greu (capítols 3 i 4).

El capítols 4, 5 i 6 descriuen resultats que suporten les troballes immunològiques sistèmiques de gossos amb dermatitis papular ja descrits anteriorment en la literatura científica. De fet, es va observar una resposta immunitària humoral baixa o absent en gossos amb dermatitis papular mentre que en els gossos amb dermatitis descamativa o ulcerativa es varen trobar nivells alts d'anticossos. A més, gossos amb dermatitis papular varen mostrar una resposta immunitària predominantment cel·lular caracteritzada per la presencia de reaccions positives a la leishmanina. No obstant això, aquesta tesi doctoral descriu per primera vegada, una producció freqüent i elevada

d'IFN-γ en sang estimulada de gossos amb dermatitis papular mentre que en gossos amb manifestacions cutànies més greus aquesta producció va ser absent o reduïda (capítols 5 i 6). A més, es va poder documentar per primer cop una lleugera correlació entre la leishmanina, la producció d'IFN-γ en sang i la dermatitis papular (capítol 5).

El capítol 5 descriu com la dermatitis papular es va diagnosticar més freqüentment en un grup de gossos ca eivissencs joves que en gossos que pertanyien a altres races.

El capítol 6 descriu un estudi en el que s'avalua l'expressió de diversos gens immunitaris en la pell clínicament lesionada in en la pell amb aspecte normal de gossos amb dermatitis papular i en gossos amb manifestacions cutànies més greus. Curiosament, aquest estudi va demostrar un patró diferent de l'expressió dels gens immunitaris a la pell de gossos amb dermatitis papular i estadi I de la leishmaniosis en comparació amb gossos malalts més greus. Es va observar una expressió superior de TLR2, TLR4, IL-10 i IFN-γ, una expressió inferior de TLR7 i una expressió similar de PD-L1 en les pàpules en comparació amb les lesions cutànies més greus. D'altra banda, la pell d'aspecte normal de gossos amb dermatitis papular presentava una expressió més baixa de TLR2, TLR7, IL-10, IFN-γ i PD-L1 que els gossos malalts més greus mentre que l'expressió de TLR4 va ser similar entre ambdós grups. Encara que les reaccions positives a la leishmanina no presentessin un patró inflamatori de dermatitis de nodular a difusa piogranulomatosa amb formació de granulomes, l'anàlisi de l'expressió gènica va presentar un patró de l'expressió dels gens immunitaris similar a l'observat en les pàpules (capítols 7 i 8).

En conclusió, aquesta tesi demostra que els gossos amb dermatitis papular i estadi I de la leishmaniosis presenten una resposta immunitària innata i adaptativa particular, així com trets clinicopatològics i parasitològics, que els protegeix contra la proliferació del paràsit i la progressió de la malaltia en comparació amb gossos malalts més greus. A més, la resposta immunitària local en les reaccions positives en la leishmanina de gossos resistents és similar a la de les lesions papulars, el que suggereix que les reaccions positives en la leishmanina poden ser un substitut de les lesions cutànies en gossos amb una immunitat protectora. A més, la major freqüència de dermatitis papular trobada en gossos ca eivissencs joves en comparació amb gossos d'altres races, suggereix que la resposta immunològica de gossos resistents els predisposa al desenvolupament de la dermatitis papular com a únic signe clínic de la infecció per *Leishmania*.

RESUMEN

La leishmaniosis canina es una enfermedad infecciosa que, en la cuenca mediterránea entre otras áreas geográficas, está causada por el protozoo *Leishmania infantum*. Esta infección puede manifestarse como una infección subclínica crónica, una enfermedad auto limitante o una enfermedad severa. Además, se ha documentado una amplia gama de manifestaciones clínicas de la enfermedad en el perro. Como en los seres humanos, la respuesta inmunológica del perro es la que dicta la amplia gama de manifestaciones clínicas asociada a esta infección. Una respuesta inmunitaria celular de tipo 1 específica y eficaz se asocia al control de la proliferación del parásito y la progresión de la enfermedad. Sin embargo, no se debe ignorar el papel de la respuesta inmune innata al instruir la polarización de la respuesta adaptativa. La manifestación cutánea está entre los signos clínicos más comunes de la leishmaniosis canina y se han diagnosticado una amplia gama de problemas dermatológicos. En un área endémica, la dermatitis papular es una manifestación típica de la leishmaniosis canina y se considera una manifestación leve de la enfermedad asociada a pronóstico favorable (estadio I de la leishmaniosis), mientras que otras manifestaciones clínicas cutáneas comunes tales como una dermatitis exfoliativa o ulcerativa aparecen asociadas a enfermedad de moderada a grave.

La hipótesis de esta tesis doctoral fue que los perros con dermatitis papular como única manifestación clínica presentasen una respuesta inmunitaria innata y adaptativa distintiva capaz de controlar la proliferación del parásito y la progresión de la enfermedad en comparación con los perros con manifestaciones cutáneas más severas. También se realizó la hipótesis que las reacciones positivas a la leishmanina en perros resistentes fueran histológica e immunológicamente similares a la dermatitis papular. La última hipótesis de esta tesis doctoral fue que la dermatitis papular fuera más común en razas resistentes a la infección por *Leishmania*. Por lo tanto, los objetivos generales de esta tesis doctoral fueron comparar los parámetros clínicos, patológicos, parasitológicos e inmunológicos de perros con dermatitis papular como única manifestación clínica *versus* perros con formas clínicas cutáneas más severas; evaluar las características histológicas e immunológicas de reacciones positivas al test de la leishmanina en perros resistentes como son los podencos ibicencos; evaluar la prevalencia de la dermatitis papular en perros que viven en una área endémica.

Los objetivos específicos fueron: 1) evaluar y comparar parámetros clínicos, patológicos, parasitológicos e inmunológicos sistémicos en perros enfermos con diferentes severidades de leishmaniosis canina con manifestaciones cutáneas incluyendo la dermatitis papular y la dermatitis exfoliativa y ulcerativa (capítulos 3, 4 y 6); 2) determinar la prevalencia de la dermatitis papular en podencos ibicencos resistentes en comparación con otras razas de perros (capítulo 5); 3) evaluar y

comparar la expresión de varios genes inmunes en la piel clínicamente lesionada y de aspecto normal de perros con diferentes severidades de leishmaniosis canina con manifestaciones cutáneas incluyendo la dermatitis papular y la dermatitis exfoliativa y ulcerativa (capítulo 6); 4) investigar las características histológicas y la expresión de varios genes inmunes en las reacciones positivas al test de la leishmanina en perros podenco ibicenco (capítulos 7 y 8).

Los estudios descriptivos presentados en esta tesis doctoral corroboraron los aspectos clínicos de la dermatitis papular descritos previamente en la literatura científica. La dermatitis papular presentó un patrón clínicopatológico homogéneo siendo la presencia de pápulas, con la apariencia de un "volcan", en piel escasamente peluda de perros jóvenes la característica más distintiva. Típicamente, y opuesto a los hallazgos encontrados en perros con más severidad, los perros afligidos únicamente con esta condición no presentaron anormalidades clinicopatológicas indicativas de severidad (capítulos 4, 5 y 7).

Esta tesis doctoral describe aspectos nuevos sobre el diagnóstico directo de la dermatitis papular. La prueba cuantitativa de la reacción en cadena de la polimerasa (qPCR) específica para *Leishmania* realizada en frotis teñidos permitió la confirmación de la naturaleza infecciosa de las pápulas en la mayoría de los casos, sugiriendo esta técnica combinada como una herramienta diagnóstica muy útil (capítulo 5). Por otra parte, los estudios histopatológicos mostraron que las lesiones papulares se caracterizaban por ser una dermatitis piogranulomatosa de nodular a difusa con formación de granulomas con escasa carga parasitaria, demostrado por medio de qPCR específica para *Leishmania*, mientras que la dermatitis exfoliativa y ulcerativa se caracterizaban principalmente por una dermatitis de perivascular a intersticial linfoplasmacítica. Además, la piel de apariencia normal de perros con dermatitis papular presentaba menos frecuentemente una inflamación microscópica y una densidad parasitaria más baja que la piel de apariencia normal de perros enfermos más severos (capítulos 3 y 4).

Los capítulos 4, 5 y 6 apoyaron los hallazgos inmunológicos descritos anteriormente en la literatura científica para la dermatitis papular y estadio I de la leishmaniosis. De hecho, los perros con dermatitis papular presentaron escasa o nula respuesta inmune humoral, mientras que en perros con dermatitis exfoliativa o ulcerativa se evidenciaron niveles altos de anticuerpos. Además, los perros con dermatitis papular mostraron una predominante respuesta inmune adaptativa mediada por células caracterizada por reacciones positivas a la prueba de la leishmanina. Sin embargo, esta tesis doctoral describió por primera vez, una producción de IFN-γ más frecuente e intensa en perros con dermatitis papular, mientras que la respuesta inmune mediada por células fue ausente o reducida en perros con manifestaciones cutáneas más severas (capítulos 5 y 6). Además,

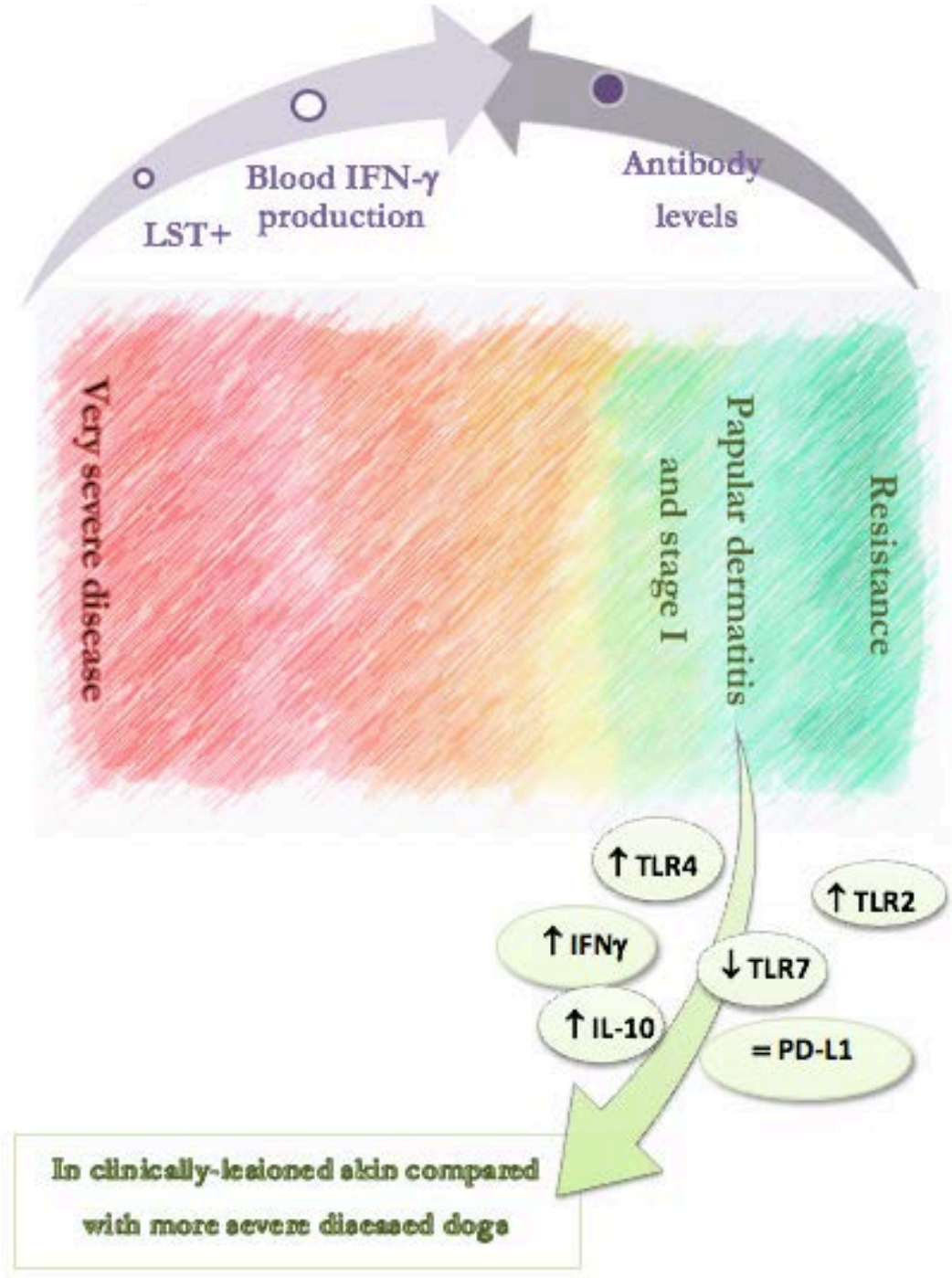
se documentó por primera vez una relación ligera entre la prueba de la leishmanina, la producción de IFN-γ y la dermatitis papular (capítulo 5).

La dermatitis papular fue diagnosticada más frecuentemente en los podencos ibicencos jóvenes en comparación con los perros pertenecientes a otras razas (capítulo 5).

En el capítulo 6 se describe un estudio destinado a evaluar la expresión de varios genes inmunes en la piel clínicamente lesionada y en la piel con apariencia normal de perros con dermatitis papular y en perros con manifestaciones cutáneas más severas. Este estudio demostró un patrón distinto de la expresión de los genes inmunes en la piel de perros con dermatitis papular y estadio I de la leishmaniosis comparados con perros enfermos más severos. Las pápulas se caracterizaron por una mayor expresión de TLR2, TLR4, IL-10 e IFN-γ, mientras que la expresión del TLR7 fue menor y la del PD-L1 similar en comparación con las lesiones cutáneas más severas. Por otro lado, la piel de aspecto normal de los perros con dermatitis papular presentó una menor expresión de TLR2, TLR7, IL-10, IFN-γ y PD-L1 que aquélla de perros enfermos más severos mientras que la transcripción del TLR4 fue similar entre ambos grupos. Aunque en las reacciones positivas a la leishmanina no se observó el mismo patrón histológico que en las lesiones papulares, el análisis de la expresión de los mismos genes inmunes mostró un patrón similar de expresión como el que se observó en las pápulas (capítulos 7 y 8).

En conclusión, esta tesis doctoral demuestra que los perros con dermatitis papular y estadio I de la leishmaniosis presentan una respuesta inmunitaria innata y adaptativa distintiva, así como características clinicopatológicas y parasitológicas, comparados con perros enfermos más severos que los protege contra la proliferación del parásito y la progresión de la enfermedad. Además, la respuesta inmune local observada en las reacciones positivas a la leishmanina en perros resistentes es similar a la observada en las lesiones papulares, sugiriendo que las reacciones positivas a la leishmanina podrían ser un sustituto de las lesiones cutáneas en perros con inmunidad protectora. Además, se encontró una mayor frecuencia de dermatitis papular en podencos ibicencos jóvenes que en perros de otras razas, lo que sugiere que el fondo inmunológico de los perros resistentes los predispone al desarrollo de la dermatitis papular como único signo clínico de la infección por *Leishmania*.

GRAPHICAL ABSTRACT



The particular place of papular dermatitis in the immunological and clinical spectrum of canine leishmaniosis

CHAPTER 1
GENERAL INTRODUCTION

The World Health Organization define leishmaniases as a group of diseases caused by more than 20 *Leishmania* species (WHO, 2018). These parasites are transmitted to humans by the bites of the infected female phlebotomine sand-fly. However, this is not just a disease of humans and *Leishmania* can infect other mammals, therefore, some *Leishmania* infections are zoonotic. Among those mammals, domestic dogs are the most important mammalian reservoir for human visceral leishmaniasis, and they also develop the disease.

Most people, as well as dogs, infected by the parasite do not develop illlness at all in their life. Therefore, the term leishmaniases refers to the fact of becoming sick due to a *Leishmania* infection and not the mere fact of being infected with the parasite (WHO, 2018).

Etiology

Leishmaniases are caused by different species of a protozoan of the genus *Leishmania* (class *Kinetoplasta*, family *Trypanosomatidae*) (Baneth and Solano-Gallego, 2012). Like other protozoan parasites, *Leishmania* have a digenetic life cycle involving a mammalian host and an insect vector (Sunter and Gull, 2017). The natural vectors of *Leishmania* parasites are phlebotomine sandflies of the genus *Phlebotomus* in the Old World and *Lutzomya* in the New World (Claborn, 2010). Though several species, and even other genera, have been suggested as potential vectors, the accepted criteria for incriminating vectors are rigorous and difficult to satisfy (Claborn, 2010). *Leishmania* have two forms, exemplified by the motile flagellated promastigote in the sand fly, being the metacyclic promastigote the infective form, and the intracellular non-flagellated amastigote in the mammalian host (Sunter and Gull, 2017).

The different species of *Leishmania* reported to be associated to human leishmaniasis are often grouped together depending on the geographical distribution and on the nature of the disease that they cause (cutaneous, mucosal, and visceral or kala-azar leishmaniasis) (Table 1.1) (Burza et al., 2018).

Leishmania infantum is the causative agent of canine leishmaniosis (CanL) in the old and new world (Baneth and Solano-Gallego, 2012). *L. infantum* MON- 1 is the most frequent and the largest extending zymodeme found in dogs in the Mediterranean basin (Aït-Oudhia et al., 2011). Other species of *Leishmania* have been documented to infect dogs in different geographical areas, including *L. donovani* (Jambulingam et al., 2017), *L. tropica* (Bamorovat et al., 2015; Baneth et al., 2017), *L. major* (Baneth et al., 2017), *L. braziliensis* (Velez et al., 2012), *L. peruviana*, *L. panamensis*

(Velez et al., 2012), and *L. amazonensis* (Baneth and Solano-Gallego, 2012). However, species different than *L. infantum* are considered rare causes of leishmaniosis in domestic dogs.

Table 1-1. The vector, origin and disease of the most common *Leishmania* species affecting human beings. Adapted from Burza et al., 2018.

Specie	***Geographical distribution***	***Clinical form***
L. infantum	China, southern Europe, Brazil, and South America for visceral and cutaneous; Central America for cutaneous form	VL, CL, and MC
L. donovani	India, Bangladesh, Ethiopia, Sudan, and South Sudan	VL and PKDL
L. braziliensis	South America	CL, ML, ADCL, and LR
L. mexicana	South America	CL, ADCL, and DCL
L. major	Iran, Saudi Arabia, north Africa, the Middle East, central Asia, and west Africa	CL
L. tropica	Eastern Mediterranean, the Middle East, and northeastern and southern Africa	CL, LR, and rarely VL
L. aethiopica	Ethiopia and Kenya	CL, ADCL, DCL, and oronasal CL
L. amazonensis	South America	CL, DCL, and ADCL
L. guyanensis	South America	CL, DCL, and ML

VL: visceral leishmaniasis; CL: cutaneous leishmaniasis; ML: mucosal leishmaniasis; ADCL: anergic diffuse cutaneous leishmaniasis; LR: leishmaniasis recidivans; DCL: disseminated cutaneous leishmaniasis; PKDL: post-kala-azar dermal leishmaniasis

Epidemiology

Human leishmaniasis affect the world's poorest population living mainly in rural and suburban areas (Burza et al., 2018). Human visceral leishmaniasis is worldwide distributed, however in 2014, more than 90% of new cases reported to WHO occurred in six countries: Brazil, Ethiopia, India, Somalia, South Sudan and Sudan (WHO, 2018). The majority of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic. Almost 90% of mucosal leishmaniasis cases occurs in the Plurinational State of Bolivia, Brazil and Peru (WHO, 2018).

Domestic dogs are considered the main reservoirs for human visceral leishmaniosis due to *L. infantum* in the Mediterranean basin, Middle East, and South America (Baneth and Solano-Gallego, 2012). Although *L. infantum* infection has been reported in many domestic and wild animals from the Old and New Worlds, the potential importance of these animals as reservoirs hosts in the epidemiology of *L. infantum* infection remains uncertain (Baneth and Solano-Gallego, 2012). Therefore, the ability to transmit *Leishmania* to feeding sandflies has been confirmed for humans, black rats, domestic cats, crab-eating foxes, and opossums (Quinnell and Courtenay, 2009; Millán et al., 2014).

Leishmania infantum infection in dogs in endemic areas is widespread (Baneth et al., 2008). Prevalence of infection determined by demonstration of specific humoral and/or cellular immune responses and/or presence of parasite DNA in several tissues ranges from 70% to 90% in the Mediterranean basin (Solano-Gallego et al., 2001; Cardoso et al., 2004; Dedet et al., 2013; Çanakçı et al., 2016). When favorable conditions for disease transmission (such as high vector sand-fly and canine densities) are present, the infection spreads quickly and extensively among the dog population (Baneth et al., 2008). On the contrary, in other areas with less favorable environmental conditions for transmission and vectors, infection is not as highly prevalent (Espejo et al., 2015). Moreover, it is important to remark that CanL is often diagnosed in non-endemic countries, such as Holland (Slappendel, 1988), the United Kingdom (Shaw et al., 2009), Germany (Mencke, 2011) and Sweden (Englund and Pringle, 2003). This mostly is due to the importation or transport of infected dogs from an endemic area to countries where no natural transmission occurs (Englund and Pringle, 2003; Baneth et al., 2008). The risk for autochthonous transmission from traveled dogs in the absence of sand-fly vectors is probably small; however, infection has been described in dogs with no travel history living together with imported infected dogs (venereal transmission), pups born from infected dams or in dogs receiving blood transfusions from infected canine donors (Diaz-Espineira and Slappendel, 1997; Rosypal et al., 2005; de Freitas et al., 2006).

Infection *versus* disease

As in human beings (Carvalho et al., 2012), in an endemic area the majority of the dog population is exposed and becomes infected without showing clinical evidence of disease or serum anti-*Leishmania* antibodies (Baneth and Solano-Gallego, 2012). Epidemiologic studies in endemic areas using polymerase chain reaction (PCR) have confirmed that the prevalence of infection in dogs is much higher than the proportion that actually develops the disease (Solano-Gallego et al., 2001).

Two extremes of outcome of natural *L. infantum* infection are illustrated in figure 1-1. The majority of dogs remains infected for a long period of time (years or lifelong) without developing any clinical disease. These dogs that are able to resist an infection by either resolving it and eliminating the parasite or restricting the infection are considered *clinically resistant*. On the opposite site, those dogs that are predisposed to developing disease after the infection are considered *susceptible* (Baneth and Solano-Gallego, 2012). Change in the health status of resistant dogs or, for instance, the administration of an immunosuppressive drug or a severe immunosuppressive disease can lead to the progression from a subclinical infection to the disease. However, there is limited evidence for it (Baneth et al., 2008).

Host control of infection is a complex interplay of innate and adaptive immune factors which are incompletely understood and that will be reviewed later.

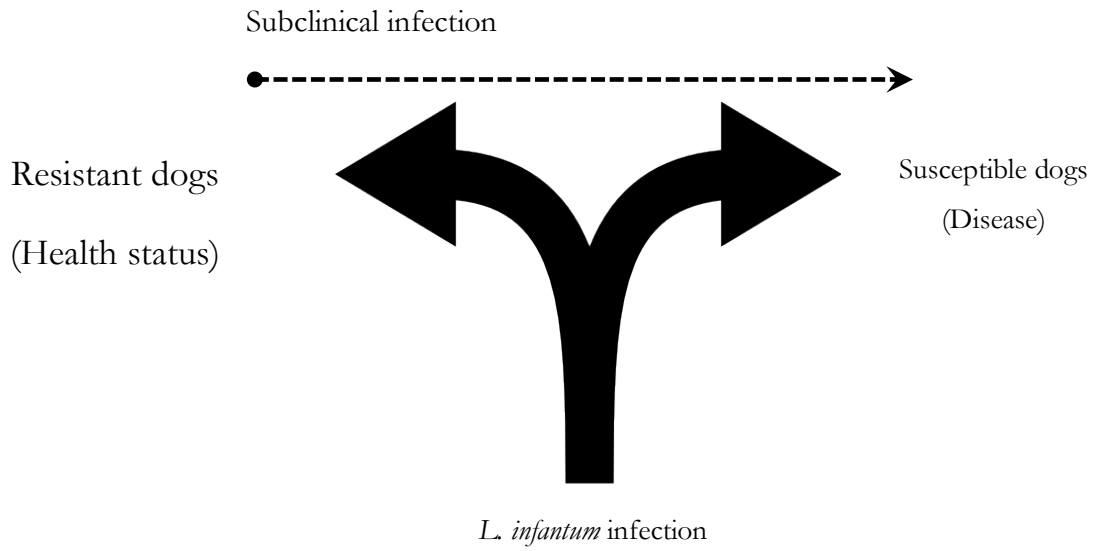


Figure 1-1. The outcome of *L. infantum* infection in dogs.

Human leishmaniasis

There are two main forms of leishmaniasis in human beings: tegumentary and visceral or kala-azar leishmaniasis. The outcome of each form is determined in part by the infecting *Leishmania* species and by the combination of inflammatory and anti-inflammatory host immune response factors that depend of other factors such as nutrition or co-infections (McGwire and Satoskar, 2014; Scorza et al., 2017).

Visceral or Kala Azar leishmaniasis

Kala Azar means black fever in Hindi. Visceral leishmaniasis (VL) is the most severe form of human leishmaniasis, which gives rise to progressive hepatosplenomegaly and bone marrow suppression. Unless treated, patients develop pancytopenia and immunosuppression and are prone to super-infections with other microbes and to dead (McGwire and Satoskar, 2014; Burza et al., 2018). Patients with VL present scarce T cell mediated *Leishmania* specific immune response, characterized by negative delayed type hypersensitivity (DTH) skin tests and interferon gamma (IFN-γ) responses (Scorza et al., 2017). Successful treatment of VL precedes the recovery of T cell reactivity to *Leishmania* antigen (Scorza et al., 2017).

Cutaneous leishmaniasis

Localized cutaneous leishmaniasis

Localized cutaneous leishmaniasis (LCL) is the most prevalent and less severe clinical manifestation of leishmaniosis (McGwire and Satoskar, 2014; Scorza et al., 2017). After *Leishmania* infection and the asymptomatic incubation period, a single or small number of painless nodular or papular lesions develop at the site of parasite deposition by the sand fly (Scorza et al., 2017). Therefore, these lesions are usually found on uncovered areas of the body such as the face, forearms and lower legs and evolve over weeks to months (McGwire and Satoskar, 2014). The lesions may progress into well-delimited ulcers with raised edges. These sloping firm margins with a prominent central crater lend a "volcanic" appearance to the ulcer, which is the most distinctive feature of this cutaneous manifestation of leishmaniosis in human beings (Uzun et al., 2018) (Figure 1-2). Although LCL often self-heals without treatment, disease resolution can take several months and leave disfiguring scars (Scorza et al., 2017). However, resolution can often be hastened by treatment. In some cases, such as those caused by *L. panamensis* and *L. braziliensis*, LCL can progress to mucosal leishmaniasis (McGwire and Satoskar, 2014). Strong cellular immune response with an IFN-γ response is observed in patients with LCL, which can be detected by a positive DTH response to *Leishmania* antigen (Scorza et al., 2017).

Disseminated cutaneous leishmaniasis

In disseminated cutaneous leishmaniasis (DCL), pleomorphic lesions (acneiform, papular, nodular, and ulcerative types) erupt at various locations often, distant from the site of inciting insect exposure (Scorza et al., 2017). Patients can respond to treatment but may require multiple or slightly longer treatment regimens than LCL (Scorza et al., 2017). As in LCL, specific cellular immune response is observed in patients with DCL, which can be detected by a positive DTH response to

Leishmania antigen. Multiple lesions over different areas of the body may be associated to an underlying deficiency in cellular immunity or it may be due to multiple bites of sandflies.

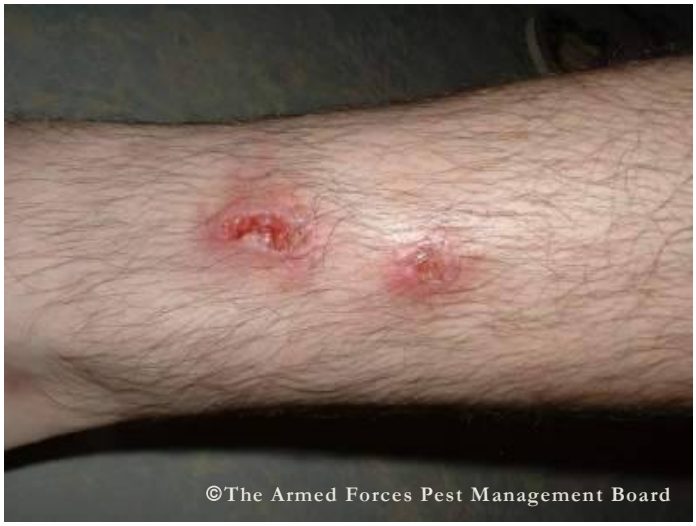


Figure 1-2. Human localized cutaneous leishmaniosis. Photo extracted from https://www.flickr.com/photos/afpmb/4709530724/

Mucosal leishmaniasis

Mucosal leishmaniasis (ML) can occur as a complication of LCL caused by *L. panamensis* and *L. braziliensis*. In regions where *L. braziliensis* is endemic, approximately 1–10% of LCL patients progress to ML. Known risk factors include sex (male > female), increased age, malnutrition, size and number of LCL lesions, lesions above the belt, and inadequate therapy (Scorza et al., 2017). ML can present months to years after resolution of primary lesions and is a horribly disfiguring infection resulting from the chronic local destruction of tissue of the nose, mouth oro- and nasopharynx and eyelids and can progress to affect respiratory function and hamper nutrition. The disease is often refractory to therapy and patients usually die from secondary super-infections and malnutrition. The underlying pathogenesis resulting in ML is not well understood; however, a hypersensitivity reaction is suggested due to a higher expression of interleukin (IL)-17, tumor necrosis factor alpha (TNF-α) and IFN-γ in circulation compared with LCL (Scorza et al., 2017). As a result, the use of pentoxifylline, which target this excess of TNF-α, has shown promise in treating patients with refractory ML when combined with standard antimonial therapy (Lessa et al., 2001).

Anergic diffuse cutaneous leishmaniasis

Anergic diffuse cutaneous leishmaniasis (ADCL) is a rare but severe form of cutaneous leishmaniasis. It is characterized by the development of multiple satellite lesions that can coalesce

into plaques covering large areas of skin. Lesions are predominantly nodular or papular in nature, contain an abundance of amastigotes, and do not ulcerate (Scorza et al., 2017). In contrast to LCL, these lesions do not self-heal over time and are often highly resistant to treatment and exhibit frequent relapse (Scorza et al., 2017). All these clinicopathological findings suggest that in these patients there is an anergic cellular immune response, which is further supported by the lack of specific *Leishmania* DTH response (Scorza et al., 2017). Moreover, and similar to VL, these patients show high levels of circulating *Leishmania* antibodies. Conventional chemotherapies affecting parasite growth have limited effects in these patients, perhaps because they do not correct the immune defects (Scorza et al., 2017).

Post-Kala Azar dermal leishmaniasis

Post kala-azar dermal leishmaniasis (PKDL) is a rare condition observed in a subset of patients from India and Sudan successfully treated for VL. Patients develop a fulminant and progressive proliferation of parasites within the skin which give rise to diffuse macular, maculo-papular or nodular lesions (McGwire and Satoskar, 2014). This manifestation occurs in patients infected mainly with *L. donovani*, and reports of PKDL due to *L. infantum* are scarce (Scorza et al., 2017). Risk factors for developing this clinical manifestation are HIV co-infection and inadequate anti-*Leishmania* treatment (Zijlstra et al., 2000; Pal and Biswas, 2018). The pathogenesis of PKDL is not fully understood but appears that these patients show intermediate level of T cell reactivity between VL and fully recovered patients (Scorza et al., 2017).

Leishmaniasis recidivans

Leishmaniasis recidivans (LR) is a rare, chronic relapsing form of cutaneous leishmaniasis. This is characterized by a relapse of cutaneous disease within the sites of previous healed lesions of LCL due to *L. major*, *L. tropica* or *L. braziliensis* (Scorza et al., 2017). This can occur decades after resolution of the primary lesions (Marovich et al., 2001; McGwire and Satoskar, 2014). These lesions are usually painless, non-ulcerated and with a granulomatous inflammation in which amastigotes are rarely seen. PCR is needed in order to confirm the infection (Scorza et al., 2017). Parasites can survive within the skin at the site of initial infection and can lead to reactivated inflammatory lesions years after initial cure, responding to an unknown stimulus. One hypothesis is that local trauma, perhaps via inflammatory changes, may play a role in reactivation of LR lesions (Wortmann et al., 2000). Based on an immunological study it seems that LR is a type IV hypersensitivity reaction to the reactivation of hidden antigens (Meymandi et al., 2009). The cellular localization of parasites during these periods of latency remains unknown.

Canine leishmaniosis

Leishmania infantum infection in dogs can manifest as chronic subclinical infection, self-limiting disease or severe illness. Moreover, the clinical manifestations and the time to disease manifestation vary extensively among diseased dogs (Solano-Gallego et al., 2009). Sick dogs can develop cutaneous and/or extracutaneous signs. However, cutaneous manifestations are perhaps the most common findings on physical examination and can be the only clinical manifestation of the disease (Saridomichelakis and Koutinas, 2014).

Cutaneous manifestations

Contrary to human leishmaniasis, where cutaneous leishmaniasis are due to dermotropic species (Burza et al., 2018), cutaneous manifestations of *L. infantum* infection in dogs are considered clinical signs of disease dissemination. Nevertheless, the cutaneous lesions are the only clinical manifestation of CanL in some cases (Baneth and Solano-Gallego, 2012). The prevalence of dermatological lesions in dogs with leishmaniosis ranges between 56% to 90% (Baneth and Solano-Gallego, 2012).

Cutaneous manifestations of CanL are very pleomorphic and vary in extent, however are rarely pruritic (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014). The exact reasons for this wide range of skin lesions associated with this disease are partially unknown. However, and as suggested for human leishmaniasis, the type and magnitude of immune response of the host may play a role (Scott and Novais, 2016).

Up-to-date review articles on the cutaneous manifestations of the disease are scarce (Saridomichelakis and Koutinas, 2014). Traditionally, skin lesions in CanL have been described as main clinicopathological presentations and miscellaneous cutaneous lesions (Saridomichelakis and Koutinas, 2014). However, and comparable to human leishmaniosis, cutaneous manifestations due to *L. infantum* infection can be explained as typical or atypical (Table 1-2) (Ordeix and Fondati, 2013). Common dermatological problems and/or highly suggestive of CanL are considered *typical* forms, whereas unusual morphological forms are considered *atypical* forms. The later, can mimic many other diseases and confound the veterinarians, which elude diagnosis in the first instance, submitting the patients to unnecessary treatments, worsening the picture, and contributing to the transmission chain of the parasite (Ordeix and Fondati, 2013; Meireles et al., 2017). In an endemic area, it is necessary for the veterinarian to be aware that any atypical lesion, especially chronic form, should be investigated for leishmaniasis.

Table 1-2. Canine cutaneous manifestations due to *L. infantum*. Adapted from Ordeix and Fondati, 2013.

	Typical	***Atypical***
More common	Exfoliative dermatitis with alopecia	Ulcerative dermatitis: • of the nasal planum • in a skin area subjected to local trauma • in areas that cover the ends of the body (tips of the pinnae, tail, digits and paw pads)
	Ulcerative dermatitis over bony prominences	Mucocutaneous ulcerative dermatitis
	Papular dermatitis in non-haired skin with central adherent crust	Mucocutaneous nodular dermatitis
	Onychogryphosis	Pustular dermatitis
		Multifocal alopecia
Less common		Naso-digital hyperkeratosis

Exfoliative dermatitis with alopecia

This cutaneous manifestation of CanL is considered the most common, with prevalence ranging between 45.7 % to 98.7 % (Saridomichelakis and Koutinas, 2014). It can be localized, regional or generalized, symmetrical or asymmetrical. Typical lesions are whitish and relatively adherent scales initially localized on the face and ears (Figure 1-3). Typically, facial scales distribute symmetrically around eyes (butterfly sign) and on the dorsal part of the nose. With the disease progression, lesions affect the trunk and extremities. This dermatological problem is usually non-pruritic and may be partially eroded under the scales. In addition, follicular casts can be occasionally seen together with the scales. When present, idiopathic sebaceous adenitis is the major differential diagnose for leishmaniosis (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014). Dogs affected by this dermatological condition typically present clinicopathological alterations and detectable *Leishmania* specific antibodies (Ordeix and Fondati, 2013). Therefore, cytological or histopathological confirmation is not always necessary from a diagnostic point of view. Nonetheless, this clinical manifestation is microscopically associated with a granulomatous or pyogranulomatous superficial and/or deep perivascular to interstitial dermatitis.

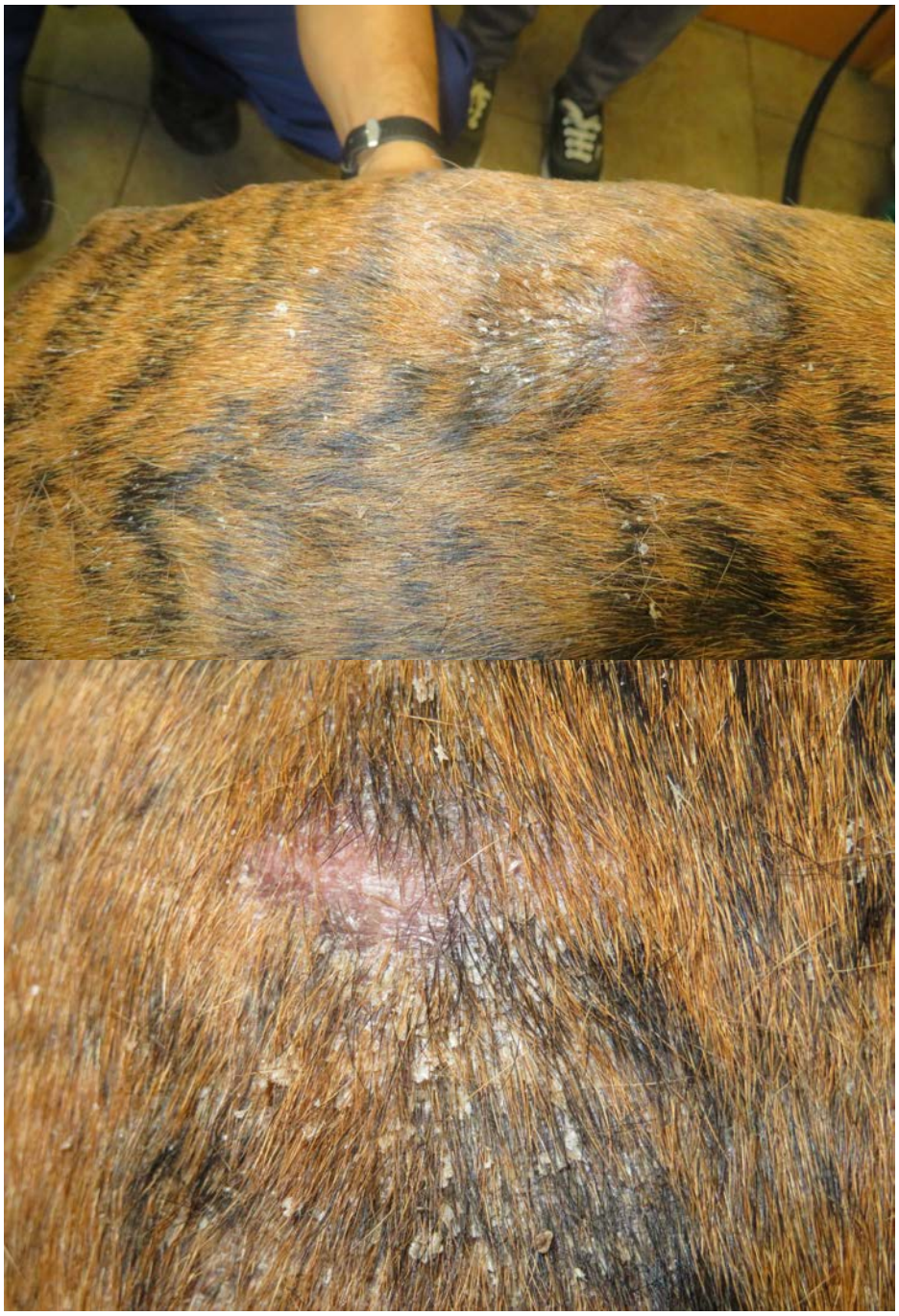


Figure 1-3. Exfoliative dermatitis in a dog with leishmaniosis. Note typical whitish and relatively adherent scales.

Sebaceous adenitis is present in half of the skin biopsies obtained from areas with exfoliative dermatitis (Saridomichelakis and Koutinas, 2014). The number of intralesional amastigotes is usually high (Papadogiannakis et al., 2005; Brachelente et al., 2005).

Ulcerative dermatitis over bony prominences

Ulcerative dermatitis is the second most common cutaneous manifestation, with prevalence ranging between 19.7% to 91.2% (Saridomichelakis and Koutinas, 2014). However, that prevalence refers to all the different ulcerative patterns described together in dogs with leishmaniosis. Therefore, the prevalence for any subset of ulcerative dermatitis is not known. Ulcerative dermatitis over bony prominences is typically characterized by indolent and persistent ulcers usually with elevated borders, commonly on the carpal and tarsal regions (Figure 1-4). It is hypothesized that continued pressure causes secondary inflammation leading to the infiltration of infected macrophages as part of the normal healing process and the formation of an ulcer in an infected dog (Saridomichelakis and Koutinas, 2014).

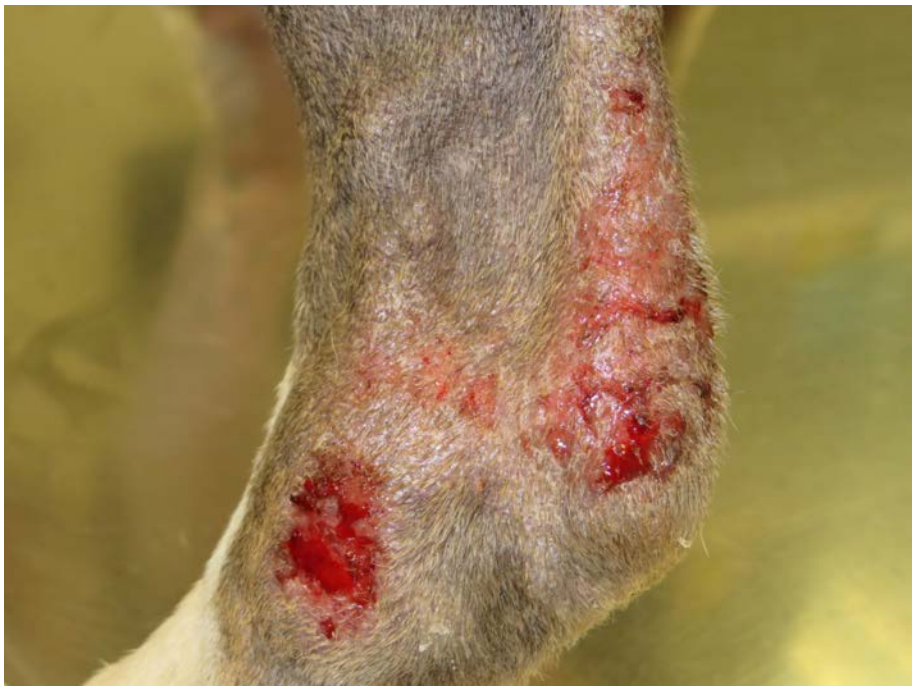


Figure 1-4. Ulcerative dermatitis over bony prominences in a dog with leishmaniosis.

Dogs affected by this dermatological condition frequently present clinicopathological alterations and detectable *Leishmania* specific antibodies (Ordeix and Fondati, 2013). However, histopathological confirmation with or without *Leishmania* specific immunohistochemistry (IHC) or molecular techniques is necessary when cytological examination do not reveal amastigotes before ruling out leishmaniosis in low seroreactive dogs (Ordeix and Fondati, 2013).

Papular dermatitis in non-haired skin with central adherent crust

In an endemic region this is a very typical cutaneous manifestation of CanL, although the exact prevalence of this condition is unknown (Ordeix and Fondati, 2013). Lesions start as a raised erythematous papule probably at the site of *Leishmania* inoculation in a less haired skin such as

inner pinnae, eyelids, dorsal part of the nose, lips and caudal abdomen (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014). They slightly grow, and sometimes tend to coalescence to reach a final size of a small plaque. A crust develops centrally, covering an ulcer with a raised edge and variable surrounding induration (Figure 1-5) (Ordeix and Fondati, 2013).

Commonly dogs with this cutaneous manifestation lack other clinicopathological abnormalities and have low or negative specific antibody levels (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014).

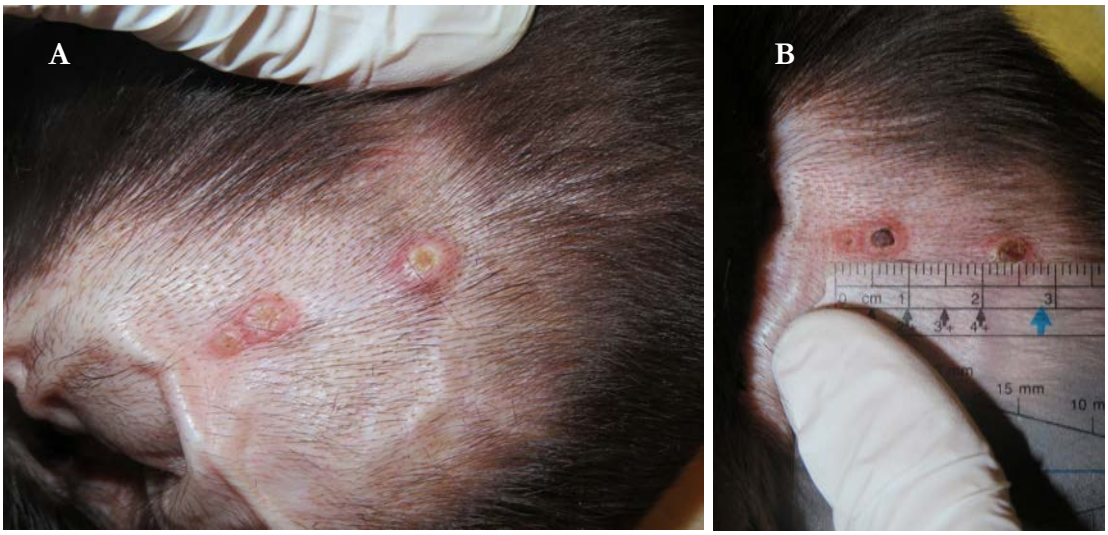


Figure 1-5. Papular dermatitis in the inner pinna from a Labrador retriever with leishmaniosis (A). Same lesions 10 days later. Note the typical crater form with the central crust and indurated margins (B).

Therefore, diagnosis usually rely on the visualization of amastigotes on skin cytology or biopsy with or without the aid of specific IHC (Ordeix and Fondati, 2013). Detection of *Leishmania* DNA on skin lesions has been also used to diagnose this condition (Lombardo et al., 2014). The histopathological picture is dominated by a nodular to diffuse pyogranulomatous dermatitis, without multinucleated giant cells and with few parasites (Saridomichelakis and Koutinas, 2014). The reduced parasite dissemination to internal organs, the low antibody levels, the positive results of the leishmanin skin test (LST) and the spontaneous resolution over 3-5 months suggest that there is strong cell-mediated immunity in these dogs against *L. infantum* that configures protection (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014).

Onychogryphosis

The prevalence of this cutaneous manifestation is between 43.4 to 54.4% (Saridomichelakis and Koutinas, 2014). It is a chronic sign; therefore, the true prevalence may be different based on the time of diagnosis of the disease in different geographical regions (Ordeix and Fondati, 2013; Koutinas et al., 2010). Classically, onycogryphosis is characterized by excessive growth and abnormal curvature of the nails. Rarely, it is the unique clinical sign, and the majority of dogs with leishmaniosis and onychogryphosis show other dermatological manifestations of the disease such as exfoliative or ulcerative dermatitis (Koutinas et al., 2010). Nail histopathology reveals lymphocytic exocytosis and mild to severe lichenoid mononuclear dermatitis, with or without hydropic degeneration of basal keratinocytes, dermo-epidermal clefting and pigmentary incontinence (Koutinas et al., 2010). Onychogryphosis is not associated with a high parasite density, in fact in one study no parasites could be found in hematoxylin and eosin-stained sections and nail tissue was PCR positive in only a few of the cases examined (Koutinas et al., 2010). Dogs with onychogryphosis usually show clinicopathological alterations and elevated levels of *Leishmania*-specific antibodies. Therefore, the final diagnosis does not rely on the histopathological examination (Ordeix and Fondati, 2013).

Ulcerative dermatitis of the nasal planum

This is an atypical form of CanL as it can be easily confused with another disease associated with ulcerative dermatosis of the nasal planum. When depigmentation, erosions or ulcers are diffuse or located at the dorsal part of the nasal planum, lupus erythematosus discoid represents the main differential diagnosis, both clinical and histologically (De Lucia et al., 2017) (Figure 1-6A). Commonly, these lesions are restricted to the lateral nasal fossa, being the main differential diagnosis a mucocutaneous pyoderma, especially in German shepherd dogs (Figure 1-6B) (Ordeix and Fondati, 2013).

Figure 1-6. Ulcerative dermatitis of the nasal planum in a Dachshund (A) and in a German shepherd dog (B) with leishmaniosis.

Ulcerative dermatitis in a skin area subjected to local trauma

The development of an ulcerative lesion with prominent borders in a skin area subjected to trauma is a "clinical pearl" that in humans beings suggests an atypical form of cutaneous leishmaniosis (Wortmann et al., 2000). The particularity of these lesions is that injury is not due to the parasite, but to other causes. However, the absence of healing or the persistence of the lesion is due to the presence of amastigotes into the cytoplasm of the macrophages present at the site of healing. In the dog leishmaniosis has been described in previous sites of cutaneous injury due to surgical wounds (Prats and Ferrer, 1995) (Figure 1-7). Moreover, leishmaniosis was suspected in a case of dermatitis similar to acral lick dermatitis (Denerolle et al., 2007).

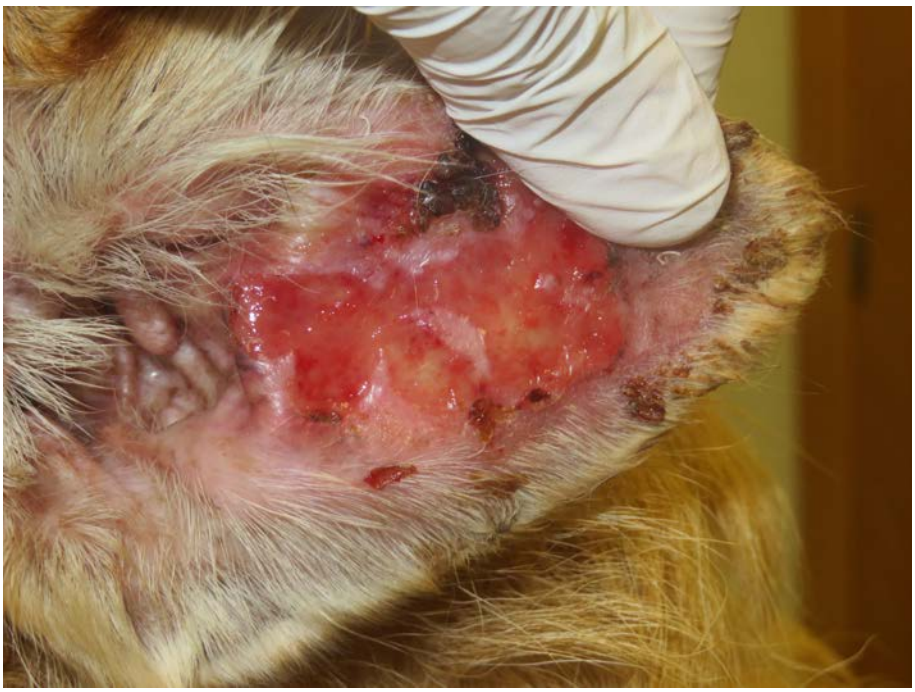


Figure 1-7. Ulcerative dermatitis in the pinna of a dog with leishmaniosis in which several surgical treatments were performed.

Ulcerative dermatitis in areas that cover the ends of the body (tips of the pinnae, tail, digits and paw pads)

Another type of ulcerative dermatitis secondary to leishmaniosis in dogs is due to cutaneous vasculitis and the deposition of immunocomplexes. In this case, ulcers are located in areas that cover the ends of the body such as tips of the pinnae, tail, digits and paw pads (Figure 1-8 and 9).

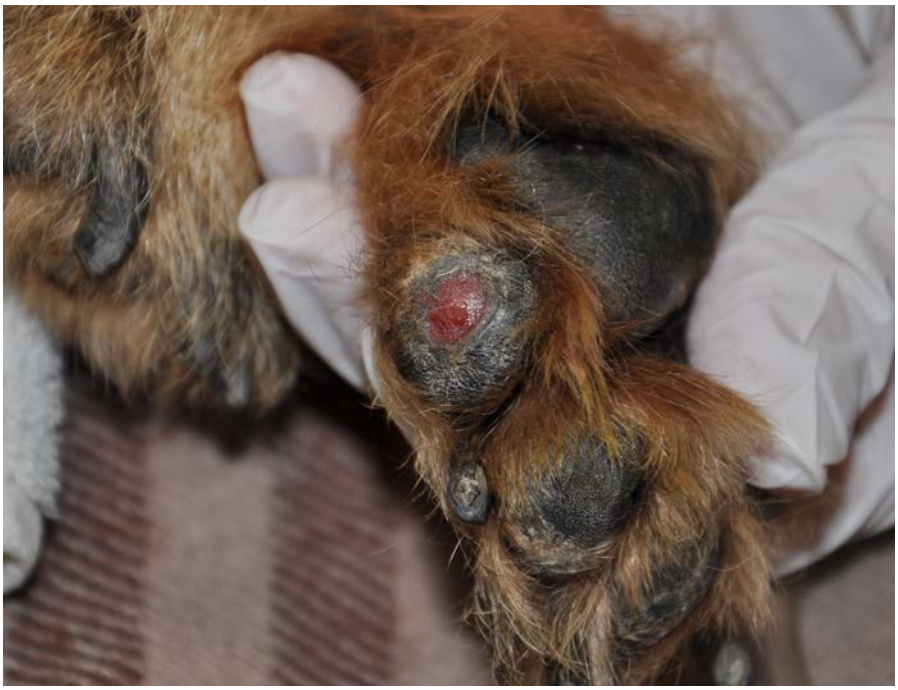


Figure 1-8. Circular area on the paw clinically suggestive of vasculitis in a dog with leishmaniosis.

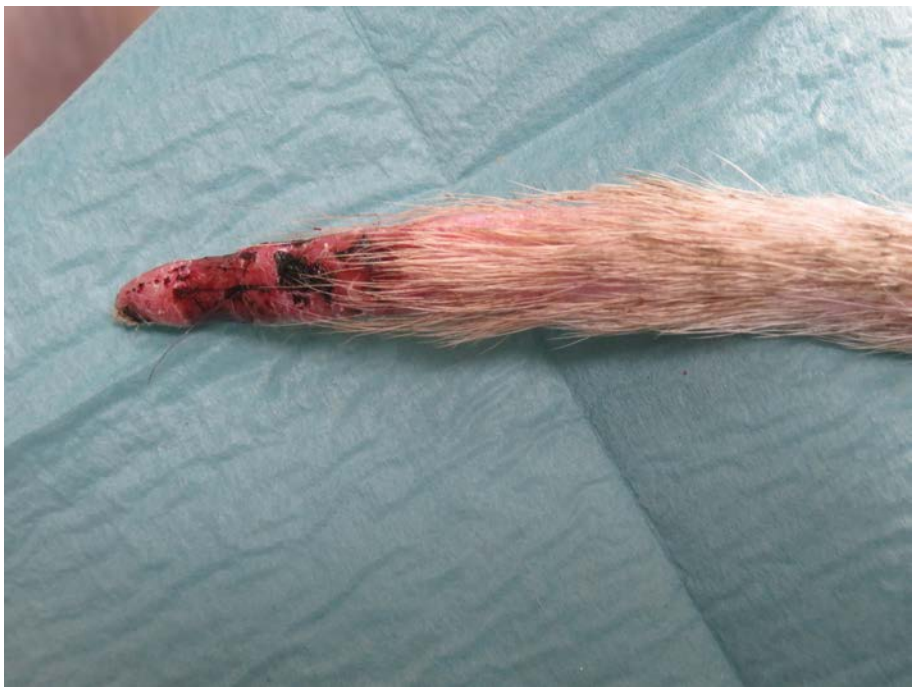


Figure 1-9. Alopecia, ulcers and crust on the tip of the tail, clinically suggestive of vasculitis, in a dog with leishmaniosis.

In these cases, the vascular damage usually remains undocumented because skin biopsies are rarely performed due to the location of the lesions. Nonetheless, as cutaneous vasculitis is clinically suggested, causes of vascular damage other than leishmaniosis (i.e. ehrlichiosis, drug

reactions) need to be rule out (Ordeix and Fondati, 2013). However, CanL is often diagnosed based on the clinicopathological alterations and detectable *Leishmania* specific antibodies.

Mucocutaneous ulcerative dermatitis

Canine leishmaniosis can lead to erosive and ulcerative lesions in the mucocutaneous junctions. All junctions may be affected. However, the periocular and perioral region, together with the nostrils, appear to be the most commonly involved (Figure 1-10). Clinical differentials include principally mucocutaneous pyoderma, mucocutaneous erythematous lupus and drug reactions (Ordeix and Fondati, 2013).

Figure 1-10. Mucocutaneous ulcerative dermatitis in a German shepherd dog with leishmaniosis. Note depigmentation, erosions and ulcerations on the eyelids (A), lips (B), nasal planum (C) and prepuce (D).

Mucocutaneous nodular dermatitis

Mucocutaneous nodular dermatitis is a relatively uncommon clinical presentation, with a prevalence up to 12% (Saridomichelakis and Koutinas, 2014). It is described more frequently in the Boxer dog. Clinically, these are single or multiple nodules of variable size (1-10 cm), usually located in the head, extremities and thorax. They are covered by hairs and sometimes ulcerate. In some cases, the lesions have been described in mucocutaneous and mucosal junctions such as oral or genital.

Pustular dermatitis

Pustular dermatitis is a clinical form described infrequently in dogs suffering from leishmaniosis (Saridomichelakis and Koutinas, 2014). The frequency of presentation ranges from less than 1 to 13% of cases (Saridomichelakis and Koutinas, 2014). Relationship between leishmaniosis and this clinical form is not completely understood. It is hypothesized that leishmaniosis is a risk factor for the development of an immunomediated, specific antibiotic non-responsive neutrophilic pustular dermatitis in dogs (Bardagí, 2012). However, if leishmaniosis indirectly causes it due to immunomediated mechanisms (causal relationship) or it is a drug reaction developed after or during anti-*Leishmania* treatment (consequential relationship) it is not known (Colombo et al., 2016). Pustular dermatitis associated with leishmaniosis is frequently generalized. Pustules are related to erythematous papules and epidermal collarets and are distributed symmetrically over the entire surface of the body, including both the skin sparsely populated by hair like the one that is not (Figure 1-11). Pruritus is variable but is often present and intense (Saridomichelakis and Koutinas, 2014).

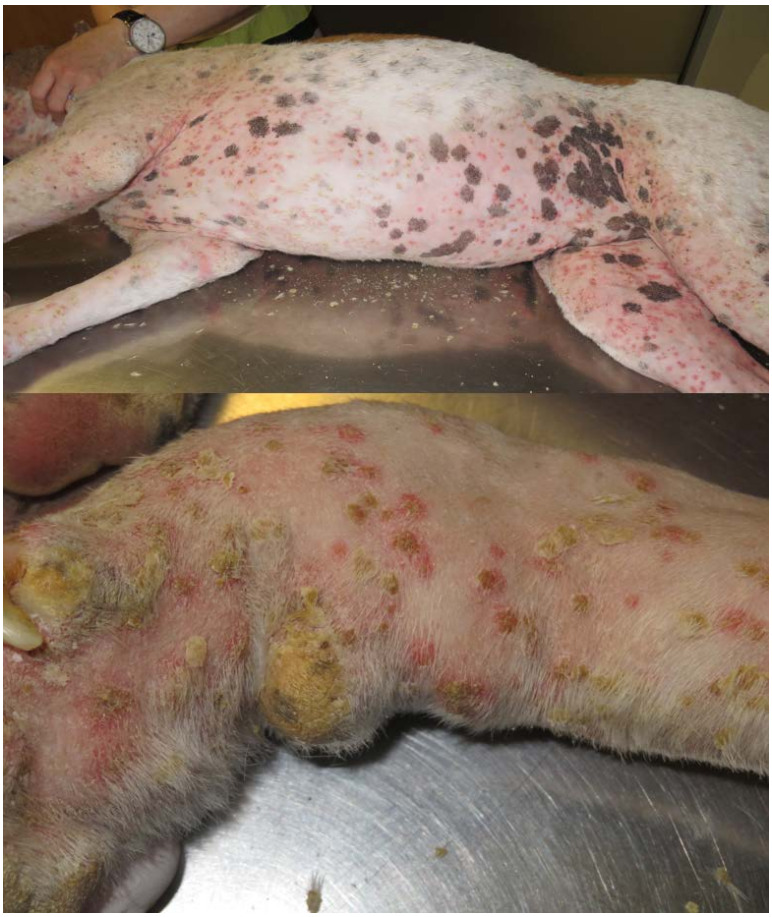


Figure 1-11. Pustular dermatitis in a Boxer with leishmaniosis. Note the atypical distribution of papulo pustular lesions in the extremities.

Multifocal alopecia

Rarely, leishmaniosis is clinically associated to a focal or multifocal alopecia consequence of an ischemic dermatopathy in dogs (Figure 1-12). Similarly, to the ulcerative dermatitis due to vasculitis, a cutaneous vascular damage due to secondary deposition of immunocomplexes has been suggested (Ordeix and Fondati, 2013). As for pustular dermatitis, it is difficult to determine the real relationship between leishmaniosis and this clinical form (Ordeix and Fondati, 2013).

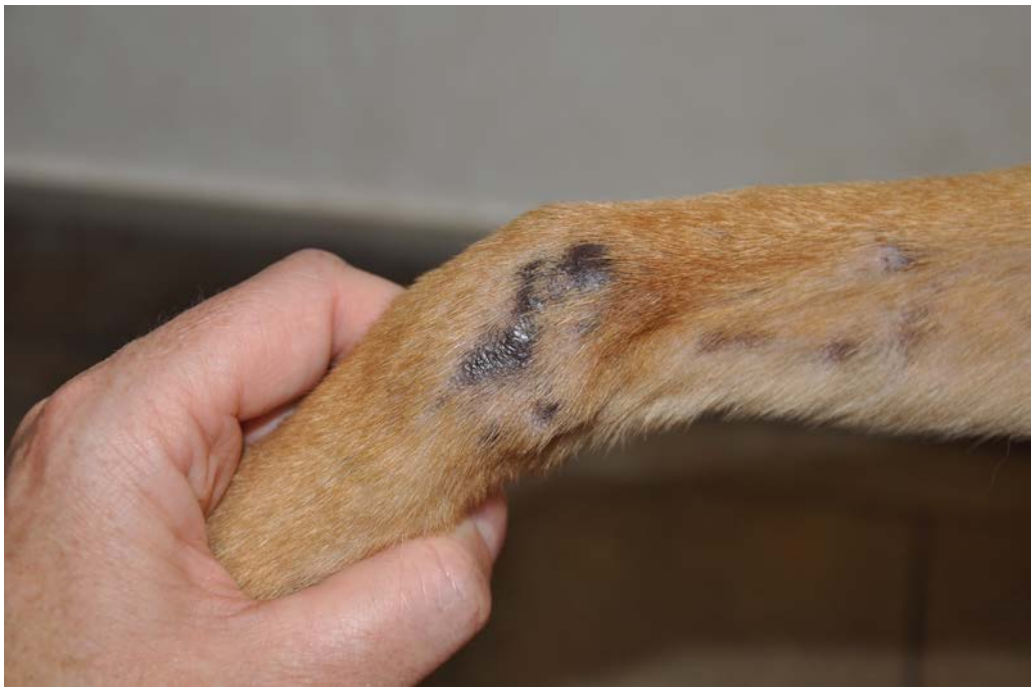


Figure 1-12. Multifocal alopecia due to an ischemic dermatopathy in a dog with leishmaniosis.

Naso-digital hyperkeratosis

This atypical problem is often associated to other clinical manifestations of leishmaniosis, both typical and atypical. Greyish, thick and dry scales characterize the lesions. These are strongly adhered to the underlying skin and sometimes are accompanied of deep fissures, which can be painful, especially on the paw pads (Ordeix and Fondati, 2013).

The normal-looking skin of infected or diseased dogs

The normal-looking skin of dogs has been scarcely studied either in diseased or in infected but clinically healthy dogs (dos-Santos et al., 2004; Solano-Gallego et al., 2004; Giunchetti et al., 2006). Normal-looking skin of dogs with leishmaniosis, with or without dermatological manifestations, frequently shows microscopic lesions along with the presence of *Leishmania* amastigotes (Solano-Gallego et al., 2004; Saridomichelakis and Koutinas, 2014). These microscopic lesions are the same than those described in clinically-lesioned skin (Saridomichelakis and Koutinas, 2014).

The presence of parasites in the normal-looking skin, therefore, supports the xenodiagnostic findings showing that dogs with CanL are infectious to the sand flies irrespective of the presence of macroscopic lesions at the site of sand-fly bites (Guarga et al., 2000). However, infectiousness to the sand-fly vector are associated with high parasite numbers in the skin (Courtenay et al., 2014). Studies of normal-looking skin samples from dogs with different stages of disease severity are lacking. These studies would help in understand if less severe clinical cases could be less infectious.

Prevalence of histological lesions and the parasite density in the normal-looking skin seems to differ between seropositive or seronegative infected but clinically healthy. In the latter, there are not histological lesions and parasites are undetectable with IHC, although their presence in the skin may be demonstrated by PCR (Solano-Gallego et al., 2004). Based on these findings, it has been suggested that such dogs may not be able to infect the sand fly vectors (Solano-Gallego et al., 2004). On the contrary, histological lesions and *Leishmania* amastigotes are present in the normal-looking skin of seropositive infected but clinically healthy dogs, although to a lesser degree compared with dogs with leishmaniosis (Solano-Gallego et al., 2004; Giunchetti et al., 2006).

Extra-cutaneous manifestations and laboratory abnormalities

CanL is a multiorgan disease that may potentially involve any organ, tissue or body fluid. Therefore, it is manifested by nonspecific systemic clinical signs and laboratory abnormalities. The most important extra-cutaneous clinical signs are listed in Table 1-3. Briefly, these include lymphadenomegaly, weight loss, anorexia, lethargy and ocular lesions amongst others (Solano-Gallego et al., 2011). Chronic renal failure is a severe organ dysfunction due to disease progression and the main cause of mortality (Solano-Gallego et al., 2011).

Diagnosis

The diagnosis of CanL is complex because of the wide and non-specific spectrum of clinical signs and clinicopathological abnormalities (Solano-Gallego et al., 2017). Moreover, two diagnostic challenges exist in an endemic area. The first is the existence of subclinical infections. The second is the use of vaccines against CanL as some vaccines elicit antibody production detected by conventional serological tests (Solano-Gallego et al., 2017). In both cases, misdiagnosis of leishmaniosis could be done in a sick dog.

Diagnosis of CanL is usually based on the compatible clinical and laboratory findings, positive serology and demonstration of the parasite in lymph node or bone marrow cytology or, in

case of cutaneous manifestations, in skin lesions or any other biological fluid or tissuea (Solano-Gallego et al., 2009; Noli and Saridomichelakis, 2014; Paltrinieri et al., 2016).

Table 1-3. Extra-cutaneous manifestations and laboratory abnormalities found in CanL due to *L. infantum* (modified from Solano-Gallego et al., 2011).

Extra-cutaneous manifestations	***Laboratory abnormalities***
General: • Generalized lymphadenomegaly • Weight loss • Decreased or increased appetite • Lethargy • Mucous membranes pallor • Splenomegaly • Polyuria and polydipsia • Vomiting • Diarrhea (including chronic colitis) • Lameness	Serum proteins and electrophoretogram: • Hyperglobulinemia • Polyclonal beta and/or gammaglobulinemia • Hypoalbuminemia • Decreased albumin/globulin ratio
Ocular: • Blepharitis (exfoliative, ulcerative, or nodular) and conjunctivitis (nodular) • Keratoconjunctivitis, either common or sicca • Anterior uveitis/Endophtalmitis	CBC/Hemostasis: • Mild to moderate non-regenerative anemia • Leukocytosis or leukopenia • Thrombocytopathy • Thrombocytopenia • Pancytopenia • Impaired secondary hemostasis and fibrinolysis
Other: • Fever • Epistaxis • Atrophic masticatory miositis • Vascular disorders (systemic vasculitis, arterial thromboembolism) • Neurological disorders • Papulo-nodular glossitis	Biochemical profile/urinalysis: • Mild to severe proteinuria • Renal azotemia • Elevated liver enzyme activities

The most common and useful diagnostic tool for the diagnosis of CanL is serology, especially indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) (Noli and Saridomichelakis, 2014). High antibody concentrations in a non-vaccinated dog with clinical signs and clinicopathological abnormalities compatible with leishmaniosis are diagnostic of the disease (Solano-Gallego et al., 2009). On the other hand, a low positive serology result may

occasionally be encountered in an infected dog that does not have leishmaniosis but another disease (Noli and Saridomichelakis, 2014). Unfortunately, there is not a serological test that discriminate between antibodies to vaccination and to natural infection with *L. infantum*. Therefore, the use of quantitative serology as the sole diagnostic technique for the diagnosis of leishmaniosis in vaccinated dogs is not recommended (Solano-Gallego et al., 2017). In general, but specially in vaccinated dogs or in dogs with compatible clinical signs and negative or low positive serology, the method of choice for diagnosing leishmaniosis is the observation of compatible pathological lesions and the parasite by cytology, histology or by IHC or detection of *Leishmania* DNA by PCR (Solano-Gallego et al., 2017).

Diagnosis of the different dermatological pictures

Figure 1-13 illustrates the diagnostic approach in dogs with cutaneous manifestations suggestive of leishmaniosis. In the majority of clinical cases, especially in typical lesions, demonstration of intralesional parasites could be sufficient to demonstrate causation of *Leishmania* infection. Cytological evaluation is a practical way to demonstrate the presence of parasites in skin lesions (Ordeix and Fondati, 2013; Noli and Saridomichelakis, 2014). Although highly specific, sensitivity of cytological examination is variable and depends on the abundance of parasites in the lesion, the time and expertise of the observer and therefore, in some cases, might may be negative. In cases where parasite visualization is not reach by means of cytological examination, determination of parasitic DNA by PCR on cutaneous cytological smears or directly from tissue aspirates has been proposed as a diagnostic method (Lombardo et al., 2014; Lima et al., 2017). Although studies aimed to evaluate its practical feasibility are lacking. It is necessary to remember that *Leishmania* positive PCR may be obtained from normal looking skin of infected dogs (Solano-Gallego et al., 2004). Therefore, a positive result simply means that the dog is infected and does not prove that this infection is the cause of the clinical signs as an infected dog may suffer from another concomitant disease. In the absence of a direct evidence of contributory factor of *Leishmania* in skin lesions, it might be necessary to finally demonstrate the etiological role of the parasites by a favorable response to anti-*Leishmania* treatment (Ordeix and Fondati, 2013).

Therefore, a skin biopsy remains an important diagnostic tool where parasite is not detected by cytology. The most common histopathological findings are periadnexial nodular to diffuse pyogranulomatous or granulomatous dermatitis, together with several epidermal changes (Baneth and Solano-Gallego, 2012). Other less common histopathological patterns such as lichenoid dermatitis with or without an interface dermatitis (De Lucia et al., 2017), subcorneal pustular dermatitis (Bardagí, 2012; Colombo et al., 2016), vasculitis and panniculitis have also been

documented. However, if organisms are not detected on routine stains, the histopathological findings may be misinterpreted as another process such as idiopathic sebaceous adenitis or discoid erythematous lupus (Bardagí et al., 2010; De Lucia et al., 2017). Therefore, visualization of the parasite may be enhanced with IHC (Ferrer et al., 1988). PCR testing may also be performed in paraffin embedded skin biopsies (Roura et al., 1999). Again, if molecular techniques are used instead of IHC, in the absence of a direct evidence of contributory factor of *Leishmania* in skin lesions, it might be necessary to finally demonstrate the etiological role of the parasites by a favorable response to anti-*Leishmania* treatment (Ordeix and Fondati, 2013).

In typical dermatological problems, such as exfoliative dermatitis, detection of high antibody levels, with or without detection of parasite in the skin, may be used as the indirect evidence that the dog suffers from a dermatological manifestation of a patent leishmaniosis (Solano-Gallego et al., 2009). However, in the absence of a direct evidence of contributory factor of *Leishmania* in skin lesions, it might be necessary to finally demonstrate the etiological role of the parasites by a favorable response to anti-*Leishmania* treatment (Ordeix and Fondati, 2013).

On the other hand, diagnosis of leishmaniosis in dogs with atypical forms only by means of a high serological result may be challenging. In fact, a dog with leishmaniosis may suffer from a concomitant dermatological disease (Ginel et al., 1993; Mozos et al., 1999). In addition, to demonstrate the etiological role of the parasite by a favorable response to anti-*Leishmania* treatment is not always possible. Actually, some atypical dermatological problems, such as ischemic dermatopathy, which sometimes is irreversible, or pustular dermatitis and vasculitis, which are cortico-responsive, do not respond to specific anti-*Leishmania* therapy alone (Ordeix and Fondati, 2013). Demonstration of intralesional parasites in atypical lesions could be insufficient to demonstrate the causal role of *Leishmania* (Ordeix and Fondati, 2013). It is necessary to remember, specifically in an endemic area, that an infected dog may suffer from a dermatological disease different than leishmaniosis.

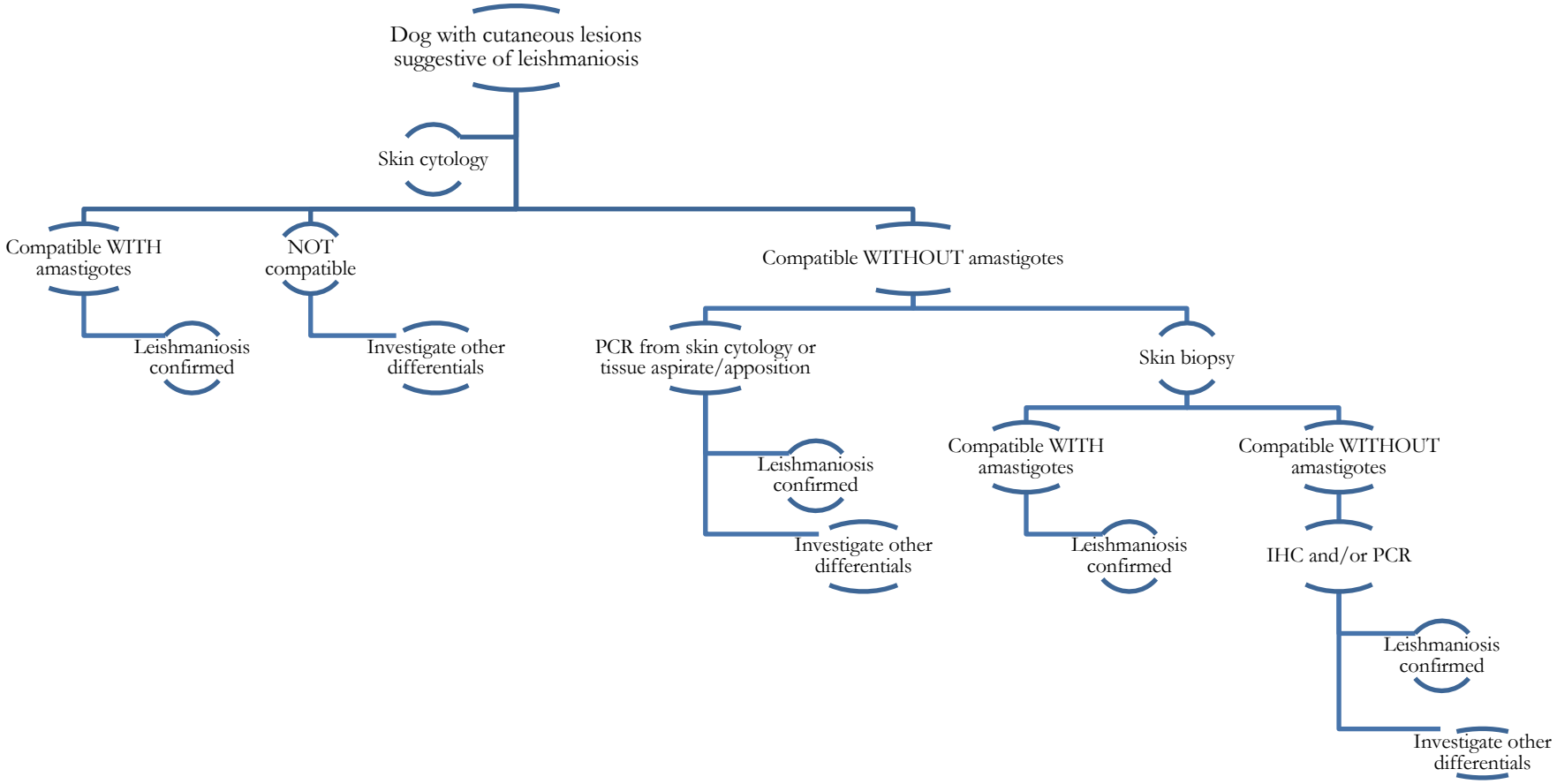


Figure 1-13. Algorithm of the direct diagnostic approach in dogs with cutaneous manifestations suggestive of leishmaniosis.

Clinical staging, treatment and prognosis

A clinical staging system based on clinicopathological abnormalities, clinical signs and serological status has been proposed and recently updated in an attempt to cover the wide spectrum of clinical manifestations found in CanL (Table 1-4) (Solano-Gallego et al., 2009; Solano-Gallego et al., 2017). Based on this system, a more appropriate treatment can be proposed, and a realistic prognosis can be established.

Table 1-4. Clinical staging of CanL (printed from Solano-Gallego et al., 2017).

Clinical stages	Serology[a]	Clinical signs	Laboratory findings	Therapy	Prognosis
Stage I Mild disease	Negative to low positive antibody levels	Dogs with mild clinical signs such as solitary lymphadenomegaly or papular dermatitis	Usually no clinicopathological abnormalities observed Normal renal profile: creatinine <1.4 mg/dl; nonproteinuric: urinary protein:creatinine ratio (UPC) <0.5	Scientific neglect[b]/ Monitoring of disease progression (see Table 2)	Good
Stage II Moderate disease	Low to high positive antibody levels	Dogs which, apart from the signs listed in Stage I, may present, for example: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), generalized lymphadenomegaly, loss of appetite, and weight loss	Clinicopathological abnormalities such as mild nonregenerative anemia, hypergammaglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome Substage (i) Normal renal profile: creatinine <1.4 mg/dl; nonproteinuric: UPC <0.5 (ii) Creatinine <1.4 mg/dl; UPC = 0.5–1	Allopurinol + meglumine antimoniate or miltefosine	Good to guarded
Stage III Severe disease	Medium to high positive antibody levels	Dogs which, apart from the signs listed in Stages I and II, may present signs originating from immune-complex lesions (e.g., uveitis and glomerulonephritis)	Clinicopathological abnormalities listed in Stage II Chronic kidney disease (CKD) IRIS[c] Stage I with UPC = 1–5 or Stage II (creatinine 1.4–2 mg/dl)[v]	Allopurinol + meglumine antimoniate or miltefosine Follow IRIS guidelines for CKD[vi]	Guarded to poor
Stage IV Very severe disease	Medium to high positive antibody levels	Dogs with clinical signs listed in Stage III. Pulmonary thromboembolism, or nephrotic syndrome and end-stage renal disease	Clinicopathological abnormalities listed in Stage II CKD IRIS Stage III (creatinine 2.1–5 mg/dl) and Stage IV (creatinine >5 mg/dl)[v] or nephrotic syndrome: marked proteinuria UPC >5	Specific treatment should be instaured individually Follow IRIS guidelines for CKD[vi]	Poor

[a]Dogs with negative to medium positive antibody levels should be confirmed as infected with other diagnostic techniques such as cytology, histology/immunohistochemistry and PCR. High levels of antibodies are conclusive of a diagnosis of CanL and are defined as a three- to fourfold increase in a well established laboratory reference cut-off.
[b]Dogs in Stage I (mild disease) are likely to require less prolonged treatment with one or two combined drugs (allopurinol, domperidone, meglumine antimoniate, or miltefosine) or alternatively monitoring with no treatment. There is limited information on dogs in this stage and, therefore, treatment options remain to be defined.
[c]Abbreviation: IRIS, International renal interest society.

Several drugs are currently available for the treatment of CanL, however although many of these drugs improve clinical signs, only rarely are *Leishmania* organisms completely eliminated (Baneth and Solano-Gallego, 2012). Relapses and retreatments are, therefore, common (Torres et al., 2011; Manna et al., 2015).

Pentavalent antimonials are the primary drug for treatment of canine and human leishmaniasis (Baneth and Solano-Gallego, 2012). Meglumine antimoniate is the most commonly used antimonials for treatment of dogs. A systematic review has concluded that the use of

meglumine antimonate for a minimum of 3-4 weeks at a minimum dose of 100mg/kg daily combined with allopruinol resulted in good clinical efficacy and a reduced relapse rate (Noli and Auxilia, 2005). Allopurinol used at a dose of 10mg/kg twice daily has become an indispensable part of the treatment of CanL (Baneth and Solano-Gallego, 2012). However, dogs receiving allopurinol therapy should be monitored for the development of urinary adverse effects, such as urolithiasis and renal mineralization (Torres et al., 2016). Moreover, resistance to allopurinol in canine leishmaniasis has been recently shown to be associated with disease relapse in naturally-infected dogs (Yasur-Landau et al., 2016).

Miltefosine administered orally at 2mg/kg daily for 4 weeks is an alternative to meglumine antimoniate when combined with allopurinol (Baneth and Solano-Gallego, 2012). However, it seems that that meglumine antimoniate might have better clinical efficacy than miltefosine (Manna et al., 2015). Conventional amphotericin B deoxycholate also has a good efficacy against CanL, however, its use is reduced because it is administered intravenously, and it is highly nephrotoxic (Baneth and Solano-Gallego, 2012). Several other drugs have been reported for the treatment of CanL with various degrees of efficacy. These include ketoconazole, pentamidine or metronidazole with spiramycin (Noli and Auxilia, 2005).

More recently, other drugs have been studied for the management of CanL. Domperidone, a dopamine D2 receptor antagonist, has been used as a sole therapy or in combination with furazolidone in the treatment of leishmaniosis (Gómez-Ochoa et al., 2009; Passos et al., 2014). Dietary nucleotides and active hexose correlated compound have been proposed as a good alternative to allopurinol in combination with meglumine antimoniate, especially for dogs suffering allopurinol-related adverse events (Segarra et al., 2017). Unfortunately, long term randomized controlled trials are lacking for these new compounds.

Prevention

Vector control

The main way to avoid *Leishmania* infection is to use topical insect repellents with proven evidence of controlling infection. These topical formulations, such as collars, spot-ons or sprays, have the dual effect of both warding off (contact repellency) and killing (insecticidal efficacy) sand flies when they contact a protected dog (Miro et al., 2017). These formulations contain different synthetic pyrethroids proven to be effective against *Phlebotomus* species. Pyrethroid compounds capable to reduce *L. infantum* infection in dogs living in endemic areas are listed in table 1-5. Unfortunately, the efficacy of these products in decreasing the rate of *Leishmania* infection has been demonstrated only through short-term follow-up studies of kenneled dogs living in endemic areas

of this infection. Therefore, real impact of these formulation in the long-term prevalence of leishmaniosis in owned dogs has not been evaluated (Miro et al., 2017).

Table 1-5. Pyrethroid compounds demonstrated to reduce *L. infantum* infection in dogs living in endemic areas.

Compund	***Formulation***	***Efficacy of prevention***	***Reference***
4% Deltamethrin	Collar	61.8%; 88%; 97.5%	Brianti et al., 2016; Papadopoulos et al., 2017; Ferroglio et al., 2008
10% imidacloprid + 4.5% flumethrin	Collar	88.3%	Brianti et al., 2016
10% imidacloprid + 50% permethrin	Spot-on	90.36% - 90.73%	Otranto et al., 2007
6.76% fipronil + 50.48% permethrin	Spot-on	100%	Papadopoulos et al., 2017
65% permethrin	Spot-on	97.5%	Ferroglio et al., 2008

Additional control measures exist in order to reduce sand fly numbers in the dogs' environment. These measures are physical such as the use of mesh of small size in windows or doors and removal of sand fly breeding locations such as compost, pruning scraps, bins, and woodpiles (Miro et al., 2017). Moreover, additional control measures include using residual insecticides sprayed at home, animal shelters and soft furnishings (Alexander and Maroli, 2003). Lastly, keeping dogs indoors from dusk to dawn during high-risk seasons (April-November), may be also an additional action to prevent contact with the vector and infection (Maroli et al., 2010).

Immunoprophylaxis

Another prophylaxis strategy for CanL is the use of vaccines. These can be used in dogs to elicit an adequate immune response that will avoid progression of disease upon infection.

The effectiveness of immunoprophyilaxis strategies has been assessed by a systematic review and metanalysis (Wylie et al., 2014). Although vaccines do not prevent establishment of infection, based on peer-reviewed evidence it was pointed that this control measure is effective in preventing CanL. However, clinical trials under fields conditions in order to demonstrate the efficacy of anti-*Leishmania* vaccines are scarce. These studies are difficult to perform due to several

issues, such as low recruitment rate of private owned dogs, the annual variability in infection rate due to changes in weather, sand fly abundance, and the necessity for a long follow-up to have measurable disease outcomes (Miro et al., 2017).

Four commercial vaccine products (Leishmune®; Leish-Tec®; CaniLeish®; Letifend®) have been licensed and marketed for the control of CanL. Leishmune® and Leish-Tec® were marketed in Brazil, although the former has been withdrawn from the market under the decision of the Brazilian health authorities (Miro et al., 2017). Canileish® has been registered in several European countries in 2011 for immunization of *Leishmania*-seronegative dogs more than 6 months old inducing immunity lasting at least 1 year (EMA/296055/2010). It contains a purified secreted/excreted antigen (LiESP) and a purified extract of *Quillaja saponaria* (QA-21), an adjuvant similar to saponin. CaniLeish® tended to significantly reduce the proportion of dogs infected with *L. infantum* based on either parasitological or serological evidence (Oliva et al., 2014). More recently a recombinant polypeptide antigen (Letifend®) has been registered in Europe (EMA/159853/2016). It contains 'protein Q' antigen, a chimeric recombinant protein made fusing parts of the *L. infantum* Lip0, Lip2a, Lip2b, and histone H2A proteins (Miro et al., 2017). Letifend® tended to significantly reduce the proportion of dogs with leishmaniosis (Fernández Cotrina et al., 2018). This vaccine does not contain an adjuvant and few side effects have been described being the most common scratching at the injection site (Fernández Cotrina et al., 2018).

Immunotherapy

The main objective of immunotherapy is to modulate the host immune system so that it can restore an immune response that can control infection (Miro et al., 2017). Although from a theorically point of view this is a desirable approach, the knowledge about the use of immunomodulatory molecules in preventing CanL is an expanding area of research with still limited evidence of efficacy.

Immunomodulators assessed to date in CanL and marketed are a dopamine D2 receptor antagonist, domperidone (Sabaté et al., 2014) and a dietary nucleotides and active hexose correlated compound (Segarra et al., 2018). Although they seem safe treatments, clinical efficacy of these products is based on very few and controversial clinical trials.

Immunology of canine leishmaniosis

As stated previously not every infected human (Carvalho et al., 2012) or dog will develop the disease (Baneth and Solano-Gallego, 2012). In fact, infection might progress into clinical disease unless the replication of the amastigotes is discontinued by immune mechanisms. Moreover, of particular interest is the wide range of clinical manifestations associated with this disease both in human beings (Burza et al., 2018) and dogs (Solano-Gallego et al., 2009). It is accepted that the wide range of clinical manifestations observed in human tegumentary leishmaniasis is dictated largely by the type and magnitude of the immune response of the host (Scott and Novais, 2016).

For decades, the development of a T helper 1 (Th1) specific immune response was thought to play a major role in controlling the disease. The differential development of Th1 and Th2-type responses translated directly to the spectrum of clinical presentations seen in patients (Scott and Novais, 2016). This reasoning was based on findings that CD4+ Th1 cells mediate resistance in *Leishmania major*-infected mice whereas CD4+ Th2 cells promote susceptibility (Scott et al., 1988). However, current understanding of the disease in both humans and mice indicate that a more complex cellular response dictates the outcome of infection and that early immune events within a critical 24–48 hours post-infection have been implicated in instructing the polarization of the Th response in leishmaniasis (Scorza et al., 2017).

Innate immune response

General overview

A number of nonspecific antimicrobial systems (e.g. phagocytosis) have been recognized which are innate in the sense that they are not intrinsically affected by prior contact with the infectious agent and are usually present before the onset of the infection. The innate response is not enhanced by previous exposure to the foreign organism and the response time is very rapid usually occurring in minutes or hours after contact with the infection agent (Delves et al., 2017). Cells associated with innate immunity such as neutrophils, dendritic cells (DCs) and macrophages directly kill pathogenic microbes via phagocytosis, or they induce the production of cytokines, which facilitate the elimination of pathogens. Moreover, the innate immune response instructs the development of long lasting pathogen specific adaptive immune responses as it is the early immunologic microenvironment which has an important role in influencing the adaptive T cell response (Scott and Novais, 2016; Delves et al., 2017).

Early immune responses to *Leishmania* infection

Innate immune response has been scarcely investigated in human leishmaniosis, and also in CanL. However, there is increasing evidence that innate mechanisms play an important role in the first line anti-*Leishmania* defenses and in instructing a long lasting specific immune response.

Once the promastigotes are injected into the skin via the bite of a sand-fly, macrophages, DCs and neutrophils that are recruited to the infection site can become infected and have important and distinct roles in shaping the immune response to infection (Scott and Novais, 2016).

Neutrophils are the first cells to encounter the parasite soon after its inoculation in the dermis by the phlebotomine vector (Scott and Novais, 2016). Their role is complicated as they may kill the parasites or protect them depending on the parasite species and the host (Scott and Novais, 2016). In an *in vitro* study performed on dog's neutrophils it was demonstrated that they were competent effector cells able to control the initial *L. infantum* infection (Pereira et al., 2017). However, some parasites evaded intracellular effector mechanisms and could be transferred to the definitive host cell, the macrophage, contributing to the development of illness in dogs (Pereira et al., 2017).

Monocytes are also recruited from the blood to the *Leishmania* lesions in a chemokine receptor 2 dependent manner; in contrast to neutrophils, tisular macrophages are efficient at killing *Leishmania* parasites by producing reactive oxygen species. Monocytes also differentiate into DCs, migrate to the lymph nodes and promote the differentiation of Th1 cells by producing IL-12. Th1 cells then migrate to the skin and eliminate the parasites by inducing nitric oxide (NO) production and/or enhancing the respiratory burst in infected macrophages (Scott and Novais, 2016). NO production is the main mediator of killing *Leishmania* by macrophages. NO is produced by nitric oxide synthase (NOS), which converts one of the terminals nitrogens of the guanidine group of L-arginine to NO, producing citrulline (Hosein et al., 2017). Canine macrophages are the main effector cell in *Leishmania* killing, and the principal effector molecule has been found to be NO as well (Holzmuller et al., 2006). IFN-γ, TNF-α and IL-2 secreted by activated T cells induce canine macrophages antileishmanial activity (Pinelli et al., 2000).

It has been demonstrated in mice models of *L. major* infection that before the development of Th1 cells, IFN-γ is primarily produced by natural killer (NK) cells within the draining lymph node, which reside in close association with DCs. Once activated by *L. major* infection, NK cells are recruited to the paracortex where they produce IFN-γ, which enhances the production of IL-12 by DCs (Scorza et al., 2017).

During the last years there has been a great interest in the involvement of Toll Like Receptors (TLRs) in the immunopathogenesis of CanL (Amorim et al., 2011; Figueiredo et al., 2013; Melo et al., 2014; Hosein et al., 2015). TLRs are one of the most important pattern recognition receptor (PRR) molecules expressed by cells of the innate immune system, which recognize molecular structures characteristic of microbial pathogens and induce an inflammatory response (Vidya et al., 2018). Upon activation, TLRs can induce inflammatory cytokines, type-1 IFN, chemokines and co-stimulatory molecules production from cells of the innate immune system (Vidya et al., 2018). Studies aimed to determine the role of TLRs in CanL are every time more numerous, however, they are mainly *in vitro* studies performed on canine macrophages (Turchetti et al., 2015) or *ex vivo* studies performed in blood (Montserrat-Sangrà et al., 2016), liver (Hosein et al., 2015), spleen (Melo et al., 2014; Hosein et al., 2015; Grano et al., 2018), intestine (Figueiredo et al., 2013), brain (Melo et al., 2014; Grano et al., 2018) or lymph node samples (Melo et al., 2014; Hosein et al., 2015) of experimentally or naturally infected dogs. Taken all this information together, it would seem that TLR2 is one of the most commonly TLR involved in the immunopathogenesis of CanL and that up regulation in several tissues seems to be associated with disease progression (Montserrat-Sangrà et al., 2016; Hosein et al., 2017). Furthermore, TLR2 transcription in un-stimulated blood is reduced during treatment and with clinical improvement (Montserrat-Sangrà et al., 2016).

TLR4 downregulation appears to be associated with disease progression in both the lymph node and spleen from experimentally infected dogs (Hosein et al., 2015). In the same model of experimental infection, TLR9 downregulation within lymph nodes appeared to correlate with disease progression (Hosein et al., 2015). Other TLRs such TLR3 or TLR7 have been studied even less (Hosein et al., 2015, Turchetti et al., 2015; Grano et al., 2018). Therefore, further studies would be required to be able to ascertain if other TLR have an important role in CanL.

Adaptive immune response

The major role against the parasite is played by the adaptive immune response. Evidence derived from the experimental model of cutaneous leishmaniosis by *L. major* in mice generated the general concept of Th cell dichotomy, in which Th1 cell response resulted in resistance or cure whereas the Th2 cell response derived in susceptibility and disease progression (Baneth and Solano-Gallego, 2012). However, this concept might not apply for all the different *Leishmania* species in different hosts. Probably, the balance between the two types of immune response appears to be important in natural CanL as it is in human and experimental murine visceral leishmaniosis (Baneth and Solano-Gallego, 2012). Control of *Leishmania* replication is associated with the development

of *Leishmania*-specific CD4+ Th1 cells. Following infection these cells are recruited to the cutaneous lesions where they produce IFN-γ to activate macrophages (Scott and Novais, 2016). As mentioned previously DCs initiate the antigen-specific immune response to *Leishmania* and are the main source of IL-12, which is essential for the development of protective CD4+ Th1 cells (Scott and Novais, 2016). By contrast, IL-4 promotes Th2 cell development and production of cytokines, such as IL-10 and transforming growth factor betta (TGF-β), that antagonize the effects of IFN-γ and are associated with lack of parasite control (Scott and Novais, 2016).

Both subclinical and sick dogs develop a mixed Th1/Th2 immune response and as in human and mice model, protective immunity is mediated by Th1 cells (Martínez-Moreno et al., 1995). Sub-clinically infected dogs present a strong cell mediated immune response, which is exemplified by strong *in vitro* lymphocyte proliferation and a DTH response with the LST or Montenegro test (Martínez-Moreno et al., 1995; Cardoso et al. 1998; Solano-Gallego et al., 2000; Strauss-Ayali et al., 2005). On the other hand, disease is classically characterized by a reduced or absent *L. infantum* specific T-cell mediated immunity in canines. Severely sick dogs lack positive LST reactions and present a humoral response, which is non-protective and denotes failure to control the infection (Hosein et al., 2017). The levels of *Leishmania*-specific immunoglobulins are greater in sick dogs compared with sub-clinical dogs (Rodríguez-Cortés et al., 2007). Moreover, high antibody levels are associated with parasite density, lack of IFN-γ production in stimulated whole blood and severe clinical status of the animal (Solano-Gallego et al., 2016; Rodríguez-Cortés et al., 2017). It has been demonstrated that IFN-γ concentration increases with long-term anti-*Leishmania* treatment together with clinical improvement in dogs that do not produce IFN-γ at diagnosis (Martínez-Orellana et al., 2017)

As stated above, the suppression of cellular immunity is the most important aspect in the pathogenesis and progression of CanL. There is evidence that the absence or decreased T-cell mediated immunity is due to T-cell exhaustion (Esch et al., 2013). This concept is defined as antigen-specific effector T cell dysfunction with sustained expression of inhibitory receptors, including programmed death 1 (PD-1) and decreased effector cytokine production such as IFN-γ (Rodrigues et al., 2014). During the last years, several studies has focused on these regulatory mechanisms in CanL and they have demonstrated that PD-1 and its ligand (PD-L1) present in regulatory IgD^{hi} B Cells are involved in the induction of T lymphocyte apoptosis via IL-10 production (Schaut et al., 2016). These studies have demonstrated an increased PD1/PD-L1 expression in peripheric mononuclear cells as well as an increase in the expression of PD-L1 in splenic macrophages in dogs with visceral leishmaniosis (Chiku et al., 2016; Schaut et al., 2016).

Skin immune responses

The skin is a central organ for *Leishmania* transmission and it shows more frequently signs of disease and plays a major role in the immunopathogenesis of this disease. However, there is limited data about immune responses in the skin of infected or diseased dogs (Hosein et al., 2017).

A mixed Th1/Th2 cytokine profile in the lesioned dermis of dogs naturally infected with *L. infantum* has been described (Brachelente et al., 2005; Menezes-Souza et al., 2011). Dogs express high levels of IL-13, TNF-α, IFN-γ and IL-4 in the dermis (Brachelente et al., 2005; Menezes-Souza et al., 2011). Moreover, dogs with high parasite burden have significantly high IL-4 expression (Brachelente et al., 2005). Th17 cytokines (i.e IL-17 and IL-22) were recently evaluated in the skin of experimental infected dogs (Hosein et al., 2015). In that study, Th17 cytokines were unchanged during experimental infection and disease progression as indicative of the well-known silent establishment of the *Leishmania* infection (Santos-Gomes et al., 2002; Hosein et al., 2015). In another experimental study, local cytokine response was absent at 6 months post infection, whereas parasite growth and onset of clinical disease both correlated with dermal up-regulation of TNF-α, IFN-γ, IL-10 and TGF-β (Rodríguez-Cortés et al., 2016). Most recently, a positive association among skin parasite load and TNF-α and IL-10 transcription was reported in the normal-looking skin of dogs naturally affected by CanL (Pereira-Fonseca et al., 2017).

The expression of TLRs in the skin of dogs with leishmaniosis has been scarcely studied. Increased transcription of TLR2 has been reported in the normal-looking skin of dogs experimentally and naturally affected by CanL (Hosein et al. 2015; Pereira-Fonseca et al., 2017). TLR2 overexpression was positively associated with skin parasite load (Pereira-Fonseca et al., 2017). Less studied, TLR9 was reported to be upregulated in the skin of dogs when compared with healthy non-infected control dogs during early stages in an experimental infection (Hosein et al., 2015). Moreover, TLR9 downregulation was observed in the skin with disease progression (Hosein et al., 2015).

Resistance to canine leishmaniosis

Susceptibility and resistance to CanL appear to have a genetic base (Baneth and Solano-Gallego, 2012). There is some evidence that autochthonous breeds from endemic areas, such as the Ibizan hound from Balearic Islands, develop various degrees of resistance to infection (Solano-Gallego et al., 2000). This breed rarely develops clinical disease and shows very effective cellular immune responses determined by means of positive DTH skin response to leishmanial antigen and

strong production of IFN-γ in stimulated blood (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017b).

Several studies have evaluated the genetic influence of some genes on the susceptibility to CanL. Variants of DLA-DRB1 and NRAMP1, also known as Slc11a1 gene, were shown to exert both a genetic influence (Altet et al., 2002; Quinnell et al., 2003). A genome-wide association (GWA) study estimated 64% heritability for CanL, and the clinical status was correctly predicted in 60% of dogs (Quilez et al., 2012). Posteriorly, another GWA study identified regions associated with susceptibility to CanL, including the genes involved in Th cells and macrophage signaling (i.e. IL2RA, IL15RA, and TLE1) (Utsunomiya et al., 2015). Recently, another GWA study identified chromosomic regions related to TNF- α, TGF- β, LST and IL-10 that potentially affected the clinical complexity and the parasite replication in canine *L. infantum* infection (Batista et al., 2016).

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CHAPTER 2

HYPOTHESES AND OBJECTIVE

Hypotheses

Although the clinical variation in CanL is well established, little is known about the reasons for that variability. It is commonly stated that each presentation might reflect a different host–parasite relationship. However, this assertion is based only in anecdotal information and not based on hypothesis driven studies. Unfortunately, immunological features that are at the basis for that assumption have not been fully addressed.

The hypothesis of this doctoral thesis was that dogs with stage I leishmaniosis and papular dermatitis as the sole clinical manifestation were dogs that presented a distinctive innate and adaptive immune responses able to protect them against parasite proliferation and disease progression in comparison with dogs with more severe cutaneous manifestations. Therefore, these dogs may be characterized by lower parasite load in skin lesions as well as different histopathological pattern suggestive of parasite control. Moreover, these patients may show a lower parasite dissemination to normal-looking skin. In terms of immune response, these dogs may develop an immune response close to that observed in resistant dogs, characterized by low humoral immune response together with IFN-γ production, and distinctive profile of cytokine and TLRs expression in the skin.

While LST reactions have been traditionally used in epidemiological studies to demonstrate *Leishmania*-specific cell-mediated immune response in human beings and in dogs, its morphological and immunological features are poorly described in the canine patient. It was also hypothesized that LST positive reaction in resistant dogs was histoimmunologically similar to papular dermatitis.

The last hypothesis of this doctoral thesis was that papular dermatitis may be more common in breeds known to be resistant to *Leishmania* infection.

Objectives

The general objectives of this doctoral thesis were:

1. to compare clinical, pathological, parasitological and immunological parameters of dogs with papular dermatitis as the sole clinical manifestation *versus* dogs with more severe cutaneous manifestations.
2. to evaluate the prevalence of papular dermatitis in dogs living in endemic areas.

3. to evaluate the histoimmunological features of positive LST reactions of resistant dogs such as Ibizan hounds.

The specific objectives were:

1. to evaluate and compare clinical, pathological, parasitological and systemic immunological parameters in sick dogs with different severities of CanL with cutaneous manifestations including papular, exfoliative and ulcerative dermatitis (chapters 3, 4 and 6).
2. to determine, the prevalence of papular dermatitis in resistant Ibizan hounds in comparison with other dog breeds (chapter 5).
3. to assess and compare the expression of several immune genes in the clinically-lesioned and normal-looking skin of dogs with different severities of CanL with cutaneous manifestations including papular, exfoliative and ulcerative dermatitis (chapter 6).
4. to investigate the histological features and the expression of several immune genes in positive LST reactions of Ibizan hound dogs (chapters 7 and 8

CHAPTER 3

Histopathological findings and detection of toll-like receptor 2 in cutaneous lesions of canine leishmaniosis

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Abstract

A broad spectrum of clinical manifestations ranging from a chronic subclinical infection to a non-self-limiting illness has been described for canine leishmaniosis (CanL). This clinical variation is determined by a variable immune response, presumably genetically determined, against the infection. Although different types of adaptive immune response in dogs with CanL have been investigated in several studies, the mechanisms that underlie and determine this variability are still poorly understood. It is currently thought that innate immune response, and particularly the role of specific mediators of the innate immune system, such as toll-like receptors (TLRs), plays a central role in this polarization. However, there is limited data available concerning the role that TLRs play in canine *Leishmania infantum* infection.

The objective of this descriptive study was to characterize and compare the inflammatory pattern, the *Leishmania* burden and expression of TLR2 in skin lesions derived from dogs with different clinical stages of leishmaniosis and cutaneous lesions.

Routine histology, *Leishmania* and TLR2 immunohistochemistry assays were performed in 11 patients with papular dermatitis (stage I – mild disease) and 10 patients with other cutaneous lesions (stage II–III – moderate to severe disease).

A significantly higher frequency of granuloma formation was demonstrated in skin samples of dogs with stage I when compared with dogs of stage II–III. Although not statistically significant, a trend for a lower parasite burden was observed for skin lesions of dogs with stage I when compared with dogs of stage II–III. A lower expression of TLR2 in skin biopsies from dogs with stage I was statistically significant compared with stage II–III. The results obtained in this study indicated an association with TLR2 in the pathogenesis of canine cutaneous leishmaniosis. Further studies are required to fully elucidate these findings.

Keywords Canine leishmaniosis, *Leishmania infantum*, papular dermatitis, granuloma, TLR2

Introduction

A broad range of clinical manifestations and immune responses have been described for canine leishmaniosis (CanL). In fact, *Leishmania infantum* infection in the dog can manifest as a chronic subclinical infection, self-limiting disease, or non-self-limiting illness (Baneth et al., 2008; Solano-Gallego et al., 2009). Therefore, a clinical staging system of CanL based on serological status, clinical signs, laboratory findings, and type of therapy and prognosis for each clinical stage has been proposed (Solano-Gallego et al., 2009). This clinical staging system ranges from stage I or mild disease to stage IV or very severe disease with different clinical outcomes, prognosis and treatment options. The two extremes of this clinical spectrum are characterized by: (1) "Resistant" dogs with a protective CD4+ T-cell-mediated immune response characterized by production of Th1 cytokines such as IFN-γ, IL-2 and TNF-α, which induce anti-*Leishmania* activity by apoptosis of parasites in macrophages via nitric oxide metabolism and, thus capable of controlling infection, and (2) severally sick dogs which are characterized by a marked humoral immune response, reduced cell mediated immunity with a mixed Th1 and Th2 cytokine (TGF-β and IL-10) pattern and high parasite burden, which is detrimental to the animal (Baneth et al., 2008).

Cutaneous clinical signs are the most common manifestation of CanL and skin lesions are very pleomorphic from a clinical and histopathological point of view (Ordeix and Fondati, 2013). The causes of this clinicopathological variability remain uncertain. However, as described for human leishmaniosis, it appears that the diversity of the immune responses against *L. infantum*, probably genetically determined, is the most important factor deciding the pathogenic mechanisms and the type of lesions observed (McCall et al., 2013). In fact, based on the recent clinical classification of CanL, a benign form characterized by a papular dermatitis is the only permissible dermatologic manifestation in stage I leishmaniosis, and therefore associated with a favorable prognosis (Solano-Gallego et al., 2009). Often dogs with papular dermatitis show no other clinicopathological findings and the level of anti-*Leishmania* antibodies are negative or weakly positive. This dermatological problem is associated with a good specific immune cell-mediated response as well as the spontaneous resolution of the lesions within 3–5 months in some cases (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014).

In human and experimental murine leishmaniosis, caused by a variety of *Leishmania* species, the type of the inflammatory infiltrate in tissues reflects the profile of the host immune response to the parasite. This is considered as morphopathological marker of Th1- or Th2-type

immune responses to *Leishmania* infection: resistance versus susceptibility to the disease (Lemos de Souza et al., 2000). Hence, the inflammatory infiltrate in lesions of resistant subjects is characterized by a mixture of lymphocytes, plasma cells, and epithelioid macrophages with granuloma formation and few parasites (Lemos de Souza et al., 2000). On the other hand, in susceptible individuals, the inflammatory infiltrate is composed almost exclusively of non-activated macrophages containing large cytoplasmic vacuoles burdened with parasites, and granulomas are not formed (Lemos de Souza et al., 2000). This aspect has not been fully investigated in canines.

It is important to highlight that although the adaptive immune response in dogs with a Th1- or Th2-type immune responses has been investigated in several studies, the mechanisms that underlie and determine these polarized responses in CanL are still poorly understood (Baneth et al., 2008). Interestingly, there is growing evidence that it is during the initial establishment of infection, when *Leishmania* species actively regulate adaptive T cell responses by confronting the innate immune system (Sacks and Sher, 2002). However, these findings arise from experimental infections in murine models with different *Leishmania* species and immune responses, and information regarding canine *L. infantum* infection is lacking.

Toll-like receptors (TLRs), which are transmembrane receptors found on the surface of cells of the innate immune system such as macrophages, mast cells and dendritic cells (Kawai and Akira, 2011), are one of the pattern recognition receptors that are activated when invading microbes confront the innate immune system. TLRs activation rapidly triggers a variety of anti-microbial immune responses like phagocytosis, maturation and microbicidal activity of phagosomes, induction of various inflammatory cytokines and development of pathogen-specific, long-lasting adaptive immunity through B and T lymphocytes (Kumar et al., 2011). Some studies confirm the importance of TLRs in the onset of leishmanial pathogenesis, susceptibility, and resistance in mice and human disease models (Tuon et al., 2008). While most reports on canine TLRs have been focused on chronic enteropathies, pyometra and osteoarthritis (McMahon et al., 2010; Chotimanukul and Sirivaidyapong, 2012), to date, TLRs have not been studied in canine *L. infantum* infection in detail (Amorim et al., 2011; Figueiredo et al., 2013). It is in this context that the characterization of TLRs needs to be clarified and further explored in CanL.

The hypothesis for this study is that dogs with papular dermatitis (mild disease, stage I) present a specific histopathological pattern in the lesional skin suggestive of a protective immune response. Moreover, these dogs with mild disease (stage I) express different levels

and/or types of TLRs allowing them to better control *Leishmania* infection when compared with dogs with more severe disease. Therefore, the objective of this descriptive study was to characterize and compare the inflammatory pattern, the parasite burden and expression of TLR2 in skin lesions derived from naturally *L. infantum* infected dogs with different stages of diseases.

Materials and methods

Study population

Skin biopsy specimens from 21 dogs with CanL diagnosed by positive specific *Leishmania* immunohistochemistry reaction in cutaneous lesions were included in the study.

Eleven patients with mild disease (stage I) characterized by papular dermatitis with negative or low antibody levels, and 10 patients with moderate or severe disease (stage II–III) (Solano-Gallego et al., 2009) with any type of cutaneous lesions other than papular dermatitis and high antibody levels were enrolled.

Six healthy Beagle dogs from a non-endemic area (United Kingdom) were included as a negative control group for TLR2 immunohistochemistry.

Only skin sections where epidermis and dermis were present were evaluated to perform quantitative analysis. Only one microscopic section for skin lesion was evaluated, and the same section was evaluated for histopathology, *Leishmania* and TLR2 immunohistochemistry assays.

Histopathology

Routine histology on skin biopsies stained with haematoxylin and eosin was performed. The distribution pattern of the infiltrate (perivascular to interstitial or nodular to diffuse with or without granuloma formation); the type of the infiltrate (macrophages, lymphocytes, plasma cells and neutrophils); the degree (none, mild, moderate, severe) of cellular infiltration in the dermis and the changes in the epidermis (hyperplasia, spongiosis, and cellular infiltration) were evaluated.

Granuloma formation was defined as a delimited aggregation of macrophages usually surrounded by a rim of lymphocytes (Dorland's Medical Dictionary for Health Consumers, 2007).

Leishmania immunohistochemistry

Cutaneous sections prepared from paraffin-embedded biopsies were cut into 4 μm sections and mounted on to glass slides. Slides were deparaffinized in xylene, and the tissue was rehydrated using graded alcohols.

Immunohistochemistry for detection of *Leishmania* antigens was performed using a standard protocol with AutostainerPlus (Dako Denmark A/S, Glostrup, Denmark) using rabbit polyclonal antibodies to *L. infantum* (kindly provided by Instituto de Salud Carlos III, Madrid, Spain). Briefly, the potassium permanganate/oxalic acid melanin-bleaching technique was used before introducing the sections into the autostainer. Endogenous peroxidase activity was then blocked using 3% hydrogen peroxide in distilled water for 30 min. Sections were then incubated for 40 min with the rabbit polyclonal anti-*Leishmania* at room temperature (RT) (1:3000 in EnVision Flex Antibody dilu- ent, cod. K8006, Dako Denmark A/S, Glostrup, Denmark). Thereafter, sections were incubated for 40 min at RT with Dako EnVision + System-HRP. Substrate used for detection was 3,3-diaminobenzidine (DAB, Sigma-Aldrich Química, Madrid, Spain) for two minutes. Sections were then counterstained with hematoxyllin and cover-sliped for their interpretation.

TLR2 immunohistochemistry

Immunohistochemistry for detection of TLR2 was made as followed. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in distilled water during 30 min in agitation and at dark environment. In order to remove melanin pigment prior to incubation with primary antibody, the potassium permanganate/oxalic acid technique was used as mentioned above.

Nonspecific staining blocking was performed using 2% bovine serum albumin (cod. A3912-10G, Sigma–Aldrich Química, S.L., Madrid, Spain) in Tris-buffered saline for 1h at RT. Sections were then incubated overnight at 4 ◦C with the rabbit polyclonal anti-TLR2 (1:250, cod. ab24192, Abcam plc, Cambridge, UK). One slide was incubated with- out primary antibody and constituted a negative control.

Biotinylated secondary antibody (polyclonal goat anti- Rabbit (1:200, Dako Denmark A/S, Glostrup, Denmark) was applied for 60 min at RT followed by an avidin-horseradish peroxidase (cod. 32020, Thermo Scientific, Rockford, IL, USA) reagent for 1h at RT. Substrate

used for detection was DAB (cod. D5637, Sigma–Aldrich Química, S.L., Madrid, Spain). Tissues were counterstained using hematoxylin, dehydrated, cleared, and mounted.

Lymph nodes were used as a positive control.

Quantitative analysis of Leishmania and TLR2 immunohistochemistries

For each specimen, *Leishmania* amastigotes and TLR2 positive cells were quantified by counting the number of immunolabeled cells in five and six consecutive high-power fields, respectively. These areas were selected from the superficial, mid and deep dermis where the inflammatory infiltrate was more prominent.

Statistical analysis

Statistical analysis was performed using the software IBM SPSS Statistics. Categorical data were expressed as percentage and statistical analysis was performed using the non-parametric Fisher's exact test to compare results between different stages of disease and the presence or absence of granuloma formation. Quantitative data were expressed as median with range or mean with standard deviation. The non-parametric Mann–Whitney test was used to compare results between different stages of disease and age, amastigote number and expression of TLR2. Samples were considered different at the 95% ($p \leq 0.05$) level of significance.

Results

Study population

From 11 patients with stage I of disease, six were female and five male dogs with a median age of 1 [0.5–4] year. The affected breeds were Rottweiler (n = 2), Boxer (n = 2), Labrador (n = 2) and Pointer, German shepherd, Belgian shepherd, Pit Bull and Spanish mastiff (n = 1). Lesions were localized in non-haired skin such as the abdomen (n = 5), face (n = 4), inner aspect of pinnae (n = 4), eyelids (n = 4), nasal planum (n = 3) and lips (n = 2). The duration of disease before biopsy was unknown.

From 10 patients with stage II–III of disease, three were female, five were males and in two patients the gender was unknown. The median age was of 4 [0.5–11] years. Several breeds were represented. Three were crossbreeds and one Shar Pei dog, Cocker Spaniel, Doberman, American Staffordshire, Labrador Retriever and Airedale Terrier and one for which the breed

was unknown. Dermatological manifestations were one or combination of the following: generalized exfoliative dermatitis (n = 3), facially oriented localized exfoliative dermatitis (n = 3), multifocal ulcerative dermatitis (n=2), muco-cutaneous ulcerative dermatitis (n = 2) and multifocal nodular dermatitis (n = 1). The duration of disease before biopsy was unknown.

The difference of age between groups was considered to be statistically significant (p = 0.019; Mann–Whitney test).

Histopathology

Moderate to severe lympho-plasmacytic and macrophagic infiltrates were noted in the dermis of all patients together with few neutrophils in some patients.

The distribution of the different inflammatory patterns in the lesions of the patients with stage I or stage II–III is shown in Table 3-1. There was a trend for granuloma formation in patients with stage I (Figures 3-1 and 3-2), whereas skin biopsies of patients with stage II–III showed a perivascular to diffuse pattern and granulomas were observed only in one case (Figure 3-3). In fact, the difference in frequency of the granuloma formation between stage I and stage II–III skin samples was statistically significant (p = 0.0237; Fisher's exact test).

Table 3-1. Distribution of the inflammatory patterns in the skin of the patients with different stages of leishmaniosis.

Inflammatory pattern		***Stage I***	***Stage II-III***
Perivascular to interstitial		36.36% (n = 4)	60% (n = 6)
Nodular to diffuse	*granuloma*	63.63% (n = 7)	10% (n = 1)
	without granuloma	0	30% (n = 3)
	Total	100% (n = 11)	100% (n = 10)

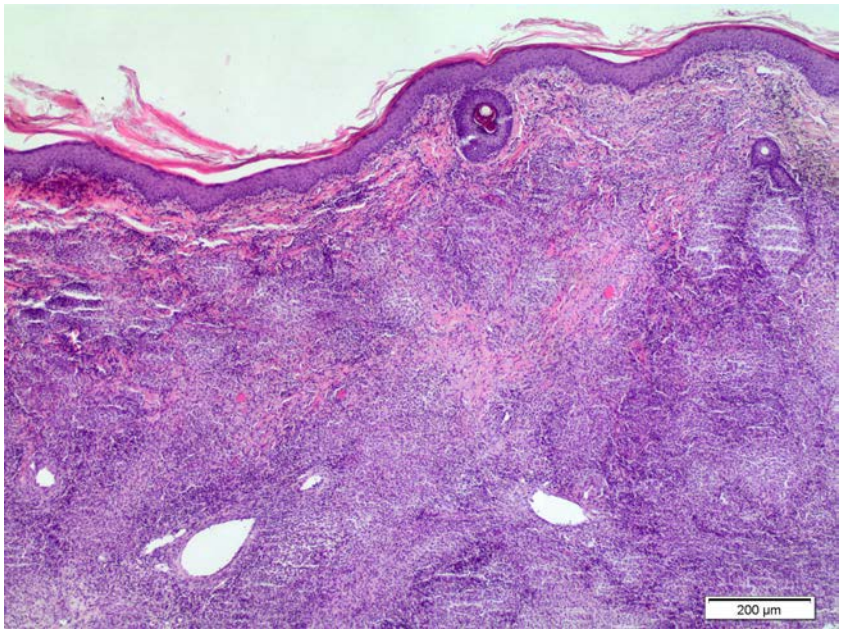

Figure 3-1. Haematoxylin and eosin stained histological image from a papular lesion. A nodular to diffuse pattern is present in the mid and deep dermis.

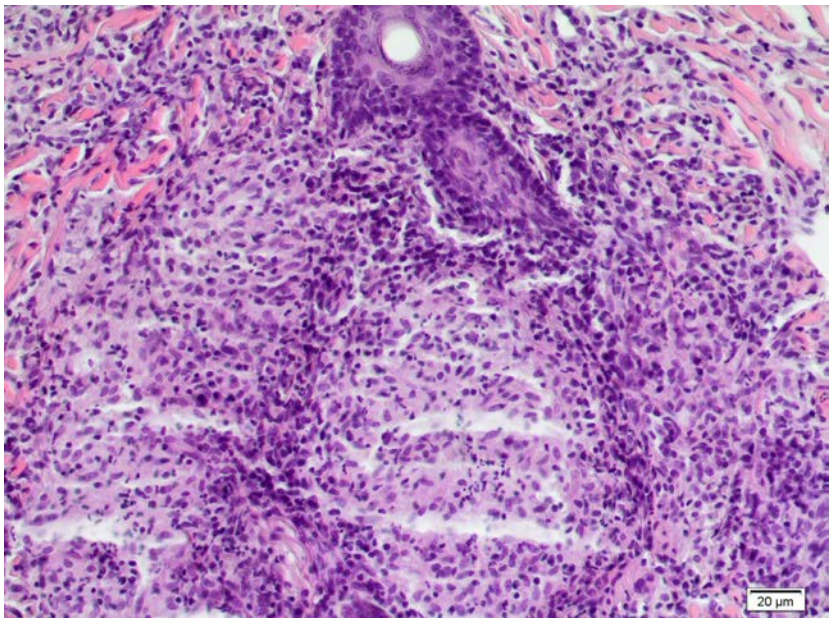

Figure 3-2. Haematoxylin and eosin stained histological image from the same patient as in Figure 3-1. Note two periadnexal granulomas characterized by central macrophages with few neutrophils and a rim of lymphocytes.

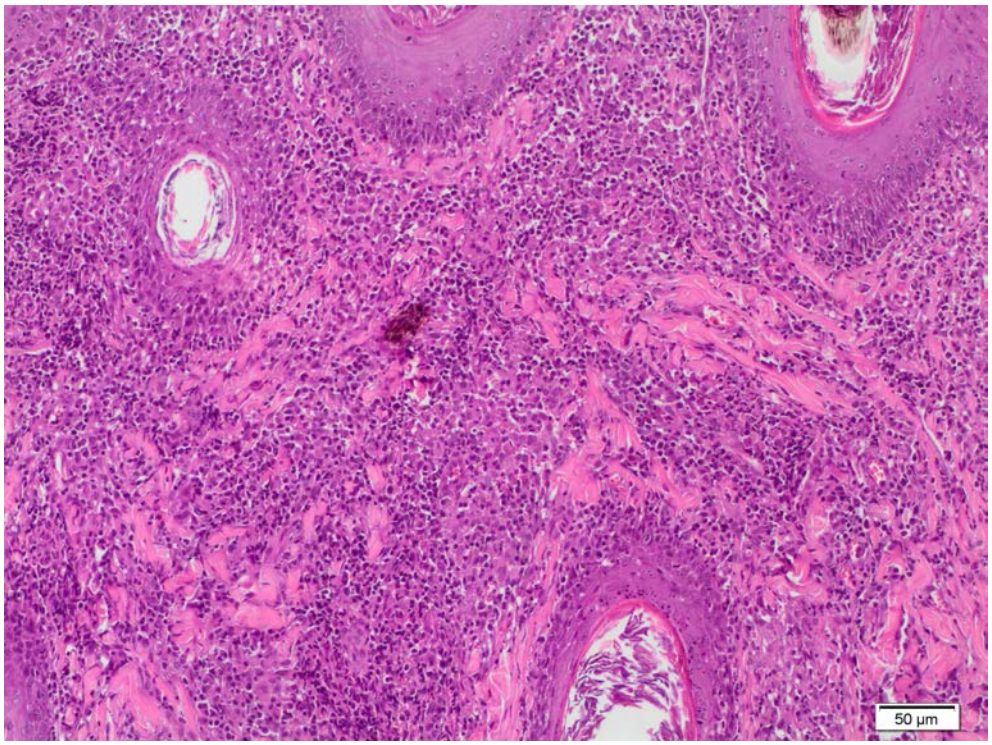

Figure 3-3. Haematoxylin and eosin stained histological image from a skin biopsy in a patient with stage II. A perivascular to interstitial pattern is present in the superficial and mid dermis.

Mild to moderate epidermal hyperplasia (n = 12) with lymphocytic exocytosis (n = 6), neutrophilic exocytosis (n = 4), crusting and ulceration (n = 3), hydropic degeneration (n = 2) and spongiosis and pustule formation (n = 1) was observed in the epidermis of patients with papular dermatitis (stage I). In patients with stage II–III, the most common epidermal lesion observed was orthokeratotic hyperkeratosis (n = 6), followed by epidermal hyperplasia (n = 5), crusting and ulceration (n = 3), spongiosis (n = 2) and neutrophilic exocytosis with pustule formation (n = 1).

Immunohistochemistry

The mean number of amastigotes in skin biopsies from patients with stages I or II–III was 53.73 ± 77.46 amastigotes/high power field (HPF) and 137.71 ± 229.13 amastigotes/HPF, respectively (Figures 3-4 and 3-5).

The difference in the means of skin parasitism between groups was not statistically significant ($p = 0.222$; Mann–Whitney test).

TLR2 immunohistochemistry of normal skin of Beagle dogs showed scattered positive basal and suprabasal epidermal and epithelial follicular cells, positive grouped cells in the follicular ostium and scattered positive perivascular mononuclear cells in superficial and mid dermis. The mean number of cells expressing TLR2 in the dermis of healthy individuals was 1.45 ± 0.4 cells/HPF (Figure 3-6).

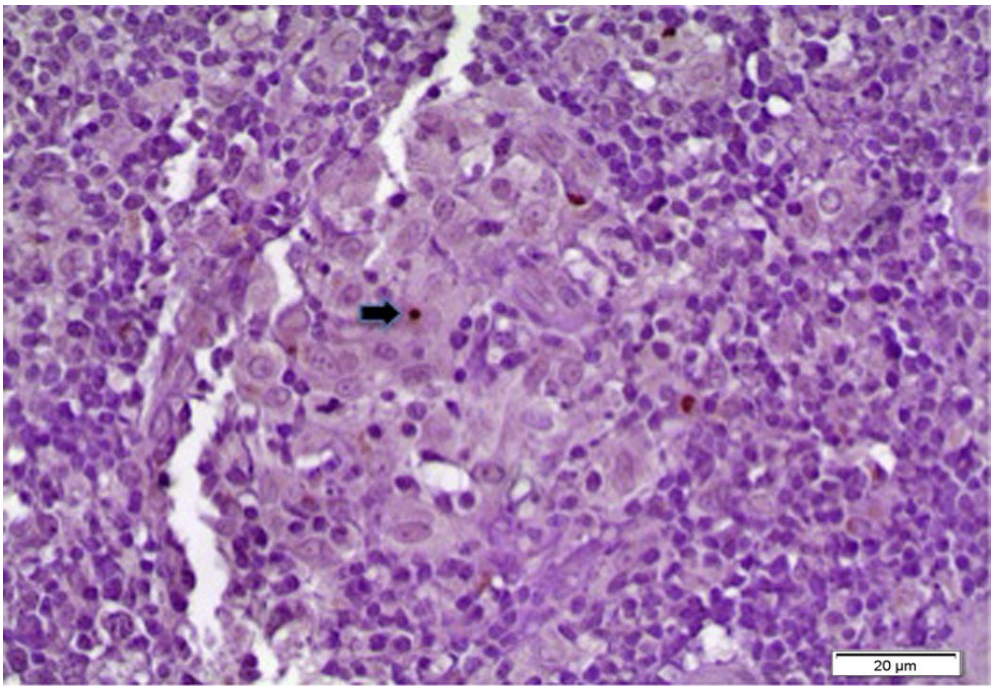

Figure 3-4. *Leishmania*-specific immunohistochemistry image from a patient with papular dermatitis. Note the low number of amastigotes. The arrow point to one amastigote at the centrum of the granuloma.

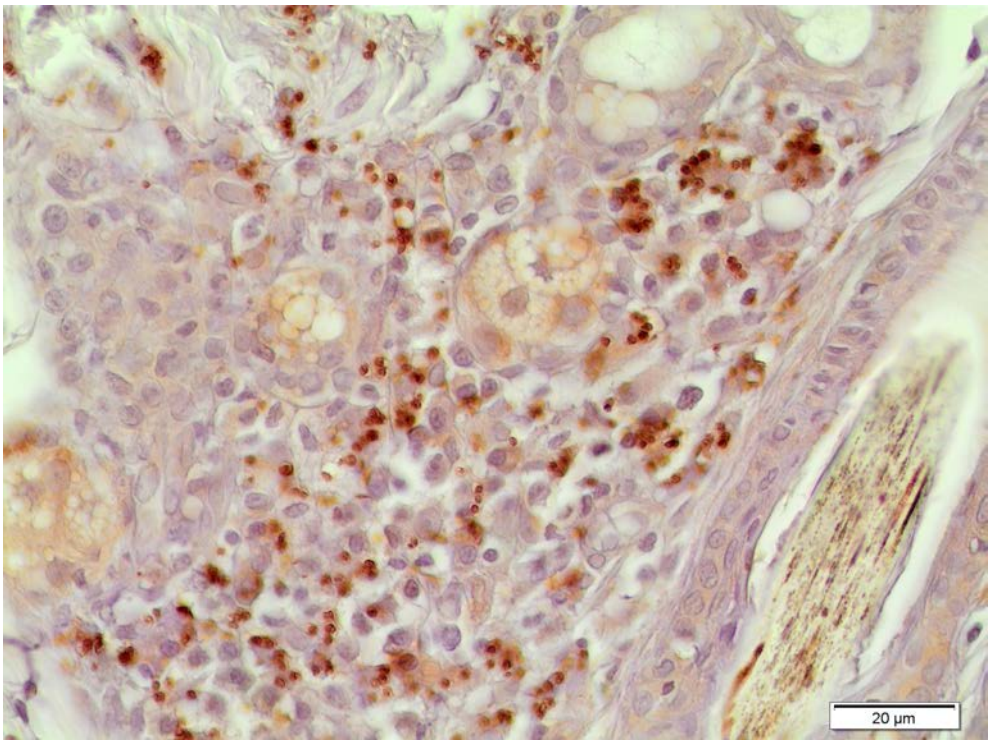

Figure 3-5. *Leishmania*-specific immunohistochemistry image from a patient of the stage II–III group. Note the high number of amastigotes present within the infiltrate.

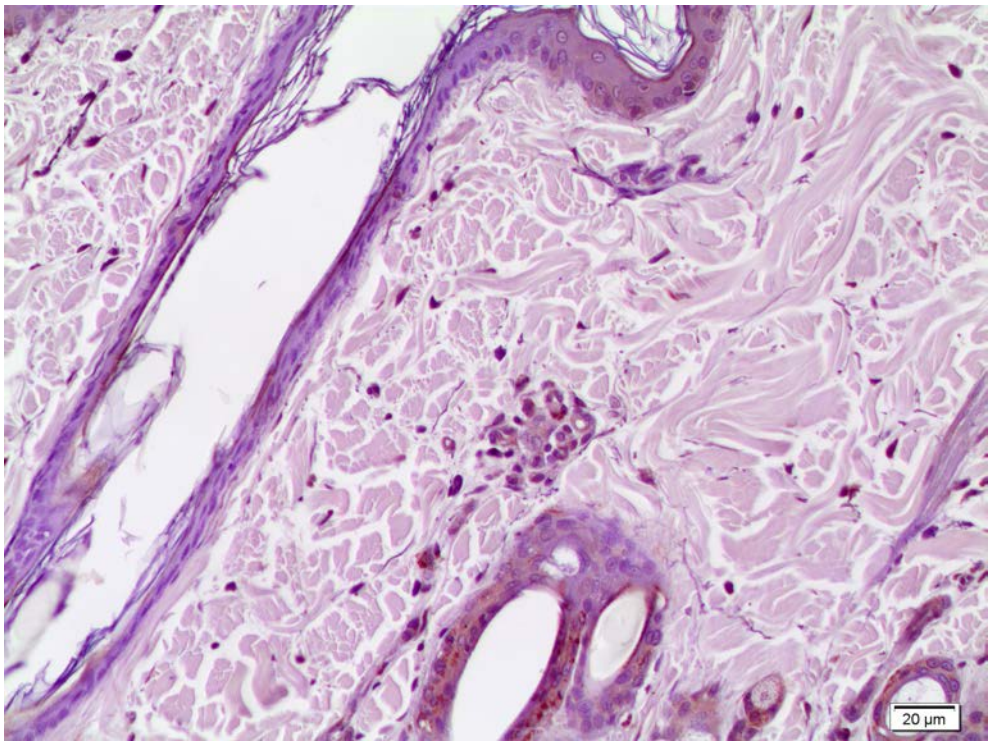

Figure 3-6. TLR2-specific immunohistochemistry image from healthy skin of a non-infected dog.

The number of mononuclear cells expressing TLR2 in the dermis of patients with papular dermatitis was 23.7±11.1cells/HPF (Figure 3-7). The expression of TLR2 in the stage II–III group was 93.3±73.8cells/HPF (Figure 3-8). The difference in the expression of TLR2 positive cells between groups was statistically significant (p=0.025; Mann–Whitney test). It was a feature of many samples of both groups that major concentration of TLR2 positive cells was present in the superficial dermis.

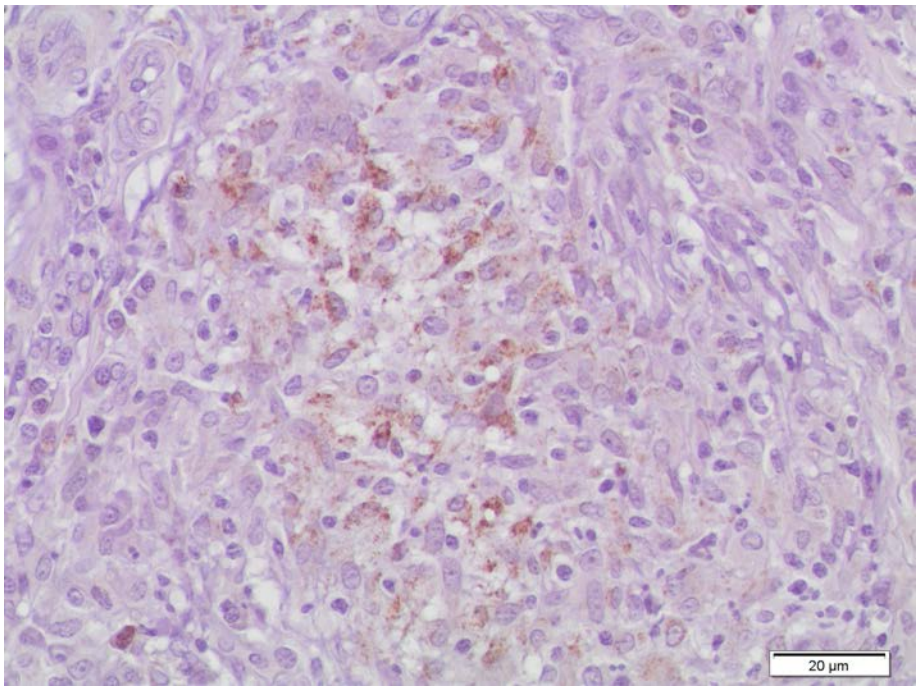

Figure 3-7. TLR2-specific immunohistochemistry image from the same patient as in Figure 1. Note the low number of TLR2 positive cells in the granuloma.

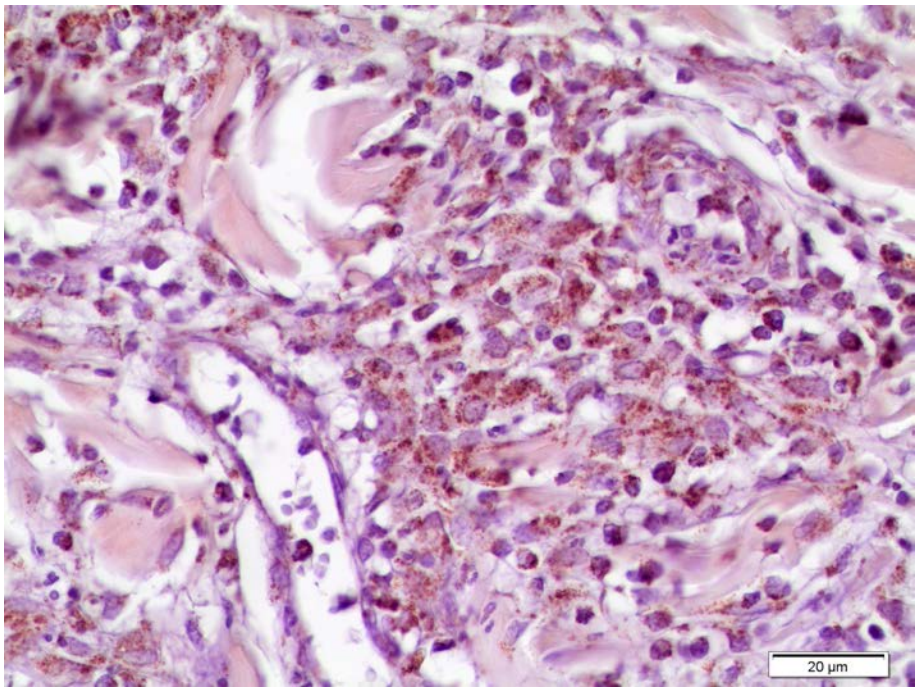

Figure 3-8. TLR2-specific immunohistochemistry image from a patient of the stage II–III group. Note the high number of positive cells present in the perivascular infiltrate.

Discussion

This descriptive study defines and compares the inflammatory pattern, the parasite burden and the expression of TLR2 in cutaneous lesions of dogs with mild disease and papular dermatitis due to *L. infantum* and in cutaneous lesions of dogs with moderate-severe disease.

Typical microscopic cutaneous lesions of CanL are consistent with inflammation of the dermis and occasionally panniculus, appearing in various histologic patterns (e.g., perivascular, interstitial, lichenoid, nodular, diffuse, peri- adnexal) (Koutinas and Koutinas, 2014). As described for any other cutaneous manifestation of CanL, papular dermatitis is predominantly characterised by a lympho-plasmacytic and macrophagic inflammatory infiltrate with variable number of neutrophils (Ordeix and Fondati, 2013). The most common inflammatory pattern described in the patients with papular dermatitis (stage I) herein described was the nodular to diffuse pattern. Moreover, one of the most relevant findings observed, for the first time, is the significantly higher frequency of granuloma formation compared with dogs with any other cutaneous manifestation (stage II–III) (Calabrese et al., 2010; Koutinas and Koutinas, 2014).

In human and experimental murine leishmaniosis, granuloma formation with few parasites characterizes the inflammatory infiltrate in lesions of resistant subjects (Lemos de Souza et al., 2000). Granuloma formation with few parasites is also a common finding in self-limiting cutaneous lesions in horses (Solano-Gallego et al., 2003). As previously described in humans, this histological finding suggests an adequate Th1 adaptive immune response with IFN-γ production and consequent control of parasite proliferation and spread (Lemos de Souza et al., 2000).

Although not statistically significant, the mean number of amastigotes per HPF was lower in skin samples of patients with stage I than in samples from patients with stage II–III in which granulomas were not formed. This lack of significance probably was the result of the high variation in the parasite concentration due to several reasons: (1) low number of cases included in this study; (2) the descriptive design of retrospective data, which preclude for selection and information bias. It is possible that some patients included in the stage I group, in fact were transitory serologically negative and displayed an immune response more typically of a stage II–III. However, this condition was not evaluated due to the retrospective analysis and lack of follow up of the patients included; (3) the duration of lesions. In human cutaneous leishmaniosis, the number of parasites present appears to be inversely proportional to the duration of the lesions (Klaus et al., 2003). Therefore, if acute lesions of papular dermatitis were included in the analysis, this could have influenced the results and (4) subjective quantitative analysis of the immunohistochemistry. Quantitative analysis of cDNA by real time polymerase chain reaction might help in performing more reliable measurements.

In conclusion, the histopathological findings observed in dogs with papular dermatitis due to *L. infantum* (i.e. *Leishmania* burden and granuloma formation), as suggested in human and experimental murine leishmaniosis (Lemos de Souza et al., 2000), are in agreement with a protective immune response and the good clinical outcome that these patients have as described elsewhere (Bottero et al., 2006; Lombardo et al., 2014).

The detection of TLR2 was increased in skin lesions of dogs with CanL compared to healthy skin of non-infected dogs. Therefore, as expected, there is more TLR2 expression in diseased skin samples where an antigenic response and consequent inflammatory infiltrate are present as described elsewhere in other tissues in dogs with leishmaniosis (Figueiredo et al., 2013) or other inflammatory diseases (McMahon et al., 2010; Chotimanukul and Sirivaidyapong, 2012). However, our study detected elevated TLR2 in skin biopsies from dogs with stage II–III disease compared with biopsies of dogs with skin papular dermatitis (stage I). The lower TLR2

expression in samples where the macrophagic infiltrate was more prominent (i.e. nodular to diffuse inflammatory pattern with granuloma formation) was unexpected. TLRs are upregulated in response to challenge by their respective agonists, therefore the elevated expression of TLR2 in skin samples of more diseased dogs could be explained by major presence of *Leishmania* antigens or even by endogenous damage-associated molecular patterns (DAMPs), also called alarmins, which includes host-derived proteins, such as degraded or increased extracellular matrix molecules and molecules released by activated and/or necrotic cells (Melo et al., 2014). Moreover, co-factors such as other inflammatory diseases, for example secondary bacterial pyoderma, could result in an overexpression of TLR2 in more severe cases with ulcerative or exfoliative dermatitis although very unlikely based on clinical and histopathological findings in the present study.

The potential role of different TLRs in generating immune responses in leishmaniosis need to be interpreted with caution depending on the *Leishmania* species and the host involved. It has been suggested, from experimental studies in human or murine models, that TLR2 plays a role in facilitating the establishment of the disease due to its role in parasite survival in macrophages upon activation by lipophosphoglycan (LPG) (Srivastava et al., 2013). Moreover, TLR2 has been associated with a regulatory function, rather than inflammatory activity of macrophages, via cytokine-mediated decrease of TLR9 expression (Srivastava et al., 2013). TLR9, which is also expressed by macrophages, was associated with the granuloma formation in humans with cutaneous leishmaniosis due to *L. braziliensis* (Tuon et al., 2010) and activation of TLR9 has been shown to promote a host-protective response (Srivastava et al., 2013). Increased frequency and expression of TLR9 has been previously associated with lower parasite load in jejunum of *Leishmania* infected dogs, whereas the colon showed a higher parasite load along with increased frequency and expression of TLR2 (Figueiredo et al., 2013). Nevertheless, higher levels of TLR2 mRNA were observed in neutrophils of mice resistant to *L. major* than those observed in susceptible mice (Charmoy et al., 2007). Moreover, the immunomodulator protein aggregate magnesium–ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) increased TLR2 expression in macrophages from dogs with visceral leishmaniosis, and induced reactive oxygen species production and nitric oxide, which are related to parasite control and elimination (Melo et al., 2013). Therefore, the regulatory role of TLR2 for the early control of lesion development and parasite burden in CanL due to *L. infantum* has to be further elucidated.

Papular dermatitis is a well-recognized clinical manifestation of CanL due to *L. infantum* (Ordeix et al., 2005; Ordeix and Fondati, 2013). In an endemic area, it is considered a typical form and highly suggestive of CanL (Ordeix and Fondati, 2013). Similar to what has been reported previously, dogs with papular dermatitis described herein were clinically characterized by the presence of persistent and multiple papules on sparsely haired skin (Ordeix et al., 2005; Bottero et al., 2006). In fact, it is suggested that as in human cutaneous leishmaniosis (Klaus et al., 2003), these lesions represent the site of parasite inoculation and multiplication. From a clinical point of view, it is noteworthy that the median age of dogs affected by papular dermatitis in this study was significantly lower than the median age observed in dogs with cutaneous manifestations of stage II–III as previously described (Lombardo et al., 2014).

Conclusion

Based on the results of this study, it can be concluded that skin biopsies from dogs with papular dermatitis (stage I) compared with more severe cutaneous manifestation of CanL (stage II–III) can be characterized by a significant higher frequency of granuloma formation, a trend for a lower parasite burden and a significant lower expression of TLR2. These results indicated an association with TLR2 in the pathogenesis of canine cutaneous leishmaniosis. Further studies are required to fully elucidate these findings.

Conflict of interest

The authors declare that they have no competing interests.

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CHAPTER 4

Histological and parasitological distinctive findings in clinically-lesioned and normal-looking skin of dogs with different clinical stages of leishmaniosis

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Abstract

Normal-looking skin of dogs with leishmaniosis frequently shows microscopic lesions along with the presence of *Leishmania* amastigotes. However, histological lesions with or without detection of amastigotes might not occur in less severe clinical cases. In addition, comparative studies between paired clinically-lesioned and normal- looking skin samples from dogs with different disease severity are lacking. The objective of this study was to compare histological and parasitological findings by *Leishmania* immunohistochemistry (IHC) and quantitative PCR (qPCR) on paired clinically-lesioned and normal-looking skin biopsies from 25 dogs with different clinical stages of leishmaniosis, 11 with stage I-mild disease (papular dermatitis) and 14 with stage II-III (ulcerative or exfoliative dermatitis).

The study demonstrated microscopic lesions in 14 out of 25 (56%) samples from normal-looking skin biopsies. In those samples, perivascular to interstitial dermatitis composed by macrophages with lymphocytes and plasma cells was observed mainly in the superficial and mid-dermis. The intensity of the dermatitis was mild to moderate and always less prominent than in the clinically-lesioned skin. In normal-looking skin samples, the presence of parasites was detected by histology, IHC and qPCR in 5/25 (20%), 8/25 (32%) and 18/25 (72%), respectively. *Leishmania* was encountered in 11/25 (44%), 23/25 (92%) and 25/25 (100%) of clinically-lesioned skin samples by histology, IHC and qPCR, respectively. Normal- looking skin from dogs with stage I-mild disease was less frequently inflamed ($p = 0.0172$). Furthermore, *Leishmania* was more easily demonstrated by histology ($p = 0.0464$), IHC ($p = 0.0421$) or qPCR ($p = 0.0068$) in normal-looking skin of dogs with stage II-III-moderate to severe disease. In addition, in the latter group, there was a significantly higher parasite load studied by means of qPCR than in dogs with less severe disease ($P = 0.043$). Clinically-lesioned skin from dogs with stage I disease was more frequently characterised by the nodular to diffuse pattern and granuloma formation ($p = 0.0166$) and by a lower parasite load studied by means of qPCR ($p = 0.043$) compared with more diseased dogs.

Normal-looking skin from dogs with stage I is less likely to present histological lesions as well as harbour the parasite when compared with dogs with moderate to severe leishmaniosis.

Keywords: *Leishmania infantum*, dog, inflammatory pattern, skin, quantitative PCR, immunohistochemistry, papular dermatitis

Introduction

Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a zoonotic vector-borne disease with a wide geographical distribution in both the Old and New World. Infected dogs are the main domestic reservoir of the parasite (Baneth et al., 2008). Dogs can manifest a chronic subclinical infection, self-limiting disease, or non-self-limiting illness (Baneth et al., 2008; Solano-Gallego et al., 2009) as previously documented in humans (Michel et al., 2011). Therefore, several degrees of disease severity are found in dogs ranging from mild disease to severe fatal disease. Two clinical staging systems are currently used in the clinical setting (Solano-Gallego et al., 2009; Paltrinieri et al., 2010). LeishVet clinical staging system ranges from stage I-mild disease to stage IV-very severe disease with different clinical outcomes, prognosis and treatment options (Solano-Gallego et al., 2009).

Cutaneous lesions are the most common clinical signs in CanL (Saridomichelakis and Koutinas, 2014) and they are very pleomorphic from a clinical and histopathological point of view as well (Ordeix and Fondati, 2013). The most common dermatological signs observed in dogs with leishmaniosis include exfoliative dermatitis, ulcerative dermatitis and onychogryphosis (Saridomichelakis and Koutinas, 2014). However, other less typical manifestations such as papular dermatitis, muco-cutaneous nodular dermatitis or sterile pustular dermatitis are also diagnosed (Saridomichelakis and Koutinas, 2014; Ordeix and Fondati, 2013). This clinical variation is due to a wide variety of pathological mechanisms occurring secondarily to the inflammation, immune complex deposition and/or autoantibody production (Koutinas and Koutinas, 2014) and to the genetically determined or acquired inability of the immune system to control parasite multiplication and tissue invasion (McCall et al., 2013).

Among the cutaneous manifestations of CanL, papular dermatitis is the only permissible dermatologic manifestation in stage I leishmaniosis (Solano-Gallego et al., 2009). Dogs with papular dermatitis commonly show no other clinicopathological abnormalities and anti-*Leishmania* antibodies are negative or weakly positive. This dermatological problem is associated with a good specific cell-mediated immune response as well as the spontaneous resolution of the lesions within 3–5 months in some cases (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014).

The normal-looking skin has been scarcely studied either in diseased or in infected but clinically healthy dogs (dos-Santos et al., 2004; Solano-Gallego et al., 2004; Papadogiannakis et al., 2005; Giunchetti et al., 2006). However, only one study evaluated both clinically-lesioned

and normal-looking skin from the same individuals (Papadogiannakis et al., 2005). In addition, to the best of our knowledge, comparative studies between paired clinically-lesioned and normal-looking skin samples from dogs with different stages of disease severity are lacking. Normal-looking skin of dogs with leishmaniosis, with or without dermatological manifestations, frequently shows microscopic lesions along with the presence of *Leishmania* amastigotes (Saridomichelakis and Koutinas, 2014). However, this might not apply in less severe clinical cases.

The objective of this study was to characterize and compare the inflammatory pattern and the parasite burden by microscopic examination, immunohistochemistry (IHC) and real-time polymerase chain reaction (qPCR) analysis in paired clinically-lesioned and normal-looking skin from the same dogs with dermatological manifestations due to CanL with different stages of disease severity (stage I-mild disease versus stage II-III-moderate to severe disease).

Materials and methods

Dogs and diagnosis of leishmaniosis

Twenty-five dogs with CanL and dermatological manifestation were prospectively enrolled at the time of diagnosis from January 2014 to February 2016. The dogs were from different Catalonian and Balearic veterinary centers from Spain: Fundació Hospital Clínic Veterinari (Bellaterra, Barcelona), Hospital Ars Veterinaria (Barcelona), Hospital Mediterrani Veterinaris (Reus, Tarragona), Consultori Montsant (Falset, Tarragona) and Hospital Mon Veterinari (Manacor, Mallorca). The diagnosis of canine leishmaniosis was made based on the results of the physical examination and cytological or dermatopathological examination of cutaneous lesions. Moreover, a complete blood count using System Siemens Advia 120 (Siemens Healthcare GmbH, Germany), a biochemical profile including creatinine, urea, total proteins, alanine transaminase and total cholesterol by Analyzer Olympus AU 400 (Olympus, Center Valley, USA), serum protein electrophoresis by Hydrasys® (Sebia Electrophoresis, Lisses, France), urinalysis with urinary protein/creatinine ratio and quantitative serology for the detection of *L. infantum* specific anti- bodies by means of a serial dilution in-house ELISA were performed (Solano-Gallego et al., 2014; Solano-Gallego et al., 2016). Dogs were classified in four different stages (stage I-mild disease, II-moderate disease, III-severe disease and IV-very severe disease) at the time of diagnosis as previously described (Solano-Gallego et al., 2009).

Collection and processing of skin samples

Two skin fragments from paired clinically-lesioned and normal-looking skin were collected from each dog. Normal-looking skin was obtained whenever possible from the lateral aspect of the neck. In cases where this region was affected, normal-looking skin was collected as far away as possible from the macroscopic lesions. Each skin sample was then immediately cut into two halves. One half was fixed in 10% formalin for routine histological and immunohistochemical examination and the other one submerged in RNA later (RNAlater® Stabilization Solution, Ambion, Inc., Austin, Texas) and kept at -80 °C until used for RNA extraction and consecutively DNA purification for qPCR analysis.

Histological examination and Leishmania immunohistochemistry

The dermal inflammatory pattern and the cell population were evaluated histologically in haematoxylin and eosin (HE)-stained sections. The distribution pattern of the infiltrate (perivascular to interstitial or nodular to diffuse with or without granuloma formation); the inflammatory cells (macrophages, lymphocytes, plasma cells and neutrophils); the degree (none, mild, moderate and severe) of cellular infiltration in the dermis and the epidermal changes (hyperplasia, spongiosis and exocytosis) were evaluated as previously described (chapter 3 and Esteve et al., 2015).

IHC for the detection of *L. infantum* amastigotes was performed as previously described (chapter 3 and Esteve et al., 2015). The parasite load in immunolabelled sections was determined as the average number of microorganisms counted in five high power fields (HPF) of areas with inflammatory infiltrate: 0, no microorganisms; 1, 1–10; 2, 11–30; and 3, > 30 (Solano-Gallego et al., 2004).

qPCR

RNA was isolated from skin biopsies using the RiboPure Kit (Ambion, Inc., Austin, Texas) and stored at −80 °C until used for future studies. DNA was purified from the interphase and organic phase generated from the RNA purification process by means of QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following the manufacturer's instructions with slight modifications. Briefly, 20 µl of proteinase K solution and 200 µl of tissue sample were used in all cases. The other steps were performed as per manufacturer's protocol. A fragment of spleen and/or skin from a clinically healthy non-infected dog from a non-endemic area (United Kingdom) was used as a control for DNA contamination during DNA extraction.

qPCR was performed with a relative quantification as previously described with minor modifications (Montserrat-Sangrà et al., 2016). Briefly, PCR mix reaction was prepared with 4 µl of DNA, 10 µl of master mix (TaqMan® Fast Advanced Master Mix, Thermo Fisher Scientific Inc.), 1 µl of *Leishmania* primers and probes (Custom TaqMan® Gene Expression Assay, ThermoFisher Scientific Inc., Waltham, USA) or 1 µl of another type of assay primers and probes [Eukaryotic 18S rRNA Endogenous Control (VICTM / MGB Probe, Primer Limited, ThermoFisher Scientific Inc., Waltham, USA)] and 5 µl of H2O.

In order to verify that the PCR was done successfully, a positive control for *Leishmania* and a negative control from a non-infected clinically healthy dog were included in the plate. PCR was carried out in a QuantStudio FlexTM 7 Real-Time PCR system (ThermoFisher Scientific Inc., Waltham, USA). Thermal cycling profile consisted of 50 °C for 2 min in order to activate the enzyme called amperase and afterwards, a total of 40 cycles were carried out. Each cycle comprised 20 s at 95 °C followed by 40 cycles of 1s at 95°C and 20s at 60°C. To compensate for variations in total DNA input, mean values of cycle threshold (CT) from duplicate determinations from the *Leishmania* and 18S rRNA-PCR were taken for the calculation of the delta CT (difference of expression between *Leishmania* CT-18S rRNA CT).

Statistical analysis

The statistical analysis was performed using the SPSS 22.0 for Windows software (SPSS Inc., USA). Categorical data were expressed as percentage and statistical analysis was performed using the McNemar's test and Fisher's exact test to compare results among related or independent variables, respectively. Quantitative data were expressed as means and standard deviations and a non-parametric Wilcoxon signed-rank test and Mann-Whitney U-test were used to compare results among related or independent variables, respectively. Differences were considered significant with a 5% significance level ($p < 0.05$).

Results

Description of clinical data of dogs

Both sexes were represented by 11 females and 14 males. The median age was 2.5 years with a range from five months to 10 years. Eleven purebred dogs belonging to ten breeds and 14 mixed-breed dogs were included. Dogs were classified in three clinical stages: stage I-mild disease characterized by persistent papular dermatitis (11 dogs, six females and five males,

median age 10 months), II- moderate disease (12 dogs, three females and nine males, median age 54 months) and III-severe disease (two female dogs, median age 54.5 months). For comparative analysis dogs were divided into two groups: group A (11 dogs with stage I) and group B (14 dogs with stage II and III). Age difference was statistically significant among groups (Mann-Whitney U-test, Z = -2.773, $p = 0.006$). In group A, six dogs were serologically negative, three were low positive and two medium positive, whereas in group B one was low positive, one was medium positive and 12 were high positive. Moreover, dogs from group A had significantly lower levels of *Leishmania* antibodies (136.8 ± 196.1 ELISA units, EU) than dogs from group B (8,892.7 ± 17,807.7 EU; Mann-Whitney U-test, Z = -3.747, $p < 0.0001$).

Descriptive histopathology

Normal-looking skin

The prevalence of microscopic lesions and presence of *Leishmania* by means of HE in normal-looking skin samples are shown in Table 4-1.

Table 4-1. Frequency of microscopic lesions and detection of *Leishmania* by means of HE, IHC and qPCR on paired skin samples from the dogs studied based on disease stage. Values with the same superscript differ significantly.

Skin samples	***Microscopic lesions***	***Detection of Leishmania***		
		HE	IHQ	qPCR
Normal-looking skin (n=25)	14/25 (56%)	5/25 (20%)	8/25 (32%)	18/25 (72%)
Stage I (n=11)	3/11 (27.3%)[a,b]	0/11 (0%)[c]	1/11 (9.1%)[d]	5/11 (45.5%)[e]
Stage II-III (n=14)	11/14 (78.6%)[b]	5/14 (35.7%)[c]	7/14 (50.0%)[d]	13/14 (92.9%)[e]
Clinically-lesioned skin (n=25)	25/25 (100%)	11/25 (44%)	23/25 (92%)	25/25 (100%)
Stage I (n=11)	11/11 (100%)[a]	1/11 (9.1%)[f]	9/11 (81.8%)	11/11 (100%)
Stage II-III (n=14)	14/14 (100%)	10/14 (71.4%)[f]	14/14 (100%)	14/14 (100%)

HE: haematoxylin and eosin stained sections; IHC: Leishmania immunohistochemistry; qPCR: quantitative PCR; [a]McNemar's test: p = 0.008; [b]Fisher's exact test: p = 0.0172; [c]Fisher's exact test: p = 0.0464; [d]Fisher's exact test: p = 0.0421; [e]Fisher's exact test: p = 0.0068; [f]Fisher's exact test: p = 0.0037

The epidermis was normal in all cases but one, with epidermal hyperplasia and ulceration. This case also showed moderate inflammatory infiltrate in the dermis with amastigotes visible with HE-stained sections. The inflammatory pattern observed ranged from perivascular to interstitial mainly in the superficial and mid-dermis in all cases (Figure 4-1). The intensity of the dermatitis was mild to moderate in all cases where inflammation was present. Macrophages with lymphocytes and plasma cells were the predominant cells. In normal-looking skin samples, the detection of intramacrophagic structures compatible with amastigotes was demonstrated in 5/25 (20%) samples, all of them from dogs from group B (Fisher's exact test, $p = 0.0464$) (Figure 4-2).

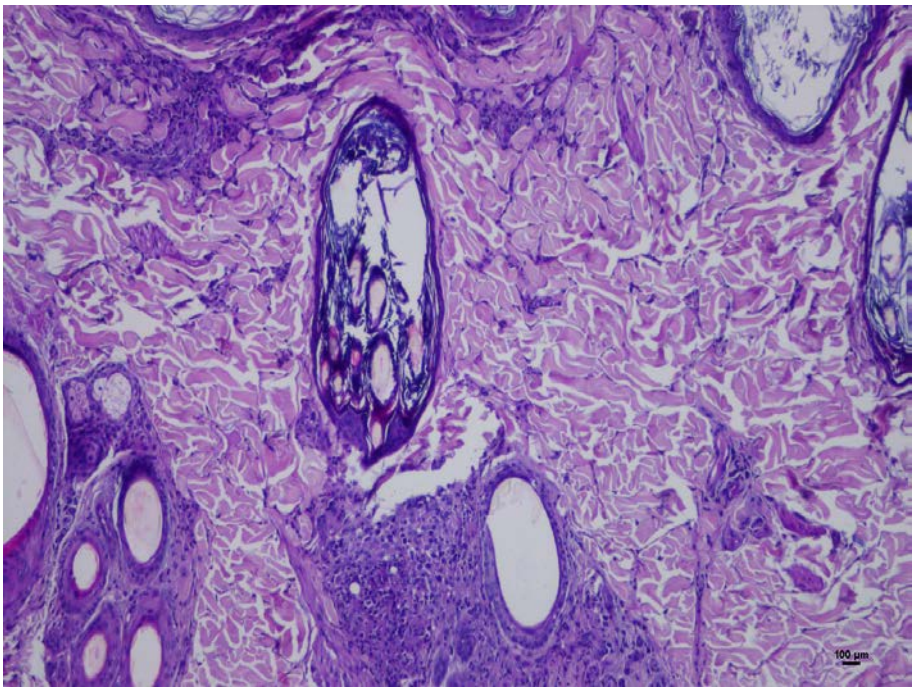

Figure 4-1. Superficial and mid perivascular to interstitial dermatitis in normal-looking skin from a dog with stage II leishmaniosis (haematoxylin and eosin staining).

Clinically-lesioned skin

The prevalence of microscopic lesions and detection of *Leishmania* by means of HE in clinically-lesioned samples are shown in Table 1. The most common epidermal changes were hyperplasia (20/25), followed by ulceration (8/25) and hyperkeratosis (7/25). Only two samples had normal epidermis. Moderate to severe lympho-plasmacytic and macrophagic infiltrates were noted in the dermis of all patients together with few neutrophils in some patients. The inflammatory pattern observed was nodular to diffuse in 13 samples (nine from group A and four from group B) and perivascular to interstitial in 12 clinically-lesioned samples (two from group A and ten from group B). Therefore, skin samples from group A were more frequently

characterized by a nodular to diffuse pattern than skin samples from group B (Fisher's exact test, $p = 0.0154$).

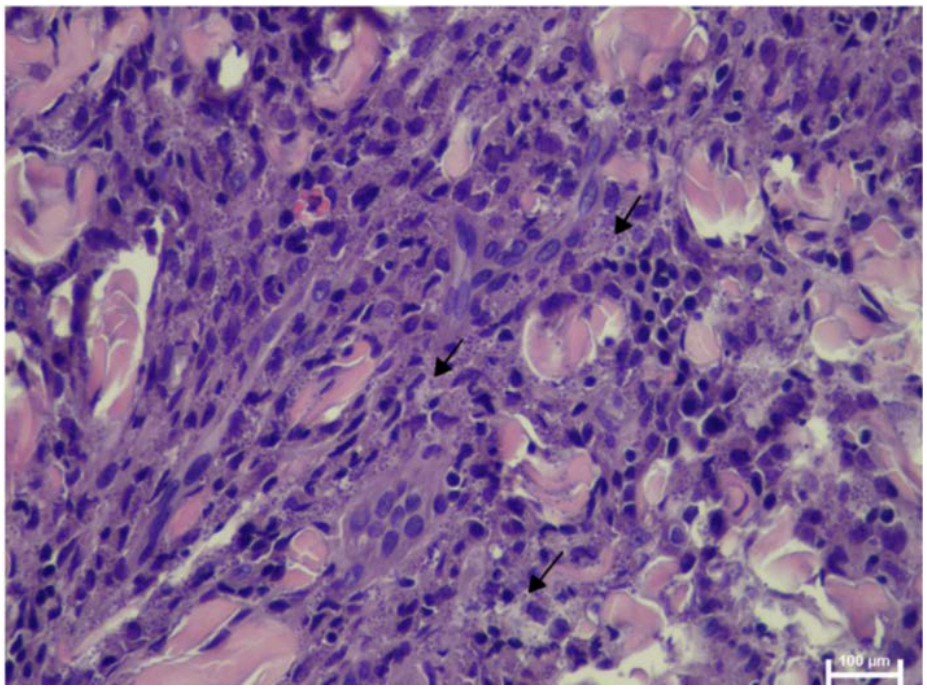

Figure 4-2. Numerous intracellular *Leishmania* amastigotes in macrophages from the inflammatory infiltrate present in the dermis of normal-looking skin sample from a dog with stage II leishmaniosis (haematoxylin and eosin staining).

Granulomas were only observed in four samples, all of them from group A (Fisher's exact test, $p = 0.0166$) (Figure 4-3). Amastigotes compatible with *Leishmania* were noted in 11/25 (44%) samples. Most of these (10/11) were samples from group B and this difference was statistically significant (Fisher's exact test, $p= 0.0037$).

Leishmania immunohistochemistry

The prevalence of positive IHC in clinically-lesioned and normal-looking skin samples are shown in Table 1. Amastigotes were noted in 8/25 (32%) normal-looking skin samples. Seven out eight of these samples were from dogs from group B (Fisher's exact test, $p = 0.0421$; Figure 4-4). The majority of positive samples (6/8) had few amastigotes (1–10 per HPF) with one between 11–30 and another with more than 30 per HPF. On the other hand, amastigotes were noted in 23/25 (92%) clinically-lesioned skin samples. Two samples with negative IHC were from dogs from group A. Although marginally statistically significant, there was a trend for a higher parasite load in clinically-lesioned skin from dogs from group B compared with group A (Mann-Whitney U-test: Z = -1,943, $p = 0.052$; Figure 4-5; Table 4-2).

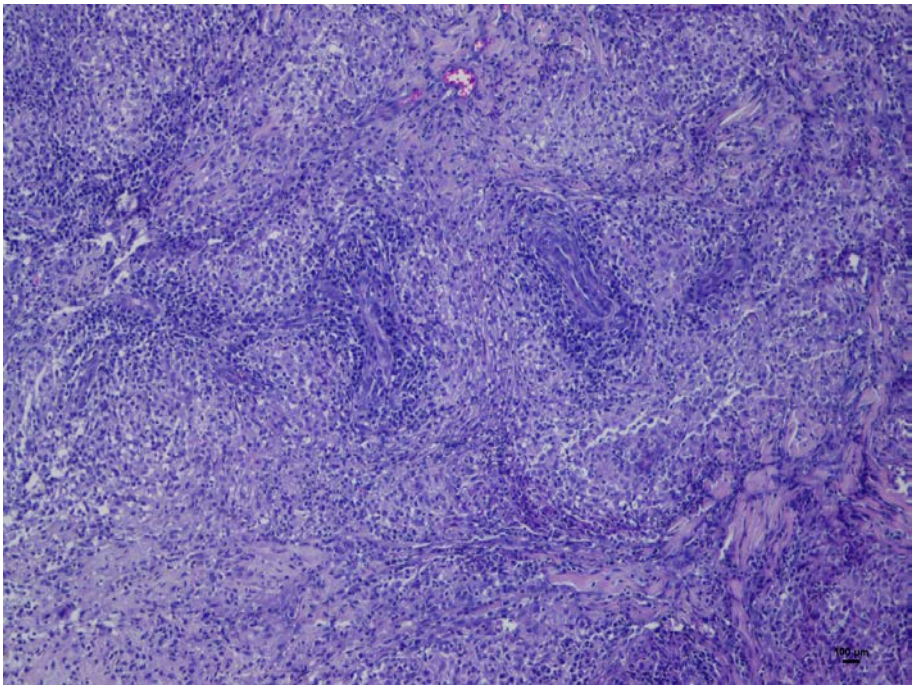

Figure 4-3. Nodular to diffuse dermatitis with granuloma formation in clinically-lesioned skin from a dog with stage I leishmaniosis (haematoxylin and eosin staining).

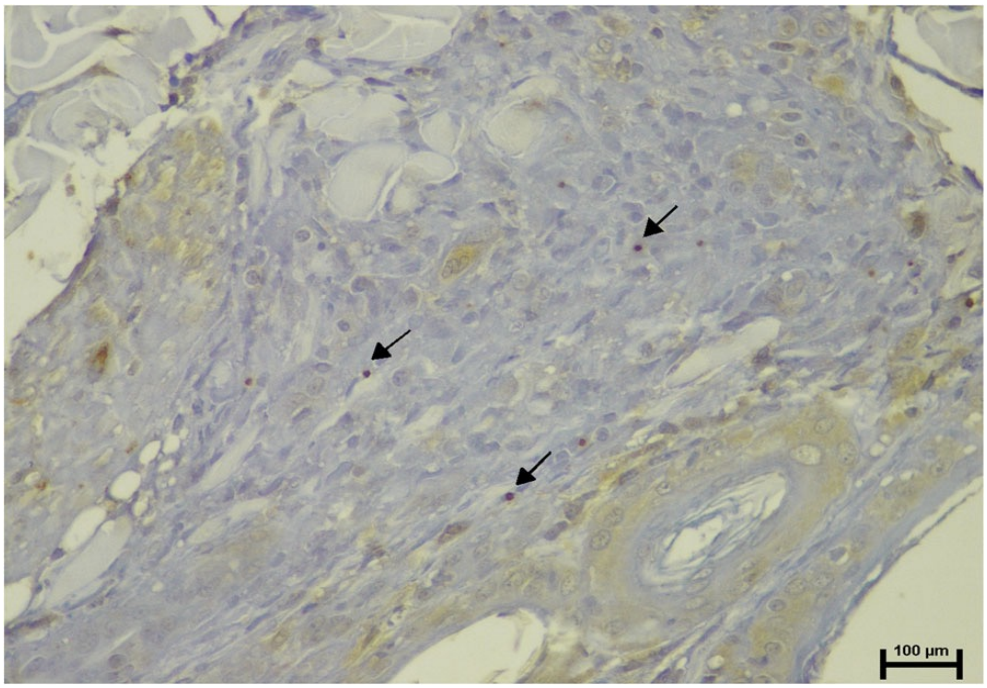

Figure 4-4. Few (1–10 per HPF) intracellular *Leishmania* amastigotes (arrows) are visualized in macrophages from the inflammatory infiltrate present in the dermis of normal-looking skin sample from the same dog as in Figure 4-1 (*Leishmania*-specific IHC staining).

qPCR

The normal-looking skin of 18/25 (72%) dogs studied was qPCR positive for *Leishmania* (Table 4-1). Negative qPCR was almost always associated with a microscopically normal skin. Only one dog presented mild perivascular dermatitis in the deep dermis and qPCR was negative. From 11 samples without histological lesions, five resulted qPCR positive. The prevalence of negative qPCR on normal-looking skin samples from dogs from group A was higher than that detected in normal- looking skin from dogs from group B (Fisher's exact test, $p = 0.0068$). The parasite load studied by means of qPCR in normal-looking skin samples was always lower than in clinically-lesioned skin whatever the stage of disease (Wilcoxon signed-rank test, group A: $Z = -2.023$, $p = 0.043$; group B: $Z = -2.691$, $p = 0.007$; Table 4-2). The relative amounts of parasites in normal-looking skin from dogs from group A was lower than in normal-looking skin from dogs from group B (Mann-Whitney U-test: $Z = -2.021$, $p = 0.043$; Table 4-2).

As expected, 25/25 (100%) of clinically-lesioned skin were qPCR positive and the parasite load was higher in samples from dogs from group B compared with dogs from group A (Mann-Whitney U-test: $Z = -2.026$, $p = 0.043$, Table 4-2).

Discussion

In this study, we demonstrated histological and parasite load differences not only among clinically-lesioned and normal-looking skin of the same dogs but also among skin samples of dogs with different clinical stages of leishmaniosis.

In agreement with previous studies, we demonstrated that the normal-looking skin of dogs with leishmaniosis frequently shows microscopic lesions (56%) and harbours the parasite, as demonstrated by routine HE staining (20%), *Leishmania*-specific IHC (32%) and, more often, by qPCR (72%). However, there are some differences among our results and those previously reported (Solano-Gallego et al., 2004; dos-Santos et al., 2004; Papadogiannakis et al., 2005; Giunchetti et al., 2006). The prevalence of microscopic lesions and detection of amastigotes either by routine histology or by IHC in our study was at the lower limit of the ranges reported in previous studies. Microscopic lesions have been noticed in 50–100% of the skin samples obtained from the normal-looking skin of dogs with CanL (Solano-Gallego et al., 2004; dos-Santos et al., 2004; Papadogiannakis et al., 2005; Saridomichelakis and Koutinas, 2014).

Table 4-2. Parasite load by means of *Leishmania*-specific IHC and qPCR on paired skin samples from the dogs studied based on disease stage.

Skin samples	***IHC[a]*** ***(mean +/-sd)***	***qPCR[g]*** ***(mean +/-sd)***
Normal-looking skin (n=25)	0.4+/-0.8[b]	3.0+/-4.7[h]
Stage I (n=11)	0.1+/-0.3[c,d]	6.1+/-4.0[i,j]
Stage II-III (n=14)	0.5+/-0.7[d,e]	1.7+/-4.5[i,k]
Clinically-lesioned skin (n=25)	1.5+/-0.9[b]	1.5+/-4.9[h]
Stage I (n=11)	1.1+/-0.8[c,f]	3.4+/-4.4[i,l]
Stage II-III (n=14)	1.7+/-0.9[e,f]	-0.4+/-4.7[k,l]

qPCR: quantitative PCR; IHC: Leishmania immunohistochemistry; SD: standard deviation; [a]For method of grading, see Methods; [b]Wilcoxon Signed-rank test: Z = -4.345, p < 0.0001; [c]Wilcoxon Signed-rank test: Z = -2.887, p = 0.004; [d]Mann-Whitney U-test: Z = -2.169, p = 0.03; [e]Wilcoxon Signed-rank test: Z = -3.274, p = 0.001; [f]Mann-Whitney U-test: Z = -1.943, p = 0.052; [g]Delta CT (difference of expression between Leishmania CT -18S CT); [h]Wilcoxon Signed-rank test: Z = -3.332, p = 0.001; [i]Wilcoxon Signed-rank test: Z = -2.023, p = 0.043; [j]Mann-Whitney U-test: Z = -2.021, p = 0.043; [k]Wilcoxon Signed-rank test: Z = -2.691, p = 0.007; [l]Mann-Whitney U-test: Z = -2.026, p = 0.043

Moreover, amastigotes were seen in up to 100% of the cases, depending on the sensitivity of the method employed (Saridomichelakis and Koutinas, 2014). These findings are probably related to the fact that in the present study about half of the dogs had mild disease, i.e. papular dermatitis. Conversely, previous studies included either dogs with more severe disease, i.e. exfoliative dermatitis (Papadogiannakis et al., 2005) or even stray dogs, which could present co-factors, such as co-infections or malnutrition, affecting the severity of disease (Solano-Gallego et al., 2004; dos-Santos et al., 2004).

In the present study, we demonstrated that dogs with different clinical stages of leishmaniosis presented differences in the frequency of microscopic lesions and parasite load in normal-looking skin. The skin biopsies from normal-looking skin from dogs with stage I-mild disease (papular dermatitis) were significantly less frequently inflamed. Furthermore, *Leishmania* was more frequently demonstrated by routine histology, immunohistochemical examination or qPCR in normal-looking skin of dogs with stage II-III-moderate to severe disease. In addition,

in the latter group, there was a significantly higher parasite load studied by means of qPCR than in dogs with less severe disease. These results suggest that dermal inflammation and cutaneous parasitism in normal-looking skin were directly related to the severity of clinical disease. Normal-looking skin of dogs with stage I-mild disease may resemble the skin of seronegative infected but clinically healthy dogs that is characterized by no histological lesions and absence of parasites by IHC, although their presence can be demonstrated by PCR (Solano-Gallego et al., 2004).

Microscopic lesions and presence of amastigotes in the inflammatory infiltrate in normal-looking skin of diseased dogs is suggestive of haematogenous dissemination of the parasite and tropism for the skin (Solano-Gallego et al., 2004). Moreover, it has been demonstrated that dissemination to the skin varies between dogs, being greater in sick and infectious dogs (Courtenay et al., 2014). Therefore, lack of these changes in the majority of dogs with normal-looking skin with stage I-mild disease would further suggest a protective immune response in these dogs able to control parasite dissemination at the site of parasite inoculation and multiplication as previously proposed (Lombardo et al., 2014; chapter 3 and Esteve et al., 2015).

Histological findings observed in clinically-lesioned skin of dogs included in this study were in accordance with the literature (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014; Esteve et al., 2015) and amastigotes were variably seen in 44 and 92% of the cases, depending on the method employed. However, the results of this study further confirm that skin biopsies from dogs with papular dermatitis (stage I-mild disease) are characterized by the nodular to diffuse pattern and a significant higher frequency of granuloma formation compared with more severe cutaneous manifestation of CanL (stage II–III- moderate or severe disease) (chapter 3 and Esteve et al., 2015). It has been proposed previously that there is a trend for a lower parasite burden in skin samples from dogs with stage I-mild disease (chapter 3 and Esteve et al., 2015). Although amastigotes were more frequently noted in HE stained slides from stage II-III diseased dogs when compared with stage I dogs, there were no statistically significant differences in prevalence between positive IHC or qPCR among both groups studied. Nevertheless, the parasite load studied by means of qPCR was lower in samples from dogs with stage I-mild disease compared with dogs with severe disease. Taken together, these data might reinforce the idea of a protective immune response that these dogs have as described elsewhere (Bottero et al., 2005; Lombardo et al., 2014; chapter 3 and Esteve et al., 2015).

Several studies have focused on the capacity of dogs to infect phlebotomine sand flies. It has been reported that the proportion of infected sand flies increases with the appearance and severity of the clinical signs and that good predictors of infectiousness are antibody levels and clinical disease, since no dogs have been found to be infectious before the detection of anti-*Leishmania* IgG antibodies (Molina, 1997; Courtenay et al., 2002). Moreover, it has been recently suggested that high parasite loads in dog ear skin, rather than the simple presence of parasites, is the most important metric to identify likely infectious individuals and potential reservoir populations (Courtenay et al., 2014). Therefore, the fact that dogs with stage I-mild disease or papular dermatitis are characterized by reduced parasite load in both normal-looking skin and clinically-lesioned skin, emphasizes the concept that these dogs do not play a significant role in *L. infantum* infection of phlebotomine sand flies as opposed to dogs with stage II-III disease.

Conclusion

In conclusion, this study confirms that normal-looking skin from dogs with stage I is less likely to present microscopic lesions as well as harbour the parasite when compared with dogs with moderate to severe CanL. Moreover, clinically-lesioned skin from dogs with stage I shows a lower parasite load than clinically-lesioned skin from more diseased dogs.

Abbreviations

CanL: Canine leishmaniosis; CT: Cycle threshold; ELISA: Enzyme-linked immunosorbent assay; IHC: Immunohistochemistry; qPCR: Quantitative polymerase chain reaction

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LO and LSG designed the research study. AD, MO, JLL and LO included all the dogs. LO and LSG coordinated the veterinary clinics enrolled. LO performed all the histological and immunohistological work. LO and SM performed the molecular work of this study. LO and LSG contributed with data analysis and interpretation. LO wrote the manuscript. LSG revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

A signed informed consent was obtained from all owners of dogs. Ethical approval was obtained by "Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona" (CEEAH 1586, February 2012).

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CHAPTER 5

High prevalence of papular dermatitis suggestive of *Leishmania infantum* infection in Ibizan hounds: A cross-sectional study from an endemic area.

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Abstract

Papular dermatitis is the mildest cutaneous form of canine leishmaniosis, however its prevalence has not been studied. The objective of this study was to investigate the prevalence of papular dermatitis in Ibizan hound dogs. Forty-seven Ibizan hounds and 46 dogs of different breeds living in a highly endemic area of leishmaniosis were included. Infection was evaluated by means of an in house ELISA, blood *L. infantum* qPCR, parasite specific IFN-γ production after blood stimulation and leishmanin skin test (LST). Diagnosis of papular dermatitis was done clinically and supported by visualization of amastigotes in skin cytology or biopsy or by molecular techniques from stained cytological smears. Papular dermatitis was observed in 31/47 (66%) Ibizan hounds and in only one dog from the other group ($p = 0.00001$). This skin condition was confirmed by means of visualization of amastigotes or by molecular techniques from skin lesions in 18 Ibizan hounds. The papule from the control dog was cytologically suggestive of an arthropod bite reaction. The percentage of infected dogs, defined by at least one positive test, was similar among groups (87.2% vs 74%, $p = 0.12$) as well as the percentage of seroreactive dogs, positive blood qPCR and positive LST reactions. There were more IFN-γ producers among the Ibizan hounds (85.7%) than in dogs of other breeds (61.5%) ($p = 0.0378$). Both groups of dogs were similarly infected, however the Ibizan hounds had a significantly higher prevalence of papular dermatitis and were more commonly characterized by increased IFN-γ production.

Keywords *Leishmania infantum*, dog, Ibizan hound, papular dermatitis, IFN-γ, cross-sectional study.

Introduction

Canine leishmaniosis (CanL) is a vector-borne disease caused by a protozoan, *Leishmania infantum*, which affects both the viscera and skin of dogs (Baneth et al., 2008). *Leishmania infantum* infection is endemic in many parts of Europe and its prevalence is high involving as much as 50%–80% of the canine population in Mediterranean countries (Solano-Gallego et al., 2001; Leontides et al., 2002). However, prevalence of disease is much lower and varies from 2 to 5% (Solano-Gallego et al., 2001; Leontides et al., 2002; Baneth et al., 2008; Noli and Saridomichelakis, 2014). Therefore, in an endemic region, many dogs remain sub-clinically infected, while others will succumb to the infection and present clincopathological alterations or even die from the disease (Baneth et al., 2008). The outcome of the infection is the consequence of a complex host-parasite interaction. In fact, it is the genetically determined or acquired inability of the canine immune system to control parasite multiplication that results in tissue invasion and progression of the disease. A resistant dog is able to control the infection due to a protective CD4+ T-cell-mediated immune response characterized by production of T helper 1 (Th1) cytokines such as interferon-gamma (IFN-γ), interleukin (IL)-2 and tumour necrosis factor-alpha (TNF-α), which induce anti-*Leishmania* activity by apoptosis of parasites in macrophages via nitric oxide metabolism (Baneth et al., 2008). On the opposite side, a severely sick dog presents a marked humoral immune response and reduced cell mediated immunity with a mixed Th1 and Th2 cytokine pattern and high parasite burden (Baneth et al., 2008). This explains why CanL is such a pleomorphic disease, since between these two extremes of the immune response there is a wide range of clinical and pathological manifestations (Baneth et al., 2008; Ordeix and Fondati, 2013; Koutinas and Koutinas, 2014; Noli and Saridomichelakis, 2014).

Among the clinical signs, cutaneous lesions are the most common finding in CanL, with 67–89% of cases showing a cutaneous involvement (Saridomichelakis and Koutinas, 2014). The frequency of each dermatological presentation in dogs with leishmaniosis due to *L. infantum* is partially unknown and defined based on descriptive studies (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014). Previous published data indicates that exfoliative dermatitis, ulcerative dermatitis and onychogryphosis are the most common cutaneous presentations with prevalence ranging from 39.6–73.1%, 15.3–40% and 24–71.1%, respectively (Saridomichelakis and Koutinas, 2014). Other less common manifestations such as papular dermatitis, muco-cutaneous nodular dermatitis or sterile pustular dermatitis are also diagnosed with varied prevalence (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014).

Each of these cutaneous clinical presentations may represent distinct clinicopathological conditions and reflect a different host-parasite relationship, as some of them had been associated with different histological lesions and varied parasite loads (Saridomichelakis and Koutinas, 2014; chapter 4 and Ordeix et al., 2017). The most studied example is papular dermatitis due to *L. infantum* as the sole clinicopathological finding, which is considered a mild clinical manifestation of this infection (Ordeix et al., 2005; Solano-Gallego et al., 2009). In fact, this clinical presentation is histologically characterised by granuloma formation with low parasite load, which denotes control of parasite proliferation (chapter 4 and Ordeix et al., 2017). Moreover, dogs with this clinical manifestation usually do not show dissemination of the infection and there is absence of parasites in other organs (Lombardo et al., 2014), lack of microscopic lesions and a low parasite load in normal-looking skin (chapter 4 and Ordeix et al., 2017). Papular dermatitis due to *L. infantum* has been more frequently documented in young dogs with no other clinicopathological alteration, negative or low antibody levels, negative or low blood parasitemia, and specific cell-mediated immune response studied by means of leishmanin skin test (LST) and high IFN-γ production, suggesting a protective immune response (Ordeix et al., 2005; Lombardo et al., 2014; chapter 4 and Ordeix et al., 2017; Montserrat-Sangrà et al., 2018). Papular dermatitis is considered to be a typical form of CanL in an endemic area (Ordeix and Fondati, 2013); however, its prevalence has not been studied.

Ibizan hound dogs are considered a resistant breed to *L. infantum* infection due to the presence of a protective immune response that is associated with a clinically healthy status and good outcome (Solano-Gallego et al., 2000; Solano-Gallego et al., 2005). A strong cell mediated immune response characterised by a high frequency of positive LST reactions as well as a potent *Leishmania*-specific IFN-γ production is reported in Ibizan hounds when compared with other breeds from the same geographical area (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017). Moreover, a low or no humoral response has been demonstrated in this breed (Solano-Gallego et al., 2000).

The hypothesis of this study was that papular dermatitis may be more common in breeds known to be resistant to *Leishmania* infection. Therefore, the general objective of the present study was to evaluate the prevalence of papular dermatitis in dogs living in endemic areas. The specific objective was to determine the prevalence of infection and of papular dermatitis in Ibizan hound in comparison with other dog breeds.

Material and Methods

Study design, setting and dogs

This cross-sectional study was conducted in December during three consecutive years (2014-2016) in the island of Mallorca, Spain. Three different herds of hunter dogs held outdoors and with similar environmental conditions and thus exposed similarly to *Leishmania* were included in this study. Forty-seven Ibizan hound dogs (Group I) were included. A control group that was recruited in parallel was composed of 46 dogs from other breeds (Group O). Signalment, including breed, sex and age, were recorded. An informed consent was obtained from all owners for participation in the study.

Evaluation of Leishmania infection status

Detection of specific *L. infantum* antibody levels

Quantitative serology using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed as previously described (Solano-Gallego et al., 2016).

DNA extraction and *Leishmania* quantitative polymerase chain reaction (qPCR) from blood.

DNA was extracted using the Gen Elute blood genomic DNA kit (Sigma-Aldrich) and parasite load was investigated by amplification of kinetoplast DNA sequence and quantified with an absolute quantification of parasites/mL of blood as previously described (Solano-Gallego et al., 2016).

Parasite specific whole blood canine IFN-γ cytokine release assay

Heparinized whole blood cytokine release assay was carried out, as previously reported (Solano-Gallego et al., 2016b). Briefly, heparinized whole blood was separately mixed with three different conditions: (i) unstimulated medium; (ii) medium with soluble *L. infantum* antigen (LSA, 5 mg/mL, Facultat de Farmacia, Universitat Autònoma de Barcelona) 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. IFN-γ was determined in supernatants obtained five days after stimulation by a commercial sandwich ELISA (DuoSet® ELISA by Development System R&D™, Abingdon, UK). Cytokine concentration from supernatants with ConA and LSA was calculated after subtracting the IFN-γ concentration obtained from unstimulated supernatants.

Leishmanin skin test

LST was carried out as described previously (Solano-Gallego et al., 2000; Solano-Gallego et al., 2005; chapter 7 and Ordeix et al., 2018). Briefly, 0.1 mL of an inactivated suspension of $3x10^8$ *L. infantum* promastigotes/mL in 0.4% phenol-saline (kindly provided by C. Chicharro, Instituto de Salud Carlos III, Madrid, Spain) was injected intradermally into the skin of the groin. The skin reaction was read at 72 h and an area of induration and/or erythema larger than 5 mm in diameter was considered positive. Moreover, diameter and thickness of the reaction observed at the site of antigen inoculation was assessed with a calliper (Mitutoyo 500-161-20) and compared between groups.

Diagnosis of papular dermatitis

Dermatological examination

A diplomate of the European College of Veterinary Dermatology (LO) and a diplomate of the European College of Veterinary Clinical Pathology (LSG) performed a complete dermatological examination in all the dogs. Cutaneous lesions represented by raised solid lesions smaller than 0.5 cm in diameter (papules) or raised solid lesions wider than higher (plaques) with or without an ulcerative or crusted central part (volcano sign) in non-haired skin were considered suggestive of papular dermatitis due to *Leishmania* and recorded (Ordeix et al., 2005; Malek et al., 2012; Ordeix and Fondati, 2013; Lombardo et al., 2014). Moreover, a sample was collected for cytological and molecular examination whenever the size of the lesion allowed this procedure.

Cytological examination

Impression smears, after removal of the adherent crust if present, or fine needle aspirates from papules or plaques were sampled and stained with a Romanowsky stain variant (Diff-Quick®) from 18 dogs. They were evaluated microscopically to assess the cellularity, the presence of an etiological agent including *Leishmania* amastigotes, and the type of inflammation or cellular infiltrate. The degree of cellularity was estimated by counting the number of nucleated cells per 10 fields under 600x. The cellularity was quantified as very low (<5 cells), low (5 – 25 cells), moderate (25 – 50), high (50 – 100) and very high (>100 cells). The inflammation or cellular infiltrate was classified by the predominance of the cell type involved. Neutrophilic inflammation was defined as lesions containing more than 80% of neutrophils. Mixed inflammation was interpreted as lesions harbouring a mixture of neutrophils and macrophages, admixed with occasional lymphocytes and/or plasma cells. An increased number of small lymphocytes, occasional intermediate-sized lymphocytes, plasma cells and macrophages was also considered as mixed inflammation.

Macrophagic inflammation was determined as lesions with a predominance of macrophages. Lymphocytic or lymphoplasmacytic inflammation was defined as lesions containing a heterogeneous lymphoid population, with predominance of small lymphocytes, admixed with occasional intermediate-sized lymphocytes and plasma cells, with low numbers of other inflammatory cells. Eosinophilic inflammation was categorized as lesions having more than 10% eosinophils in addition with other inflammatory cells.

Impression smears or fine needle aspirates from the lesions were interpreted as positive for *Leishmania* infection when at least one *Leishmania* amastigote was observed intracellularly or extracellularly.

DNA extraction and *Leishmania* qPCR from cytological stained smears

Genomic DNA was extracted with QIAamp® DNA Mini and Blood (Qiagen) following the manufacturer's instructions from cytological stained smears in 11 dogs as previously reported (Lima et al., 2017). Beforehand, each sample was scrapped using a scalpel blade number 11 (Braun, Tuttlingen, Germany) and the material was deposited in an eppendorf tube. After adding 20 µL of protease and 200 µL of PBS, the recommended protocol was carried out (Lima et al., 2017). qPCR was performed with primers targeting *Leishmania* kinetoplast DNA and 18s RNA (Solano-Gallego et al., 2016).

Dermatopathological examination and *Leishmania*-immunohistochemistry

In three dogs skin biopsies were obtained under sedation based on clinician's criteria and fixed in 10% neutral buffered formalin and process routinely. The dermal inflammatory pattern and the cell populations were evaluated histologically in hematoxylin and eosin-stained sections. Moreover, a deparaffinization step was performed on the paraffin blocks of skin biopsies before *Leishmania* immunohistochemistry (IHC) was performed with a standard staining protocol with AutostainerPlus (Dako Denmark A/S, Glostrup, Denmark) using rabbit polyclonal antibodies to *L. infantum,* as previously described (chapter 3 and Esteve et al., 2015).

Statistical methods

Standard descriptive statistics (mean ± standard deviation (SD) and median with ranges) were calculated for quantitative variables, whereas categorical data were reported in percentages. A non-parametric Mann-Whitney U-test and Fisher's exact test were used to compare quantitative and categorical data, respectively, between groups. Cohen's kappa coefficient was used to calculate the agreement between parasite specific IFN-γ cytokine production and LST in evaluating *Leishmania* specific cellular immune response. Moreover, agreement between those techniques and

papular dermatitis was also calculated with this index. The kappa agreement was determined as follows: no agreement ($k < 0$), slight agreement ($0 < k < 0.2$), fair agreement ($0.2 < k < 0.4$), moderate agreement ($0.4 < k < 0.6$), substantial agreement ($0.6 < k < 0.8$) and almost perfect agreement ($k > 0.8$). Differences were considered significant with a 5% significance level ($p < 0.05$). Statistical analysis was performed using the IBM SPSS 22.0 programme for Windows software (SPSS Inc., Armonk, New York, USA).

Results

Dogs

Group I was composed of 47 Ibizan hound dogs: 36 intact females and 11 intact males and their median age was 12 months [6-90]. In group O there were 46 dogs of which 15 were mixed breed dogs, 12 English setter dogs, 11 Brittany dogs, two Weimaraner dogs, two shorthaired pointer dogs, and one dog of the following breeds: border collie, beagle, Rhodesian ridgeback dog and Mallorca shepherd dog. There were 26 females and 20 males of which the majority was intact and their median age was 39.5 months old [4-132]. Differences in the frequency of sex and age between groups were considered statistically significant ($p = 0.049$, Fisher's exact test and $p = 0.014$, Mann-Whitney U-test, Z-Score = 2.45457, respectively). The percentage of young dogs equal or less than 1.5 years old was similar between groups (Group I = 30% and group O = 28%, $p = 1$, Fisher's exact test). Moreover, the percentage of adult dogs equal or older than 4 years old was statistically different between groups (Group I = 9% and group O = 41%, $p = 0.0247$, Fisher's exact test).

Diagnosis of papular dermatitis

Dermatological examination revealed that 31 Ibizan hound dogs (66%) presented papular dermatitis clinically suggestive of leishmaniosis. These dogs did not present other cutaneous lesions but mild crusting dermatitis in pinna in two dogs suggestive of fly dermatitis. Papules were single or multiple and always located in the inner aspect of one or both pinna (Figure 5-1). Table 5-1 shows the results of the diagnostic tests performed for the diagnosis of *Leishmania* infection and papular aetiology in 31 Ibizan hound dogs with papular dermatitis. From 16 Ibizan hound dogs without papules, 15 were infected and in one *Leishmania* infection could not be demonstrated. In contrast, dermatological examination performed in dogs from group O evidenced varied cutaneous problems including ulcers on the tip of the ear pinnae and/or tail (n= 5), ventral erythema and/or

hyperpigmentation (n = 4); otitis externa (n = 2); multifocal alopecia (n = 2); dorso-lumbar pruritus (n = 1) and nasal hyperkeratosis (n = 1). Only one dog from group O presented a papulo-crusting lesion on the bridge of the nose (2.2%) (p = 0.00001, Fisher's exact test). This lesion was not highly suggestive of leishmaniosis as it was non-erythematous and without the characteristic volcano sign (Figure 5-2). This dog was infected as demonstrated by a low positive serology and LST positive reaction. However, it was IFN-γ non-producer and presented a negative *Leishmania*-qPCR in blood.

Leishmaniosis was confirmed in 18 out 31 Ibizan hounds with papular dermatitis by means of visualization of amastigotes on cytological or histological samples or by molecular techniques from stained smears. Cytological evaluation was performed in 17 out 31 Ibizan hounds with papules. The type of inflammation was varied. Ten out 17 dogs presented a mixed inflammation, whereas neutrophilic inflammation was observed in four and three out 17 dogs, respectively. Cytological examination was not diagnostic in three cases out of 17. Six of 17 dogs presented amastigotes on cytological evaluation (five dogs with mixed inflammation and one with neutrophilic inflammation). Therefore, qPCR was performed in the remaining 11 dogs in which amastigotes were not observed on cytological examination. Nine out 11 samples yield a positive result. One negative sample was characterised by low cellularity with mixed inflammation whereas the other was cytologically non-diagnostic.

Dermatopathological examination from three Ibizan hound dogs revealed a nodular to diffuse lympho-plasmacytic and macrophagic dermatitis. Amastigotes were revealed in the IHC staining in these three dogs.

Cytological examination of the unique papulo-crusting lesion observed in a non-Ibizan hound dog (one Weimaraner) was characterized by the presence of mixed inflammation with numerous eosinophils in the absence of *Leishmania* amastigotes.

There was and slight agreement between papular dermatitis and IFN-γ assay or LST (Cohen's k = 0.099 and 0.055, respectively). However, when considering only dogs equal or younger than 1.5 years old, the agreement between papular dermatitis and IFN-γ assay or LST (Cohen's k = 0.479 and 0.42, respectively) was moderate.

Table 5-1. Results of the diagnostic tests performed for the diagnosis of *Leishmania* infection and papular etiology in Ibizan hound dogs with papular dermatitis clinically suggestive of CanL.

ID	*Cytology*	*Smear qPCR*	*Biopsy*	*IHC*	*ELISA*	*Blood qPCR*	*IFN-γ*	*LST*
1	-	+	ND	ND	-	-	Producer	+
2	-	+	ND	ND	-	ND	Producer	+
3	-	+	ND	ND	-	ND	Producer	+
4	-	+	ND	ND	-	Low +	Producer	+
5	-	+	ND	ND	-	Low +	Non-producer	ND
6	-	+	ND	ND	-	-	Producer	+
7	-	+	ND	ND	-	-	Producer	+
8	-	+	ND	ND	-	ND	Producer	ND
9	-	+	ND	ND	-	-	Producer	ND
10	-	-	ND	ND	-	ND	Non-producer	ND
11	-	-	ND	ND	Low +	ND	Producer	+
12	+	ND	ND	ND	-	-	Producer	-
13	+	ND	ND	ND	-	Low +	Producer	+
14	+	ND	ND	ND	-	ND	ND	ND
15	+	ND	ND	ND	Low +	Low +	Producer	ND
16	+	ND	ND	ND	-	ND	Non-producer	ND
17	+	ND	ND	ND	-	Low +	Producer	ND
18	ND	ND	YES	+	-	Low +	Producer	+
19	ND	ND	YES	+	-	Low +	Producer	+
20	ND	ND	YES	+	-	Low +	Producer	+
21	ND	ND	ND	ND	-	-	Non- producer	-
22	ND	ND	ND	ND	-	Low +	Producer	+
23	ND	ND	ND	ND	-	-	Producer	+
24	ND	ND	ND	ND	-	-	Non-producer	ND
25	ND	ND	ND	ND	-	-	Producer	+

Table 5-1. continuation

ID	*Cytology*	*Smear qPCR*	*Biopsy*	*IHC*	*ELISA*	*Blood qPCR*	*IFN-γ*	*LST*
26	ND	ND	ND	ND	-	-	Producer	+
27	ND	ND	ND	ND	-	-	Producer	+
28	ND	ND	ND	ND	-	-	ND	+
29	ND	ND	ND	ND	-	ND	Producer	+
30	ND	ND	ND	ND	Low +	-	Producer	+
31	ND	ND	ND	ND	-	-	Producer	+

qPCR: quantitative polymerase chain reaction; IHC: immunohistochemistry; ELISA: enzyme linked immunosorbent assay; IFN-γ: interferon gamma; LST: leishmanin skin test; ND: not done; -: negative; +: positive

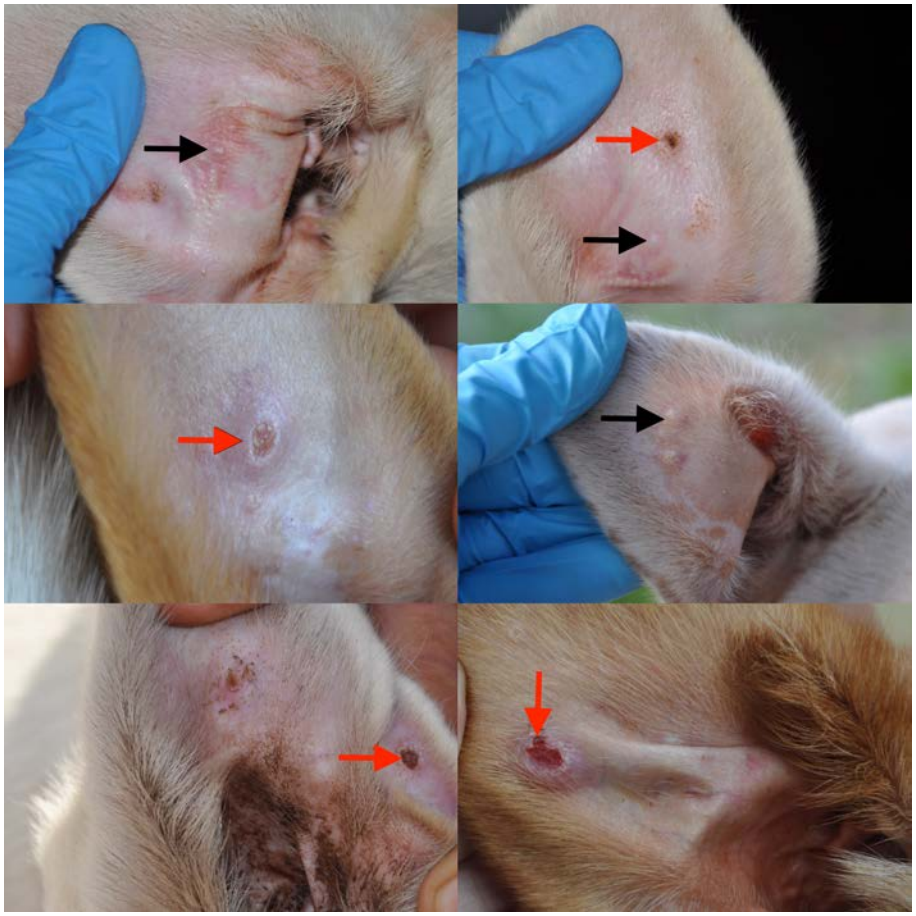

Figure 5-1. Papular dermatitis clinically suggestive of CanL located in the inner aspect of pinna in six Ibizan hound dogs. Note, erythematous and slightly scaling papules (black arrows) indicative of acute or early lesions and ulcerated and/or crusted papules (late lesions) with the characteristic "volcanic" appearance (red arrows).

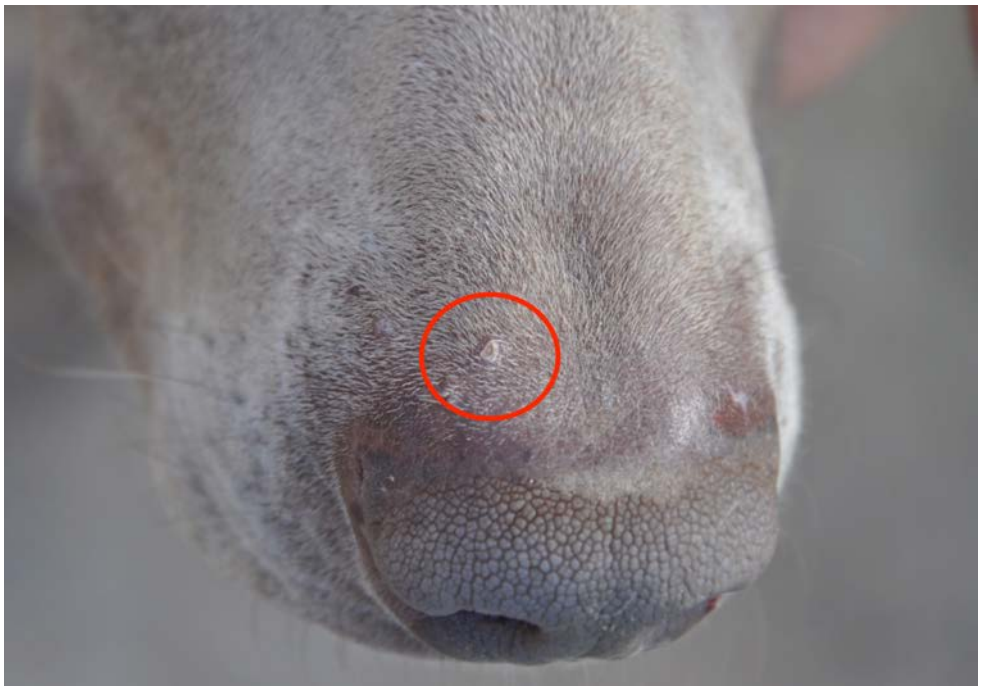

Figure 5-2. Non-erythematous and small papule (inside the red circle) on the bridge of the nose of one weimaraner dog. This lesion was not clinically suggestive of CanL and showed an eosinophilic infiltrate in the cytological evaluation.

Leishmania infection status

Overall data

Table 5-2 shows a brief account of the results of the diagnostic tests performed to identify *Leishmania* infection in the included dogs. In six Ibizan hound dogs and in 12 control dogs, infection could not be determined due to negative results in all tests (case 21 and three control dogs) or a combination of negative results and not performed tests (five and nine dogs, respectively). However, from these six Ibizan hound dogs five presented papular dermatitis (cases 10, 14, 16, 21 and 24), confirmed by cytological examination in two of them (case 14 and 16).

The percentage of dogs with at least one positive test was 87.2% and 74% in group I and O, respectively ($p = 0.12$, Fisher's exact test).

Table 5-2. Summary of the results of the diagnostic tests performed to evaluate *Leishmania infection* status.

Diagnostic tests	***Group I (n = 47)***	***Group O (n = 46)***
Nº of positive ELISA/total	10/47 (21.3%)	10/46 (21.7%)
Nº of positive blood qPCR/total	12/35 (34.3%)	21/42 (50%)
Nº of IFN-γ producers/total	36/42 (85.7%)[a]	16/26 (61.5%)[a]
Nº of positive LST/total	31/33 (94%)	16/21 (76.2%)

ELISA: enzyme linked immunosorbent assay; qPCR: quantitative polymerase chain reaction; IFN-γ: interferon gamma; LST: leishmanin skin test; [a]*Fisher's exact test, p = 0.0378*

Detection of specific *L. infantum* antibody levels

Dogs from group I were serologically classified as follows: negative (n = 37) and low positive (n = 10). On the other hand, dogs from group O were classified as negative (n = 36), low positive (n = 7) and medium positive (n = 3). The percentage of seroreactive dogs was similar between groups (Table 1, $p = 1$, Fisher´s exact test). Moreover, levels of antibodies were similar among groups (Group I, mean ± SD: 24.85 ± 22.09 EU and group O, mean ± SD: 30.08 ± 44.96 EU; $p = 0.18$, Mann-Whitney U-test, Z = 1.35248).

Quantitative polymerase chain reaction from blood

Based on blood *Leishmania* qPCR, dogs from group I were classified as negative (n = 23) or low positive (n = 12) while 21 dogs from group O were classified as negative or low positive (n = 21). The parasite load evaluation was not possible in 12 and 4 dogs from group I and O, respectively, due to lack of sample. A similar percentage of positive *Leishmania* qPCR in blood were found between groups ($p = 0.25$, Fisher´s exact test). Parasite load was considered similar among groups (Group I, mean ± SD: 0.12 ± 0.20 parasite/mL and group O, mean ± SD: 0.30 ± 0.37 parasite/mL; $p = 0.19$, Mann-Whitney U-test, Z = 1.30992).

Determination of *L. infantum* specific IFN-γ production after blood stimulation

IFN-γ assay was performed in 42 out of 47 dogs from group I and in 26 out of 46 dogs from group O. Unfortunately, this *ex-vivo* test was not performed in 5 and 20 dogs from group I and O, respectively, due to lack of sample. There were 36 IFN-γ producers in group I (85.7%), whereas only 16 dogs had detectable levels of specific production of IFN-γ in group O (61.5%)

(Table 1, $p = 0.0378$, Fisher's exact test). Moreover, there was a higher IFN-γ production in group I dogs when compared with dogs from group O (Group I, mean ± SD: 3165.33 ± 4571.30 pg/mL and group O, mean ± SD: 1225.96 ± 2458.67 pg/mL; $p = 0.023$, Mann-Whitney U-test, $Z = 2.26605$).

Leishmanin skin test

LST was performed in 33 out of 47 dogs from group I and in 21 out of 46 dogs from group O. Thirty-one of 33 dogs (94%) from group I and 16 out 21 (76.2%) from group O presented a positive LST result (Table 1, $p = 0.09$, Fisher's exact test). Although there was a trend for larger and thicker positive reactions in Ibizan hound dogs, this difference was not statistically different (Group I, mean ± SD: 18,6 ± 7.3 mm and group O, mean ± SD: 9.5 ± 6 mm, $p = 0.23$, Mann-Whitney U-test, $Z = 1.20974$ and Group I, mean ± SD: 9.02 ± 3.51 mm and group O, mean ± SD: 5.57 ± 2.29, $p = 0.2$, Mann-Whitney U-test, $Z = 1.27048$, respectively).

In relation to Kappa agreement, there was substantial agreement between LST and IFN-γ assay (Cohen's $k = 0.61$).

Discussion

Prevalence of the different cutaneous manifestations of CanL are defined based on anecdotal reports and they are still largely incomplete (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014). Although cutaneous involvement is the most common clinical presentation of CanL, and a wide range of cutaneous lesions exist due to *L. infantum* infection, studies intended to investigate the frequency of the diverse dermatological problems are lacking. The aim of the current study was to assess the prevalence of papular dermatitis in Ibizan hound dogs living in a highly endemic area for leishmaniosis and to compare it with the prevalence observed in other breeds. This study demonstrated that in areas where leishmaniosis is highly endemic, such as on the Island of Mallorca, Ibizan hound dogs presented a statistically significant higher rate of papular dermatitis suggestive of CanL when compared with dogs of other breeds. In particular, 66% of Ibizan hounds were presented with single or multiple papules, whereas only 2.2% (1/46) of the dogs belonging to other breeds presented one papulo-crusting lesion.

One limitation of this study was that final definitive diagnosis of papular dermatitis due to *Leishmania* was not reached in all the cases. Therefore, diagnosis was mostly assessed by dermatological examination. Actually, papular dermatitis due to *Leishmania* has some clinical features that make it characteristic in dogs. Firstly, and similarly to old world localized cutaneous

leishmaniasis in human beings (Malek et al., 2012; Uzun et al., 2018), these lesions are commonly presented as indurated papules or plaques ulcerated from their center and covered by a crust tightly adhered to the base. The sloping firm margins with a prominent central crater lend a "volcanic" appearance to the ulcer, which is the most distinctive feature of this cutaneous manifestation of leishmaniosis in dogs and human beings (Malek et al., 2012; Ordeix and Fondati, 2013; Uzun et al., 2018). More acute lesions are presented as a painless, persistent, erythematous papule, which rapidly become ulcerated. Secondly, these lesions are typically located presumably at the site of the sand fly bite in scarcely haired skin such as the inner aspect of pinnae, bridge of the nose, lips, eyelids and abdominal skin of short haired-dogs (Ordeix et al., 2005; Ordeix and Fondati, 2013; Lombardo et al., 2014). Thirdly, papular dermatitis clinically suggestive of CanL is typically observed in young dogs, usually less than one year-old (Ordeix et al., 2005; Lombardo et al., 2014; chapter 3 and Esteve et al., 2015; chapter 4 and Ordeix et al., 2017; Montserrat-Sangrà et al., 2018).

Nonetheless, clinical diagnosis was confirmed by microscopic examination of stained smears and biopsies stained with *Leishmania*-specific IHC or qPCR from stained smears in 18 out of 31 Ibizan hounds. Cytological examination yield positive results in only 6 out of 17 (35.3%) dogs in which it was performed. Although, this method is a simple, rapid, reliable and inexpensive method that directly reveals *Leishmania* amastigotes, the parasite may not be detected in all cases. In particular, PCR methods are especially useful and offer superior sensitivity in the diagnosis of leishmaniosis in those cases where organisms are scarce. In fact, it has been previously reported that a low parasite load studied by qPCR in papular dermatitis and stage I leishmaniosis compared with other cutaneous manifestations such as ulcerative or exfoliative lesions in more diseased dogs (chapter 4 and Ordeix et al., 2017). In the present study, nine out 11 (81.8%) Ibizan hound dogs with negative cytology presented a positive qPCR from skin lesions. Only two dogs presented negative results on *Leishmania* qPCR from cutaneous smears (case 10 and 11). It is possible that the sensitivity of qPCR could have been influenced by the poor quality of the smear and very low cellularity observed in both dogs. In fact, cytological examination was not diagnostic in case 10 with a mean cell number per 600x of magnification of < 2 cells and a diagnosis of mixed inflammation was made in case 11 with low nucleated cellularity (mean number of cells per 600x of magnification < 5 cells). Less probable, these dogs might have had a different cutaneous disease.

Only one dog from the other group showed one papulo-crusting lesion on the bridge of its nose. This dog was infected as it was serologically classified as low positive and presented a positive LST. However, this lesion was not clinically suggestive of CanL based on the absence of the characteristic clinical feature (i.e. erythematous papule or volcaniform lesion). Moreover, although qPCR could not be performed on this stained smear, cytological examination was considered highly

suggestive of an arthropod bite reaction due to the presence of numerous eosinophils. Actually, eosinophils were not observed in any of the cutaneous smears from the Ibizan hound dogs.

Possible reasons for the discrepancy between prevalence of papular dermatitis among groups are varied. One could make the hypothesis that difference among prevalence of papular dermatitis suggestive of CanL could be the result of different *Leishmania* infection's rates. However, both groups evaluated in the present study presented similar rates of infection (87.2% in Ibizan hound *vs* 74% in control dogs), defined by at least one positive test. This was not an unexpected finding, as both groups of dogs were from the same geographical area and were hunting dogs living outdoors and thus similarly exposed to *Leishmania*. However, prevalence of the infection calculated by adding all animals that were positive at least in one test (80.6%) was higher than that previously reported (67%-77%) in studies performed in the same island (Mallorca) (Solano-Gallego et al, 2000; Solano-Gallego et al., 2001). This is in part due to the fact that in those studies prevalence of infection was estimated using only serological and LST or molecular techniques, whereas in our study parasite specific IFN-γ production was also evaluated. In six Ibizan hound dogs and in 12 control dogs, infection could not be determined due to negative results in all tests (case 21 and three control dogs) or a combination of negative results and not performed tests (five and nine dogs, respectively). It is worth noting that case 21, an apparently non-infected dog, showed one papule. Unfortunately, this lesion was not sampled and etiology could not be confirmed. From five Ibizan hound dogs with unknown infection status, four presented papular dermatitis (cases 10, 14, 16 and 24), confirmed by cytological examination in two of them (case 14 and 16).

As mentioned previously in the introduction, clinical presentations of CanL may reflect different host immune responses against the parasite (Montserrat-Sangrà et al., 2018). Therefore, the high prevalence of papular dermatitis demonstrated in Ibizan hound dogs might be related to a different immune response mounted in this breed against *Leishmania* infection. The percentage of seroreactive dogs was comparable between groups. Seroprevalence observed in this study (21% in both groups) agrees with that obtained (26%) previously (Solano-Gallego et al., 2001). Conversely, the frequency of IFN-γ producers and amount of IFN-γ produced was significantly higher in Ibizan hounds. This was not surprising because a marked IFN-γ response to LSA after blood stimulation has been previously described in healthy Ibizan hounds when compared with control and sick dogs (Martínez-Orellana et al., 2017). Therefore, a high production of specific *L. infantum* IFN-γ seen in Ibizan hound dogs studied herein supports the previous findings demonstrating a predominance of *L. infantum* specific cellular immunity by means of LST and specific IFN-γ in Ibizan hounds living in a highly endemic area of leishmaniosis (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017). Moreover, a slight agreement between IFN-γ

production and presence of papular dermatitis was obtained in the present study, advocating that this clinical manifestation of CanL is suggestive of cellular immune response.

The percentage of positive LST reactions was similar between groups. In addition, positive reactions were similar in terms of diameter and thickness between groups. This was an unexpected finding as a higher frequency of LST reactions were anticipated in Ibizan hounds (Solano-Gallego et al., 2000). However, there was a trend for a higher frequency and larger positive reactions in Ibizan hounds, and probably a higher number of tested control dogs would yield statistically significant differences. Moreover, there was a substantial agreement between this test and IFN-γ production in assessing cellular immunity and a slight agreement between LST and papular dermatitis.

As stated above, papular dermatitis clinically suggestive of CanL is typically observed in young dogs, suggesting that this is a clinical problem observed after the first contacts with *Leishmania* (Ordeix et al., 2005; Lombardo et al., 2014; chapter 3 and Esteve et al., 2015; chapter 4 and Ordeix et al., 2017; Montserrat-Sangrà et al., 2018). Age does not seem to play a role in the presence of papular dermatitis in the groups studied. In the present study, the number of young dogs (i.e. less than 1.5 years) included in both groups was similar. Finally, in human old world localized cutaneous leishmaniosis these lesions appear at the site of sand fly bites (Malek et al., 2012). The erected and hairless pinnae of Ibizan hound dogs, with a large contact surface, may be a predisposing factor of this breed to a major exposure to *Phlebotomus* bites and papular dermatitis development and visualization of the cutaneous lesions. Further studies aimed to evaluate *Phlebotomus* exposure through analysis of anti-sand fly saliva antibodies in these dogs would be of interest (Kostalova et al., 2017).

Altogether, these findings indicate that the protective anti-*Leishmania* immune response mounted by the Ibizan hound dog, characterised by a combination of high production of specific *L. infantum* IFN-γ, positive LST reactions and seronegative result predispose them to the development of the mildest cutaneous form of CanL, named papular dermatitis.

Conclusion

Papular dermatitis, clinicopathologically suggestive of CanL, was more prevalent in Ibizan hound dogs when compared with other breeds in a highly endemic area for leishmaniosis. Although both groups were similarly infected, Ibizan hound dogs were more frequently *Leishmania* specific-

IFN-γ producers. These findings might point out papular dermatitis, in combination with a seronegative result, as a clinical marker for cellular specific immune response.

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CHAPTER 6

Toll-like receptors 2, 4, and 7, interferon-gamma, interleukin 10 and programmed death ligand 1 transcripts in skin from dogs with different clinical stages of leishmaniosis

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Original study

Abstract

Canine leishmaniosis is a zoonotic disease caused by *Leishmania infantum* that affects domestic dogs and can have several dermatological manifestations. Progression of *Leishmania* infection and disease development is dependent on the type of immune response elaborated against the parasite. T cell immunity is characterized by effective and protective cellular response. However, there is increasing evidence that innate mechanisms play an important role as well in anti-*Leishmania* defenses. The objective of this study was to determine and compare the transcription of TLR2, TLR4, IFN-γ, IL-10 and PDL-1 in paired clinically-lesioned and normal-looking skin from 25 diseased dogs (mild disease-stage I (n=11) and moderate to severe disease-stage II and III (n=14)) as well as in normal-looking skin from healthy dogs (n=10) from a non-endemic area and infected Ibizan hound dogs (n=9). Moreover, another objective was to correlate the transcripts with clinicopathological, immunological and parasitological findings.

Clinically-lesioned skin from mild affected dogs was characterized by a significantly upregulation of TLR2 and IL-10 and downregulation of TLR7 and a trend for an upregulation of TLR4 and IFN-γ when compared with more severe affected dogs. On the other hand, normal-looking skin of mild affected dogs was characterized by a significant lower expression of TLR7, IFN-γ and PD-L1 and a trend for lower expression of TLR2 and IL-10 when compared with more severe affected dogs. In this study, TLR7 and PD-L1 transcripts were determined, for the first time, in canine skin of dogs with leishmaniosis and they appeared to be associated with disease severity while IL-10 did not. Altogether, the results of the present study provide further insights into the TLR and cytokine profile in the clinically-lesioned and normal-looking skin of dogs with canine leishmaniosis

Keywords Leishmaniosis, canine, *Leishmania infantum*, skin, cytokine, toll-like receptors, gene expression

Introduction

Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a zoonotic and an endemic disease in the Mediterranean basin among other areas such as south America, Middle East and Asia (Solano-Gallego et al., 2009).

The complex immune response against the parasite is crucial for the outcome of *Leishmania* infection (Papadogiannakis and Koutinas, 2015). In fact, subclinical infection is the result of an effective T helper 1 (Th1) cellular immunity, with the activation of macrophages by IFN-γ and TNF-α and the elimination of intracellular amastigotes via the L-arginine nitric oxide pathway (Pinelli et al., 2000; Papadogiannakis and Koutinas, 2015). On the other side, disease development and progression are often correlated with increased parasite burdens together with a strong but non-protective humoral immune response and reduced or absent T cell mediated immunity (Solano-Gallego et al., 2009).

CanL is a systemic disease with varied clinical signs that range from a self-limiting disease to severe illness or even death (Solano-Gallego et al., 2009). Therefore, a clinical staging system for CanL that classifies the disease into four stages (stage I or mild disease, stage II or moderate disease, stage III or severe disease and stage IV o very severe disease) based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies was previously proposed (Solano-Gallego et al., 2009) and recently updated (Solano-Gallego et al., 2017). Among the different clinical manifestations of CanL, dermatological disease is the most frequent (Ordeix and Fondati, 2013; Saridomichelakis and Koutinas, 2014). Cutaneous lesions are very pleomorphic from a clinical and histopathological point of view (Ordeix and Fondati, 2013) and this clinicopathological variation might reflect a different host-parasite relationship and immune interactions (Saridomichelakis and Koutinas, 2014; chapter 4 and Ordeix et al., 2017). This is the particular case of papular dermatitis (Saridomichelakis and Koutinas, 2014). Papular dermatitis is a typical dermatological manifestation of CanL in an endemic area (Ordeix and Fondati, 2013), which is classified as a stage I or mild disease in the absence of other clinicopathological abnormalities (Solano-Gallego et al., 2017). The lack of other clinicopathological abnormalities, the reduced parasite dissemination to internal organs, the low or negative specific antibody levels, the positive results of the leishmanin skin test (LST) and the spontaneous resolution over 3-5 months observed in these dogs suggest that there is strong T-cell mediated immunity in these dogs against *L. infantum* that configures protection (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014).

Immune response in CanL has been the focus of many investigations during the last years. However, much of this work focused on adaptive immune response and in particular in T-cell

mediated immunity. Therefore, there is limited data on the importance of innate immune responses in CanL (Hosein et al., 2017). Moreover, most research has focused on the analysis of the immune response in peripheral blood. It is currently accepted that the immune response to the parasite is compartmentalized and different among organs (Maia and Campino, 2012; Rodríguez-Cortés et al., 2016). While the skin plays a major role in CanL immunopathogenesis, very little evidence is obtained from studies performed in normal-looking or lesioned skin from infected or diseased dogs (Hosein et al., 2017).

A mixed Th1/Th2 cytokine profile in the dermis of dogs naturally infected with *L. infantum* was originally described (Brachelente et al., 2005; Menezes-Souza et al., 2011). Sick dogs expressed high levels of IL-13, TNF-α, IFN-γ and IL-4 in the dermis (Brachelente et al., 2005; Menezes-Souza et al., 2011). Moreover, lesioned dermis of dogs with high parasite burden had significantly high IL-4 expression (Brachelente et al., 2005). Afterward, Th17 cytokines (i.e IL-17 and IL-22) were evaluated in the skin of experimental infected dogs (Hosein et al., 2015). In that study, progressive downregulation of IL-22 transcription in the skin was noted although not statistically significant (Hosein et al., 2015). In another experimental study, local cytokine response was absent at 6 months post infection, whereas parasite growth and onset of clinical disease both correlated with dermal upregulation of TNF-α, IFN-γ, IL-10 and TGF-β (Rodríguez-Cortés et al., 2016). Most recently, increased transcription of TNF-α and IL-10 has been reported in the normal-looking skin of dogs naturally affected by CanL associated positively with skin parasite load (Pereira-Fonseca et al., 2017).

Innate immune response has been scarcely investigated in leishmaniosis, and in particular in CanL. However, there is increasing evidence that innate mechanisms play an important role in anti-*Leishmania* defenses. Lately, there has been a great interest in the involvement of Toll Like Receptors (TLRs) in the immunopathogenesis of CanL (Amorim et al., 2011; Figueiredo et al., 2013; Melo et al., 2014; Hosein et al., 2015). TLRs are one of the most important pattern recognition receptor (PRR) molecules which recognize molecular structures characteristic of microbial pathogens and induce an inflammatory response (Vidya et al., 2018). Studies aimed to determine the role of TLRs in CanL are every time more numerous, however, they are mainly *in vitro* studies performed on canine macrophages (Turchetti et al., 2015) or studies performed in blood (Montserrat-Sangrà et al., 2016), liver (Hosein et al., 2015), spleen (Melo et al., 2014; Hosein et al., 2015; Grano et al., 2018) intestine (Figueiredo et al., 2013), brain (Melo et al., 2014; Grano et al., 2018) or lymph node samples (Melo et al., 2014; Hosein et al., 2015). TLR2 is one of the most TLRs associated with the pathogenesis of cutaneous lesions in CanL. Especially, an immunohistochemical study in the skin of dogs showed a lower expression of TLR2 in skin

biopsies from dogs with mild disease (stage I leishmaniosis and papular dermatitis) compared with dogs with moderate or severe disease (chapter 3 and Esteve et al., 2015). Recently, increased transcription of TLR2 has been reported in the normal-looking skin of dogs naturally affected by CanL associated positively with skin parasite load (Pereira-Fonseca et al., 2017). TLR9, which has been less studied, was upregulated in the skin of dogs when compared with healthy non-infected control dogs during early stages of an experimental infection (Hosein et al., 2015). Moreover, in the same study, TLR9 downregulation was observed in the skin with disease progression (Hosein et al., 2015).

As stated above, the suppression of cellular immunity is the most important aspect in the pathogenesis and progression of CanL. This Th1 response is dampened by regulatory immune responses when infection is not controlled by the initial IFN-γ based response (Esch et al., 2013). During the last years, several studies has focused on these regulatory mechanisms and they have demonstrated that PD-1, and its ligand (PD-L1), present in regulatory IgD^{hi} B cells are involved in the induction of T lymphocyte apoptosis via IL-10 production (Schaut et al., 2016b). These studies have determined an increased PD1/PD-L1 expression in peripheric mononuclear cells as well as an increase in the expression of PD-L1 in splenic macrophages in dogs with visceral leishmaniosis (Schaut et al., 2016; Schaut et al., 2016b; Chiku et al., 2016). However, to the best knowledge of the authors, PD-L1 expression in the skin of diseased or infected dogs has not been investigated.

Based on previous literature, few studies have investigated the immunological response in the cutaneous lesions in naturally infected dogs with CanL, and none of them has compared it between dogs with different degrees of disease severity. Therefore, the objective of this study was to determine and compare the transcription of TLR2, TLR4, IFN-γ, IL-10 and PDL-1 in paired clinically-lesioned and normal-looking skin from dogs with different clinical stages of CanL. Moreover, another objective was to correlate the transcripts with clinicopathological, immunological and parasitological findings.

Material and Methods

The Ethical Committee of the Autonomous University of Barcelona approved the study's protocol. The dog's tutors were informed about the study objectives and the procedure and they signed an informed consent.

Study groups

Twenty-five dogs with CanL and dermatological manifestation were prospectively selected from different Catalonian and Balearic veterinary centers from Spain. These patients were the same as those previously included in the study reported in chapter 4. Diagnosis was based on the observation of *Leishmania* on cytological and/or dermatopathological examination with or without *Leishmania* specific immunohistochemical examination of cutaneous lesions (chapter 4 and Ordeix et al., 2017). Moreover, a complete blood count using System Siemens Advia 120 (Siemens Healthcare GmbH, Germany), a biochemical profile by Analyzer Olympus AU 400 (CLIAwaived, USA), serum protein electrophoresis by Hydrasys® (Sebia Electrophoresis, USA), urinalysis with urinary protein/creatinine ratio and quantitative serology for the detection of *L. infantum* specific antibodies by means of a serial dilution in-house ELISA were performed (Solano-Gallego et al., 2016). Blood *Leishmania* quantitative polymerase chain reaction (qPCR) was also performed (Solano-Gallego et al., 2016). Based on clinicopathological data and according to Solano-Galego and collaborators these dogs were classified in three clinical stages: stage I-mild disease characterized by persistent papular dermatitis (n=11), II-moderate disease (n=12) and III-severe disease (n=2) (Solano-Gallego et al., 2009). However, for comparative analysis dogs were divided into two groups: group A (11 dogs with stage I) and group B (14 dogs with stages II and III). Normal-looking skin samples from 10 clinically healthy non-infected Beagles dogs from a non-endemic area (United Kingdom) (group C) and nine Ibizan hounds (group D) were used as control dogs. Ibizan hound dogs were the same as those included in the study reported in chapters 7 and 8. They showed positive LST reactions and specific IFN-γ production in stimulated cultured blood (chapter 7 and Ordeix et al., 2018).

Skin biopsies

For all patients two-skin fragments ≤ 0.5 cm from clinically lesioned skin and skin with normal appearance were collected. Normal-looking skin was obtained whenever possible from the lateral aspect of the neck. In cases where this region was affected, the biopsy was obtained from an area as far as possible from the macroscopically affected lesions. Each skin sample was then immediately cut into two halves. One half was fixed in 10% formalin for descriptive histopathology and analysis of the dermal inflammatory pattern as described previously (chapter 4 and Ordeix et al., 2017) and the other one submerged in RNA later (RNAlater® Stabilization Solution, Ambion, Inc., Austin, Texas), stored at 4°C overnight and then keep at -80ºC until used. The main histological features obtained in the histopathological descriptive described in chapter 4 study are summarized in table 6-1.

Table 6-1. Frequency of microscopic lesions and type of inflammatory pattern noticed in clinically-lesioned and normal-looking skin of sick dogs.

Histological analysis			***Group A (n =11)***		***Group B (n =14)***	
			Clinically-lesioned	Normal-looking	Clinically-lesioned	Normal-looking
Microscopic lesions			11/11 (100%)	3/11[a] (27.3%)	14/14 (100%)	11/14[a] (78.6%)
Inflammatory pattern	*Perivascular to interstitial*		2/11[b] (18.2%)	3/3 (100%)	10/14[b] (71.4%)	11/11 (100%)
	Nodular to diffuse	*With granuloma*	4/11[c] (36.4%)	0/3 (0%)	0/14[c] (0%)	0/11 (0%)
		Without granuloma	5/11 (45.4%)	0/3 (0%)	4/14 (28.6%)	0/11 (0%)

[a]Fisher's exact test; p = 0.0172; [b]Fisher's exact test, p = 0.015; [c]Fisher's exact test, p = 0.0166

RNA extraction

Before RNA isolation protocol, skin samples were thawed on ice and placed in lysis solution (TRI Reagent, RiboPure™ Kit, Ambion, Inc., Austin, Texas) and homogenized with a rotor-stator homogenizer (T 10 basic ULTRA-TURRAX 230V IKA 3420000) using standard procedures. Total RNA was then isolated using RiboPure™ Kit (Ambion, Inc., Austin, Texas) under strict RNase-free condition according to the manufacturer's protocol. In order to remove contaminating DNA, a DNase digestion step was included using TURBO DNA-free™ DNase Treatment and Removal Reagents (Ambion, Inc., Austin, Texas) following the manufacturer's instructions. RNA concentration was determined by a Nanodrop device (Thermo Fisher Scientific Inc) and RNA integrity and quality were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies) in some biopsies. Samples had a final concentration of 9.4–881.2 ng /µl. The majority of samples included in this study had a RNA integrity number value greater than 7. The recovered RNA was stored at –80°C until cDNA synthesis.

cDNA synthesis

cDNA was generated using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was aliquoted and stored at −20◦C until used for quantitative PCR (qPCR).

Quantitative PCR

Primers used in this study are listed in table 6-2. Moreover, two suitable reference genes (Succinate dehydrogenase complex; subunit A; Flavoprotein (SDHA), and similar to CG14980-PB (CG14980) were selected as reference genes (Peters et al., 2007; Wood et al., 2008; Montserrat-Sangrà et al., 2016).

Table 6-2. Canine reference and target genes used in the present study.

Assay ID	*Gene symbol*	*Gene name*	*Genebank mRNA*	*Genebank reference sequence*	*Amplicon pairwise*
CF02625049_S1	TLR2	Toll-like receptor 2	AF328930.1	NM_001005264.2	69
CF02622203_G1	TLR4	Toll-like receptor 4	AB080363.1	NM_001002950.1	120
CF02710573_S1	TLR7	Toll-like receptor 7	AB248956.1	NM_001048124.1	124
CF02624265_M1	IL-10	Interleukin 10	AF328930.1	NM_001003077.1	83
CF02623316_M1	IFN-γ	Interferon gamma	AF091130.1	NM_001003174.1	117
APG2FND	PD-L1	Programmed dead ligand 1	NM_001291972.1	NM_001291972.1	164
CF02643820_M1	LOC479750	Similar to CG14980-PB	XM_536878.2	-	78
CF02664981_M1	SDHA	Succinate dehydrogenase complex; subunit A; flavoprotein	XM_535807.2	DQ402985.1	64

PCR amplification was performed using the QuantStudioTM 12K Flex System Real-Time PCR (Thermo Fisher Scientific, Carlsbad, California, US) using TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Foster City, California, US). Plates (96 wells/plate) were filled with 0.35 µL nuclease free water (Sigma, San Luis, Missouri, US), 7.50 µL TaqMan Universal Master Mix (2×), 0.75 µL TaqMan assay 20, 6.4 µL 1/5 cDNA. Plates were closed with an optical film (Applied Biosystems) centrifuged in order to mix the samples and were placed into a laboratory pipetting robot (Epmotion 5057 Liquid-handlingrobot, Eppendorf, Hamburg, Germany) to generate a 384 wells/plate. Then, the generated 384 wells plates were transferred into a real time PCR device. The PCR components and the PCR cycler conditions were identical for the all target and reference genes. Denaturation program (95ºC, 10 min), amplification and quantification program repeated 40 times (95ºC for 15 s, 60ºC for 10 s, 72ºC for 60 s) with a single fluorescence measurement. The baseline and threshold were automatically defined for the program in each run. Each sample was performed in triplicate for all the target and reference genes and a calibrator

sample (one sample from group C) was employed as control in each plate. All target genes per each dog were run the same day and within the same plate. Data were processed while applying the relative quantification method comparable to the delta-delta-cycle threshold value (ddCt)-method. For normalization of target genes expression, the arithmetic mean of the two reference genes were taken for the calculation of a reference gene index (Montserrat-Sangrà et al., 2016). Quantitative PCR data analyses was done by the Cloudsuite software (Life technologies™, Thermo Fisher Scientific).

Skin parasite load

DNA was purified from the interphase and organic phase generated from the RNA purification process by means of QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following the manufacturer's instructions with slight modifications. Briefly, 20µl of proteinase K solution and 200µl of tissue sample were used in all cases. The other steps were performed as per manufacturer's protocol. A fragment of skin from a control dog was used as a control for DNA contamination during DNA extraction. qPCR was performed with a relative quantification as previously described in chapter 4 (Ordeix et al., 2017). The parasite load was measured with the calculation of the dCt (the difference of expression between mean values of duplicate determination of *Leishmania* Ct and 18S rRNA Ct). Therefore, low or negative values of dCt represented higher parasite load than elevated dCt.

Whole blood cytokine release assay and determination of canine IFN-γ

IFN-γ release whole blood cultured assay was performed as described previously (Solano-Gallego et al., 2016b). Briefly, heparinized whole blood was separately mixed with three different conditions: (i) unstimulated medium; (ii) medium with soluble *L. infantum* antigen (LSA, 5 mg/mL, Facultat de Farmacia, Universitat Autònoma de Barcelona) 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. IFN-γ was determined in supernatants obtained five days after stimulation by a commercial sandwich ELISA (DuoSet ELISA by Development System R&D™, Abingdon, UK). Cytokine concentration from supernatants with ConA and LSA was calculated after subtracting the IFN-γ concentration obtained from unstimulated supernatants.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 1.0.0.1032) (SPSS Inc., USA). Categorical data were expressed as percentage and statistical analysis was performed using

the Fisher's exact test to compare results among independent variables. Quantitative data were expressed as means and standard deviations and the non-parametric Wilcoxon signed-rank test and Mann-Whitney U-test were used to compare results among related or independent variables, respectively. The Spearman's rank order correlation between transcripts in skin samples and immunological (*L. infantum* specific antibody levels and blood IFN-γ production), clinicopathological and parasitological data was also calculated. Differences were considered significant with a 5% significance level ($p < 0.05$).

Results

Immunological, parasitological and clinicopathological data

Control dogs from a non-endemic area (group C) were deemed clinically healthy seronegative non-infected dogs and were not included in comparisons reported in this section. The most relevant evaluated parameters studied in diseased and infected dogs are listed in table 6-3**.**

As expected dogs classified in group A were in a less severe disease status than dogs classified in group B as they had significantly lower values of total proteins, beta and gamma globulins and higher values of albumin/globulin ratio, hematocrit and hemoglobin. Moreover, dogs from group B had significantly higher levels of specific antibodies and skin parasite load either in clinically-lesioned and normal-looking skin than dogs from group A.

Dogs from group A showed significantly lower levels of creatinine, total protein and albumin and increased levels of *Leishmania* antibodies when compared with Ibizan hound dogs. Moreover, skin parasite load in normal-looking in dogs from group A was similar than that detected in normal-looking skin from Ibizan hounds. On the other hand, dogs from group B had all clinicopathological parameters related with disease severity altered, except UPC ratio, creatinine and urea. Moreover, they presented higher levels of specific antibodies and higher parasite density either in clinically-lesioned and normal-looking skin when compared with Ibizan hound dogs. In addition, this group showed a tendency for a lower blood IFN-γ production and higher blood parasite load than Ibizan hound dogs.

Transcripts in clinically-lesioned skin in comparison with healthy skin from control dogs

Mean and standard deviation of relative quantifications of the expression of the immune response genes analyzed in the present study are listed in table 6-4.

Relative quantification of TLR2, TLR4, IFN-γ and PD-L1 transcripts were significantly higher in clinically-lesioned skin from sick dogs (groups A and B analyzed together) when compared with healthy skin from control dogs (group C). When the groups A and B were analyzed separately, it was determined that all transcripts except TLR7 and IL-10 were significantly increased in clinically-lesioned skin from dogs from group A when compared with group C. In this group, TLR7 was significantly downregulated and, although IL-10 was higher than in the skin from dogs from group C, this difference was not statistically significant. On the other hand, although all the transcripts were higher in clinically-lesioned skin from dogs from group B when compared with group C, only TLR2, IFN-γ and PD-L1 were significantly upregulated.

Transcripts in clinically-lesioned skin in comparison with healthy skin from Ibizan hound dogs

When clinically-lesioned skin from sick dogs (groups A and B) was compared with healthy skin from Ibizan hound dogs (group D), it was determined that all transcripts except that of TLR7 were significantly higher. When studied separately, group A presented upregulation of all immune genes except TLR7, and group B, presented upregulation of all immune genes except TLR4.

Transcripts in clinically-lesioned skin in sick dogs with different clinical staging

Clinically-lesioned skin from dogs from group A showed significant upregulation of TLR2 and IL-10 and downregulation of TLR7 in comparison with clinically-lesioned skin from dogs from group B. Although non-statistically significant, a trend for an upregulation of TLR4 and IFN-γ was also observed in group A (Figure 6-1).

Transcripts in normal-looking skin from sick dogs in comparison with healthy skin from control dogs

Transcripts of TLR2, IL-10, IFN-γ and PD-L1 were significantly increased in normal-looking skin of sick dogs (groups A and B) when compared with skin from dogs from group C. However, when the analysis was done separately for the different sick groups, it was determined that for group A only relative quantification of TLR7 was significantly downregulated when compared with the skin from dogs from group C. Although all the transcripts were higher in normal-looking skin from dogs from group B when compared with group C, only TLR2, IFN-γ and PD-L1 were significantly upregulated.

Table 6-3. Clinicopathological data, antibody levels, IFN-γ production in stimulated blood and skin parasite load of sick (groups A and B) Ibizan hound dogs (group D).

Parameters		*Group A (n = 11)*	*Group B (n = 14)*	*Group D (n = 9)*
UPC		0.2±0.1	0.7±0.7	ND
Creatinine (mg/dL)		0.8±0.1[a]	0.9±0.2	1±0.1[a]
Urea (mg/dL)		43±14[b]	31.3±7.8[b]	33.8±7.4
Total protein (g/dL)		5.7±0.6[c,d]	9.1±2.4[c,e]	6.3±0.3[d,e]
Albumin (g/dL)		3±0.4[f]	2.3±0.8[g]	3.5±0.3[f,g]
Beta globulin (g/dL)		1.3±0.3[h]	1.9±0.6[h,i]	1.3±0.1[i]
Gamma globulin (g/dL)		0.7±0.9[j]	3±2.6[j,k]	0.5±0.1[k]
Albumin/globulin ratio		1.1±0.2[l]	0.5±0.4[l,m]	1.2±0.2[m]
Hematocrit (%)		45.6±8.5[n]	33±9.8[n,o]	49.3±5.9[o]
Hemoglobin (g/dL)		15.4±2.4[p]	11.1±3.8[p,q]	16.1±1.8[q]
***Leishmania infantum* specific antibody levels (ELISA units)**		136.8±196.1[r,s]	8892.7±17807.7[r,t]	18.3±10.3[s,t]
Blood *L. infantum* specific IFN-γ (pg/mL)		2046±2746.6	713.7±832.8	3486±5291.1
Blood parasite load		7.8±10.5	6.7±11.1	0.9±2.2
Skin parasite load (dCt)	*Clinically-lesioned*	3.6±4.4[u,v,w]	-0.4±4.7[u,x,y]	-
	Normal-looking	6.1±4[w,z]	1.7±4.5[y,z,1]	8.9±1.9[v,x,1]

UPC: urinary protein/creatinine ratio; ND: not done; ELISA: enzyme linked immunosorbent assay; dCt: delta-cycle threshold; *[a]Mann-Whitney U-test, Z = -3,235, p = 0.001; [b]Mann-Whitney U-test, Z = -2,083, p = 0.037; [c]Mann-Whitney U-test, Z = -3,686669, p < 0.0001; [d]Mann-Whitney U-test, Z = -2,117, p = 0.034; [e]Mann-Whitney U-test, Z = -3,529, p< 0.0001; [f]Mann-Whitney U-test, Z -2,503, p = 0.012; [g]Mann-Whitney U-test, Z = -3,498, p < 0.0001; [h]Mann-Whitney U-test, Z = -2,572, p = 0.01; [i]Mann-Whitney U-test, Z -3,038, p = 0.002; [j]Mann-Whitney U-test, Z -3,079, p = 0.002; [k]Mann-Whitney U-test, Z = -3,442, p = 0.001; [l]Mann-Whitney U-test, Z = -2,899, p = 0.004; [m]Mann-Whitney U-test, Z = -3,775, p < 0.0001; [n]Mann-Whitney U-test, Z = -2,251, p = 0.024; [o]Mann-Whitney U-test, Z -2,929, p = 0.003; [p]Mann-Whitney U-test, Z = -2,148, p = 0.032; [q]Mann-Whitney U-test, Z = -2,539, p = 0.011; [r]Mann-Whitney U-test, Z = -3,887, p < 0.0001; [s]Mann-Whitney U-test, Z = -2,090, p = 0.037; [t]Mann-Whitney U-test, Z = -3,970, p < 0.0001; [u]Mann-Whitney U-test, Z = -2,026, p = 0.043; [v]Mann-Whitney U-test, Z = -2,773, p = 0.0006; [w]Wilcoxon Signed Ranks Test Z = -2,803, p = 0.005; [x]Mann-Whitney U-test, Z = -3,717, p < 0.0001; [y]Wilcoxon Signed Ranks Test Z = -2,856, p = 0.004; [z]Mann-Whitney U-test, Z = -2,869, p = 0.004; [1]Mann-Whitney U-test, Z = -3,465, p = 0.001*

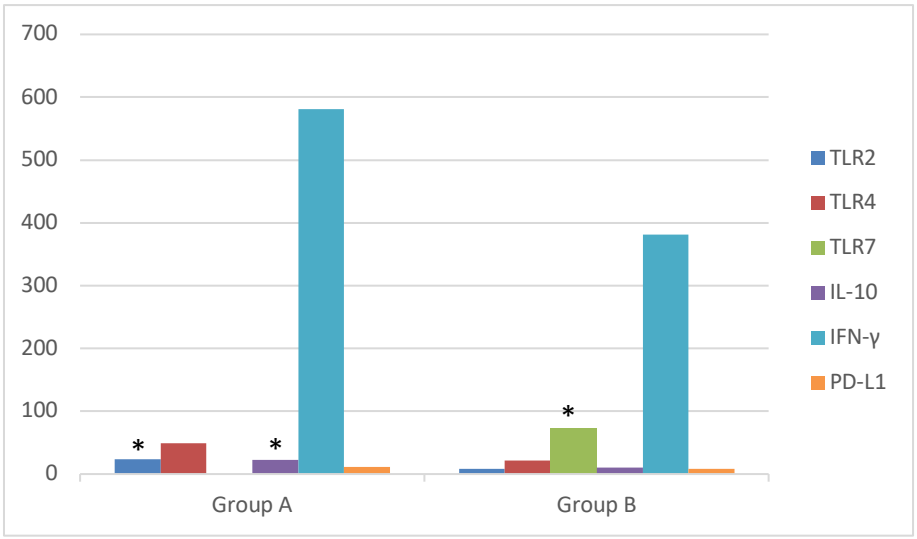


Figure 6-1. Relative quantification of the immune genes studied in clinically-lesioned skin from mild affected dogs (group A) and more severe affected dogs (group B). Differences with significance at $p < 0.05$ are indicated by *.

Transcripts in normal-looking skin in comparison with healthy skin from Ibizan hound dogs

Normal-looking skin from sick dogs (groups A and B) had significant upregulation of TLR2, TLR4 and TLR7 when compared with healthy skin from group D. When studied separately, group A presented downregulation of both IL-10 and IFN-γ and group B presented upregulation of TLR2, TLR7 and IL-10 when compared with group D.

Transcripts in normal-looking skin in sick dogs with different clinical staging

Normal-looking skin from dogs from group A showed significant downregulation of TLR7, IFN-γ and PD-L1 in comparison with normal-looking skin from dogs from group B. Although non-statistically significant, a trend for a downregulation of TLR2 and IL-10 was also observed in group A when compared with group B (Figure 6-2).

Table 6-4. Gene transcripts of sick (groups A and B) and control dogs (groups C and D).

Target genes	Sick dogs (Group A + B)		Group A		Group B		Control dogs	
							Group C	Group D
	Clinically-lesioned	*Normal-looking*	*Clinically-lesioned*	*Normal-looking*	*Clinically-lesioned*	*Normal-looking*	*Healthy skin*	
TLR2	14.7±12.3[a,b,c]	3.4±3.9[c,26,27]	23.3±13.2[n,o,p,q]	2.4±2.1[q]	7.9±5.9[n,5,6,7]	4.1±4.6[7,20,21]	0.8±0.7[a,o,5,20,26]	0.9±1.3[b,p,6,21,27]
TLR4	33.7±44.8[d,e]	9.5±11.8[28]	49.5±49.4[r,s]	10.2±10.8	21.3±38.1	9.1±12.8	1.6±1.8[d,r]	2.4±6.2[e,s,28]
TLR7	40.5±108.3	14.8±24.6[29]	0.001±0.002[t,u]	0.04±0.1[14,15]	72.3±138.5[t,8]	24.4±27.9[14,22]	13.9±14.4[u,15]	0.0±0.0[8,22,29]
IL-10	15.9±13.1[f,g]	7.9±8.3[g,30]	22.8±16.5[v,w,x]	3.6±2.3[x,16]	10.5±5.9[v,9]	10.6±9.7[23]	0.5±0.6[30]	4.9±1.6[f,w,9,16,23]
IFN-γ	469.3±467.2[h,i,j]	129.4±278.7[j,31]	581.5±574.5[y,z,1]	0.5±0.2[1,17,18]	381.1±353.5[10,11]	212.2±335.5[17,24]	1.5±2.2[h,y,10,24,31]	4.3±7.2[i,z,11,18]
PD-L1	9.5±6.1[k,l,m]	5.2±10.6[m,32]	11.4±6.4[2,3,4]	0.8±0.6[4,19]	8±5.7[12,13]	8.2±12.8[19,25]	0.6±0.4[k,2,12,25,32]	4.8±3[l,3,13]

[a]Mann-Whitney U-test, Z = -4,163, p < 0.0001; [b]Mann-Whitney U-test, Z = -4,079, p < 0.0001; [c]Wilcoxon Signed Ranks Test Z = -3,680, p< 0.0001; [d]Mann-Whitney U-test, Z = -2,538, p = 0.011; [e]Mann-Whitney U-test, Z = -2,616, p = 0.009; [f]Mann-Whitney U-test, Z = -4,231, p < 0.0001; [g]Wilcoxon Signed Ranks Test Z = -2,281, p = 0.023; [h]Mann-Whitney U-test, Z = -4,564, p < 0.0001; [i]Mann-Whitney U-test, Z = -4,392, p < 0.0001; [j]Wilcoxon Signed Ranks Test Z = -3,224, p = 0.01; [k]Mann-Whitney U-test, Z = -4,017, p < 0.0001; [l]Mann-Whitney U-test, Z = -2,674, p = 0.007; [m]Wilcoxon Signed Ranks Test Z = -2,798, p = 0.005; [n]Mann-Whitney U-test, Z = -3,613, p < 0.0001; [o]Mann-Whitney U-test, Z = -3,874, p < 0.0001; [p]Mann-Whitney U-test, Z = -3,761, < 0.0001; [q]Wilcoxon Signed Ranks Test Z = -2,666, p = 0.008; [r]Mann-Whitney U-test, Z = -3,206, p = 0.001; [s]Mann-Whitney U-test, Z = -3,229, p = 0.001; [t]Mann-Whitney U-test, Z = -2,905, p = 0.004; [u]Mann-Whitney U-test, Z = -4,099, p < 0.0001; [v]Mann-Whitney U-test, Z = -2,300, p = 0.021; [w]Mann-Whitney U-test, Z = -3,775, p < 0.0001; [x]Wilcoxon Signed Ranks Test Z = -2,547, p = 0.011; [y]Mann-Whitney U-test, Z = -3,873, p < 0.0001; [z]Mann-Whitney U-test, Z = -3,761, p < 0.0001; [1]Wilcoxon Signed Ranks Test Z = -2,666, p = 0.008; [2]Mann-Whitney U-test, Z = -3,591, p < 0.0001; [3]Mann-Whitney U-test, Z = -2,545, p = 0.011; [4]Wilcoxon Signed Ranks Test Z = -2,666, p = 0.008; [5]Mann-Whitney U-test, Z = -3,455, p = 0.001; [6]Mann-Whitney U-test, Z = -3,465, p = 0.001; [7]Wilcoxon Signed Ranks Test Z = -2,354, p = 0.019; [8]Mann-Whitney U-test, Z = -2,261, p = 0.024; [9]Mann-Whitney U-test, Z = -3,917, p < 0.0001; [10]Mann-Whitney U-test, Z = -4,099, p < 0.0001; [11]Mann-Whitney U-test, Z = -3,969, p < 0.0001; [12]Mann-Whitney U-test, Z = -3,455, p = 0.001; [13]Mann-Whitney U-test, Z = -2,205, p = 0.027; [14]Mann-Whitney U-test, Z = -2,923, p = 0.003; [15]Mann-Whitney U-test, Z = -3,818, p < 0.0001; [16]Mann-Whitney U-test, Z = -3,657, p < 0.0001; [17]Mann-Whitney U-test, Z = -3,970, p < 0.0001; [18]Mann-Whitney U-test, Z = -3,578, p < 0.0001; [19]Mann-Whitney U-test, Z = -3,466, p = 0.001; [20]Mann-Whitney U-test, Z = -2,226, p = 0.026; [21]Mann-Whitney U-test, Z = -2,269, p = 0.023; [22]Mann-Whitney U-test, Z = -2,437, p = 0.015; [23]Mann-Whitney U-test, Z = -3,916, p < 0.0001; [24]Mann-Whitney U-test, Z = -3,806, p < 0.0001; [25]Mann-Whitney U-test, Z = -2,811, p = 0.005; [26]Mann-Whitney U-test, Z = -2,272, p = 0.0023; [27]Mann-Whitney U-test, Z = -2,411, p = 0.016; [28]Mann-Whitney U-test, Z = -2,678, p = 0.007; [29]Mann-Whitney U-test, Z = -2,322, p = 0.020; [30]Mann-Whitney U-test, Z = -4,309, p < 0.0001; [31]Mann-Whitney U-test, Z = -2,272, p = 0.023; [32]Mann-Whitney U-test, Z = -2,802, p = 0.005

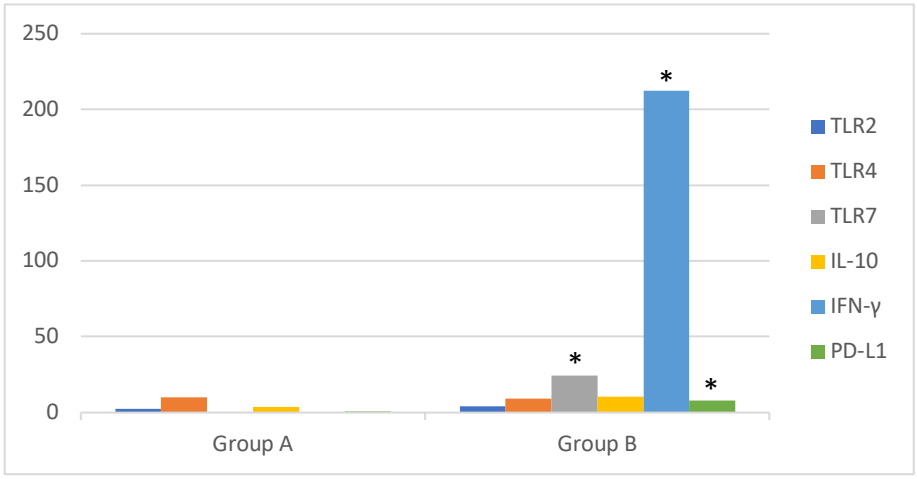


Figure 6-2. Relative quantification of the immune genes studied in normal-looking skin from mild affected dogs (group A) and more severe affected dogs (group B). Differences with significance at $p < 0.05$ are indicated by *.

Transcripts in clinically-lesioned skin in comparison with normal-looking skin from sick dogs

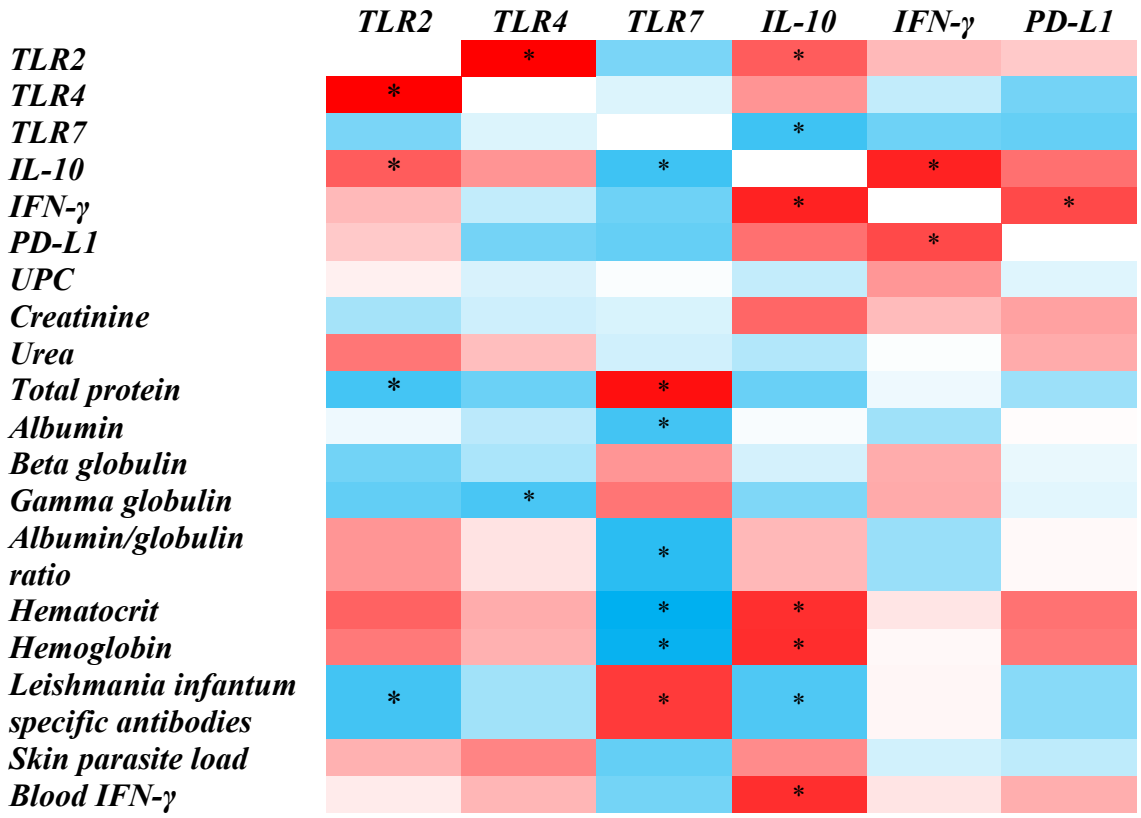
Clinically-lesioned skin from dogs with cutaneous manifestations of leishmaniosis (groups A and B) showed significant upregulation of TLR2, IL-10, IFN-γ and PD-L1 in comparison with paired normal-looking skin. When sick dog groups were analyzed separately, dogs from group A showed the same pattern of transcripts upregulation as described above, whereas in group B only TLR2 transcript was significantly higher than paired normal-looking skin.

Correlations with transcripts in clinically-lesioned skin and clinicopathological, immunological and parasitological findings.

Correlations between transcripts in clinically-lesioned skin from sick dogs and the different parameters are all illustrated in the heat map shown in table 6-5. A significant positive correlation was noted between TLR2, TLR4 and IL-10 transcripts, whereas a significant negative correlation was observed between TLR2 and total protein and specific *L. infantum* antibodies. TLR4 in addition was negatively correlated with gamma globulin concentration. TLR7 was the transcript with more significant correlations obtained. In fact, it was positively correlated with total protein and specific antibody levels, whereas it was negatively correlated with IL-10, albumin, albumin/globulin ratio, hematocrit, and hemoglobin. IL-10 was, in addition, positively correlated to skin IFN-γ transcript, hematocrit, hemoglobin and blood

IFN-γ production, whereas it was observed a negative correlation between IL-10 and specific antibodies. Skin IFN-γ transcript, in addition to the aforementioned correlations, was positively correlated with PD-L1.

Table 6-5. Heat map illustrating the positive (red) and negative (blue) correlation values between immune genes transcripts in clinically-lesioned skin and clinicopathological, immunological and parasitological findings (Correlations with $p < 0.05$ are indicated by *).



Correlations with transcripts in normal-looking skin and clinicopathological, immunological and parasitological findings.

More significant correlations were determined between transcripts in normal-looking skin and clinicopathological and serological data, IFN-γ production in blood and skin parasite load than in clinically-lesioned skin (Table 6-6). TLR2 transcript was positively correlated with all the immune genes' transcripts and beta globulins. In addition, TLR2 transcript was negatively correlated with albumin, albumin/globulin ratio, hematocrit, hemoglobin and skin parasite load. TLR4 was negatively correlated with al albumin/globulin ratio, hematocrit and hemoglobin. A significant positive correlation was obtained between TLR7 and IL-10, IFN-γ, PD-L1, total

protein, beta and gamma globulins and specific *L. infantum* antibodies. TLR7 was negatively correlated with albumin, albumin/globulin ratio, hematocrit, hemoglobin, and skin parasite load. IL-10 showed similar correlation than TLR7, except for a positive correlation with UPC ratio and negative correlation with blood IFN-γ production. Skin IFN-γ was positively correlated with PD-L1, UPC ratio, total protein, beta and gamma globulins and specific *L. infantum* antibodies. In addition, there was a negative correlation between skin IFN-γ and albumin, albumin/globulin ratio, hematocrit, hemoglobin and skin parasite load. Finally, PD-L1 was positively correlated with UPC ratio, total protein, beta and gamma globulins and antibody levels, whereas a negative correlation was observed between this transcript and hemoglobin and skin parasite load.

A lower delta CT means higher skin parasite load. Therefore, negative correlations determined between parasite load and TLR2, TLR7, IL-10, IFN-γ and PD-L1 mean an association between an upregulation of these transcripts and higher parasite density in normal-looking skin.

Table 6-6. Heat map illustrating the positive (red) and negative (blue) correlation values between immune genes transcripts in normal-looking skin and clinicopathological, immunological and parasitological findings (Correlations with $p < 0.05$ are indicated by *).

	TLR2	***TLR4***	***TLR7***	***IL-10***	***IFN-γ***	***PD-L1***
TLR2		*	*	*	*	
TLR4	*					
TLR7	*			*	*	*
IL-10	*		*		*	*
IFN-γ	*		*	*		*
PD-L1			*	*	*	
UPC				*	*	*
Creatinine						
Urea						
Total protein			*	*	*	*
Albumin	*	*	*	*	*	
Beta globulin	*		*	*	*	*
Gamma globulin			*	*	*	*
Albumin/globulin ratio	*	*	*	*	*	
Hematocrit	*	*	*	*	*	
Hemoglobin	*	*	*	*	*	*
Leishmania infantum specific antibodies			*	*	*	*
Skin parasite load	*		*	*	*	*
Blood IFN-γ				*		

Discussion

The skin is a central organ in CanL and plays a major role in its immunopathogenesis, however, there is limited data about immune responses in the skin of infected or diseased dogs (Brachelente et al., 2005; Papadogiannakis et al., 2005; Menezes-Souza et al., 2011; Hosein et al., 2015; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017). Furthermore, comparative studies on skin immune responses in dogs with varied clinical presentations or disease severity are lacking. This study aimed to investigate, for the first time, the transcription of TLR2, TLR4, IFN-γ, IL-10 and PDL-1 in paired clinically-lesioned and normal-looking skin from the same dogs with different clinical stages and degrees of disease severity.

TLR2 was significantly upregulated in clinically-lesioned skin of sick dogs with different clinical stages (stage I and stage II-III), when compared with paired normal-looking skin and also with healthy skin of non-infected and infected Ibizan hound dogs. This was an anticipated finding. TLR2 is one of the most commonly TLR studied and involved in the immunopathogenesis of CanL. TLR2 overexpression has been demonstrated in several tissues, including the intestine (Figueiredo et al., 2013), brain (Melo et al., 2014; Grano et al., 2018), peripheral lymphoid organs (Melo et al., 2014; Hosein et al., 2015), liver (Hosein et al., 2015), blood (Montserrat-Sangrà et al., 2016) and skin (Hosein et al., 2015; Pereira-Fonseca et al., 2017) of naturally or experimentally diseased dogs and it seems to be associated with disease severity and progression.

In the present study, dogs with mild disease presented an upregulation of TLR2 in clinically-lesioned skin compared with dogs with moderated and severe disease. This, in fact, was an unexpected finding as a lower expression of TLR2 in skin biopsies of dogs with stage I-papular dermatitis was documented compared with dogs with more severe disease (chapter 3 and Esteve et al., 2015). That discrepancy might be related to the retrospective design of that study and that TLR2 expression was measured by means of immunohistochemistry, a technique that is less accurate and sensitive than qPCR. Similar to the present study, TLR2 expression in cutaneous lesions is different among the spectrum of tegumentary leishmaniasis in human beings (Campos et al., 2018). In fact, TLR2 expression is greater in localized cutaneous leishmaniasis and borderline disseminated cutaneous leishmaniasis than in mucosal leishmaniasis caused by *L. braziliensis* (Campos et al., 2018). Moreover, no significant changes were seen for TLR2 transcripts in normal-looking skin of diseased Beagle dogs 15 months after an experimental infection (Hosein et al., 2015). The authors suggested that the trend for

downregulation of the TLRs with disease progression was suggestive of an inhibitory role where the parasite may be facilitating the onset of disease by reducing or limiting the transcription of these TLRs which would otherwise play a protective role (Hosein et al., 2015). In addition, TLR2 transcript was negatively correlated with total protein and specific antibody levels in clinically-lesioned skin, rendering our finding, that dogs with stage I presented higher TLR2 transcript in clinically-lesioned skin, reasonable. Contrary to our study, TLR2 expression in non-stimulated blood from dogs with stage I leishmaniosis was similar to healthy control dogs and lower, although not statistically significant, than that observed in dogs with more severe disease (Montserrat-Sangrà et al., 2018). Nonetheless, gene expression of the TLRs may vary according to the compartment evaluated (Melo et al., 2014).

Interestingly, TLR2 was only upregulated in normal-looking skin of dogs with moderate and severe disease when compared with healthy skin of non-infected and Ibizan hound dogs. Although non-statistically significant, TLR2 transcript in normal-looking skin of dogs with stage I was lower than that determined in normal-looking skin from dogs with moderate and severe disease. Major upregulation of TLR2 in normal-looking skin of more affected dogs might be related to the evidence previously published that demonstrated that normal-looking skin in more severe diseased dogs present more frequently microscopical lesions than normal-looking skin of mild affected dogs (chapter 4 and Ordeix et al., 2017). Accordingly, positive correlation was revealed in normal-looking skin among TLR2 transcript and beta globulin and skin parasite load, whereas negative correlation was noted with parameters indicative of disease severity (albumin, albumin/globulin ratio, hematocrit and hemoglobin). These results agree with those published previously for TLR2 transcripts in non-stimulated blood of diseased dogs (Montserrat-Sangrà et al., 2016) and healthy skin of naturally infected dogs (Pereira-Fonseca et al., 2017).

TLR4 was significantly upregulated in clinically-lesioned skin of dogs with CanL (group A and B) when compared with healthy skin of non-infected and Ibizan hound dogs. TLR4 transcription has been evaluated in several studies, which have revealed diverse results according to the tissue assessed. TLR4 upregulation was found in spleen and lymph nodes whereas TLR4 was unchanged in brain and choroid plexus in diseased dogs naturally infected with *L. infantum* (Melo et al., 2014). However, inconsistent results were obtained in later studies. Hosein and collaborators evidenced a TLR4 downregulation with disease progression in both the lymph node and spleen together with no changes in normal-looking skin from experimentally infected dogs (Hosein et al., 2015). Although not statistically significant, TLR4 expression in non-

stimulated blood from dogs with leishmaniosis was higher than that of serologically negative healthy dogs (Montserrat-Sangrà et al., 2016). Moreover, in another study, TLR4 was only upregulated in the brain of a small subpopulation of diseased dogs, whereas it was downregulated in the spleen (Grano et al., 2018). In the present study, when diseased dogs were analyzed separately, it was concluded that only dogs with stage I showed TLR4 upregulation in clinically-lesioned skin when compared with healthy non-infected and Ibizan hound dogs. Moreover, although not statistically significant, dogs with mild disease presented higher TLR4 transcripts in clinically-lesioned skin compared with dogs with moderate and severe disease. As these dogs were less clinically affected, this result agreed with previous data that associated TLR4 downregulation in spleen and lymph nodes with disease progression (Hosein et al., 2015). However, it is interesting to note that in a previous published study no differences were found in TLR4 relative quantification in non-stimulated blood between mild and more severe affected dogs, against pointing out a compartmentalization of TLR genes expression in different organs during CanL (Montserrat-Sangrà et al., 2018). In addition, TLR4 transcript in clinically-lesioned skin was negatively correlated with gamma globulin, rendering our finding, that dogs with stage I tended to present a higher TLR4 transcription in clinically-lesioned skin, reasonable.

TLR4 was upregulated in normal-looking skin of dogs with leishmaniosis (group A and B) when compared only with healthy skin of Ibizan hound dogs. Therefore, similar relative quantification between normal-looking skin of diseased dogs and healthy non-infected dogs agreed with previously published data (Hosein et at., 2015). As for blood, TLR4 quantification in normal-looking skin did not allow to differentiate among disease severity (Montserrat-Sangrà et al., 2018). However, TLR4 transcript was negatively correlated with albumin, albumin/globulin ratio, hematocrit and hemoglobin in normal-looking skin. Taking all these findings together, major gene expression in clinically-lesioned skin of mild affected dogs and similarities between TLR4 gene expression in normal-looking skin in dogs with different clinical stages, it would seem that TLR4 expression is related to a protective role.

There are limited studies that determine TLR7 transcripts in CanL (Turchetti et al., 2015; Grano et al., 2018). This TLR has been rarely studied on canine skin (Okui et al., 2008) and it has never been studied in the skin of dogs infected with *L. infantum*. In one *ex vivo* study, the transcription of that receptor was similar among canine monocyte-derived macrophages with higher or lower resistance to intracellular survival of *L. infantum* (Turchetti et al., 2015). In another study, a slightly increased relative quantification of TLR7 was observed in the brain and the spleen of dogs with CanL, although this increase was not statistically significant (Grano et

al., 2018). TLR7 relative quantification was very variable between dogs evaluated in the present study. However, and as observed in the brain and spleen of diseased dogs (Grano et al., 2018), there was an increased transcription of this receptor in clinically-lesioned skin from diseased dogs compared with healthy skin of non-infected dogs and Ibizan hound dogs, although it was not statistically significant. Surprisingly, in clinically-lesioned skin from mild affected dogs this receptor was similar to Ibizan hound dogs and significantly downregulated compared with healthy skin of non-infected dogs. On the other hand, there was a significant TLR7 upregulation in clinically-lesioned skin from more severe affected dogs compared with healthy skin of Ibizan hound dogs. Accordingly, TLR7 transcription was downregulated in clinically-lesioned skin of mild affected dogs when compared with clinically-lesioned skin of moderate and severe affected dogs.

Relative quantification of TLR7 in normal-looking skin of diseased dogs (group A and B) parallel that of clinically-lesioned skin. This receptor was significantly downregulated and there was a trend for an upregulation in normal-looking skin of mild and more severe affected dogs, respectively when compared with healthy skin of non-infected dogs and Ibizan hound dogs. As in clinically-normal skin, TLR7 transcript was significantly lower in normal-looking skin from milder affected dogs than in severe dogs. Based on this transcript profile, it would seem that TLR7 is associated with moderate to very severe disease. In agreement with this suggestion is the fact that upregulation of TLR7 appears to be positively associated with pathological findings indicative of disease severity. In fact, higher relative quantification of its mRNA was positively associated with total protein and *L. infantum* antibodies in both clinically-lesioned and normal-looking skin. Moreover, TLR7 upregulation was associated with a higher parasite density in normal-looking skin and there was a trend for a higher parasite density in clinically-lesioned skin. In addition, a negative significant correlation was observed among this transcript and albumin, albumin/globulin ratio, hematocrit and hemoglobin in both clinically-lesioned and normal-looking skin. TLR7 is located in endosomal compartments (Vidya et al., 2017). It has been proposed that endosomal TLR7 activation in B cells by *L. donovani* is responsible for disease exacerbation in mice through IL-10 and IFN-I production (Silva-Barrios et al., 2016). Moreover, TLR7 signaling has been recently associated with cell death of protective CD4 T cells in an experimental model of *L. donovani* infection in mice (Fabié et al., 2018). It has been demonstrated that apoptotic cell material, derived from inflammatory tissue damage, triggers TLR7 in CD4 T cells during chronic infection. Signaling via TLR7 induces the upregulation and activation of interferon regulatory factor-5, which promotes the cell death of

protective CD4 T cells (Fabié et al., 2018). Hence, local tissue damage mediated by persistent inflammation leads to suppression of protective T cell responses during chronic visceral leishmaniosis.

IL-10 was significantly upregulated in clinically-lesioned skin of dogs with CanL, both in mild and more severe affected dogs, when compared with healthy skin of Ibizan hound dogs and with normal-looking skin. Although IL-10 transcripts were higher in clinically-lesioned skin of dogs with CanL, both in mild and more severe affected dogs, when compared with healthy skin of control dogs, these differences were not statistically significant. There is paucity of studies on skin IL-10 response, and all of them have been performed in normal-looking skin of naturally or experimentally infected dogs (Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016). In the present study, clinically-lesioned skin of mild affected dogs presented an upregulation of IL-10 compared with clinically-lesioned skin of more severe affected dogs. Furthermore, a significant negative correlation was revealed among IL-10 transcript in clinically-lesioned skin and *Leishmania*-specific antibody levels. IL-10 was also positively correlated with hematocrit, hemoglobin and blood IFN-γ production. In addition, in the present study a correlation of IL-10 transcription and skin parasite loads could not be obtained in clinically-lesioned skin. This was an unexpected finding considered that in human leishmaniosis IL-10 seems to play significant role in the suppression of cellular immunity (Scott and Novais, 2016). Therefore, IL-10 transcript in clinically-lesioned skin does not appear to be a marker of disease severity as previously demonstrated in whole blood cytokine release assays in dogs with clinical leishmaniosis (Solano-Gallego et al., 2016).

Relative quantification of IL-10 in normal-looking skin of sick dogs was statistically higher than in healthy skin of non-infected dogs. Our results agree with those previously published, in which dermal upregulation of IL-10 was determined in normal-looking skin of natural or experimentally infected dogs (Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017). Moreover, in those studies, and in agreement with the present findings, a correlation with parasite density was concluded in normal-looking skin (Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017). Although, not statistically significant IL-10 transcripts in normal-looking skin from mild affected dogs was lower than in more severe affected dogs. In agreement with that was the observation of a positive correlation of IL-10 transcripts in normal-looking skin with clinicopathological parameters associated with disease severity (UPC ratio, total protein, beta and gamma globulins and specific antibody levels), whereas negative correlation with albumin,

albumin/globulin ratio, hematocrit, hemoglobin and blood IFN-γ production was concluded. Therefore, Il-10 might be a marker of disease severity in normal-looking skin with early inflammatory process (chapter 4 and Ordeix et al., 2017), whereas in clinically-lesioned skin not. In fact, it has been recently suggested that IL-10 has a specific temporal role for susceptibility to visceral leishmaniosis in a mice model of *L. donovani* infection (Mesquita et al., 2018). This study demonstrates that an increase in IL-10 production has an impact early but not later after infection. Overexpression of IL-10 during the initial steps of the infection impacts host ability to control *L. donovani* infection by limiting the development of a protective adaptive immune response (Mesquita et al., 2018).

As expected, an upregulation of IFN-γ transcript was observed in clinically-lesioned skin from dogs with CanL, both mild and more severe affected dogs, when compared with healthy skin of non-infected dogs and Ibizan hound dogs. This result agrees with those previously published (Brachelente et al., 2005). According with the assumption that IFN-γ is a protective Th-1 associated cytokine (Hosein et al., 2017), relative quantification of IFN-γ in clinically-lesioned skin from mild affected dogs was higher than in clinically-lesioned skin from more sever affected dogs, although this difference was not statistically significant. Interestingly, IFN-γ transcript in clinically-lesioned skin from mild affected dogs was higher than that obtained in normal-looking skin, whereas transcripts among clinically-lesioned and normal-looking skin from dogs with moderate or severe disease were similar. This result agrees with the fact that normal-looking skin from dogs with mild disease present less frequently microscopic histological lesions when compared with dogs with moderate to severe leishmaniosis, as reported previously (chapter 4 and Ordeix et al., 2017). It is noteworthy, that IFN-γ transcripts in clinically-lesioned skin was not correlated with any clinicopathological, serological or parasitological parameter, neither with blood IFN-γ concentration.

In the present study, an upregulation of IFN-γ in normal-looking skin of diseased dogs was detected in comparison with healthy skin of non-infected dogs in agreement with previous studies (Menezes-Souza et al. 2011; Rodríguez-Cortés et al., 2016). Although not statistically significant, IFN-γ transcript in normal-looking of diseased dogs was higher than in Ibizan hound dogs. Dogs from group D were infected dogs, therefore an IFN-γ production was expected. This result agrees with the assumption that Ibizan hounds present a protective *L. infantum* specific cellular immunity, as has been demonstrated by means of LST and marked IFN-γ response to LSA after blood stimulation (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017). Relative quantification of IFN-γ in normal-looking skin from mild affected dogs was

significantly lower than that observed in normal-looking skin from more diseased dogs. As stated above, this result agrees with the fact that normal-looking skin from dogs with mild disease is less inflamed than normal-looking skin of dogs with moderate to severe leishmaniosis (chapter 4 and Ordeix et al., 2017). Accordingly, upregulation of this cytokine in normal-looking skin appeared to be positively (UPC ratio, total protein, beta and gamma globulins, specific antibody levels and parasite density) and negatively (albumin, albumin/globulin ratio, hematocrit and hemoglobin) associated with pathological findings indicative of disease severity.

To the best knowledge of the authors, PD-L1 expression has never been investigated in the skin of dogs with leishmaniosis. In the present study, we found an upregulation of PD-L1 in clinically-lesioned skin of diseased dogs, both mild and more severe affected, in comparison with healthy skin of non-infected dogs and Ibizan hound dogs. Therefore, this overexpression may suggest a role of PD-L1 in the immunopathogenesis of CanL. In mild affected dogs, PD-L1 transcript in clinically-lesioned skin was higher than in normal-looking skin, whereas in more severe diseased dogs this difference was not observed. As mentioned previously for IFN-γ, this finding may be the result of a lack or less prominent microscopic inflammatory lesions in normal-looking skin of mild diseased dogs when compared with dogs with moderate to severe leishmaniosis (chapter 4 and Ordeix et al., 2017). PD-L1 transcripts were similar among mild and more severe affected dogs in clinically-lesioned skin. In fact, this transcript was not correlated with any clinicopathological, serological or parasitological parameter in clinically-lesioned skin. Moreover, this result is in concordance with still unpublished study conducted by our group, which resulted in a lack of difference in PD-L1 transcripts in LSA stimulated blood between IFN-γ producer and non-producer dogs with CanL (Montserrat-Sangrà et al., 2018b). The increase in the PD-L1 expression observed in the clinically-lesioned skin of dogs with CanL might be a mechanism that parasites exploit to avoid the immune response, as suggested in human leishmaniasis (Barroso et al., 2018).

PD-L1 relative quantification in normal-looking skin of diseased dogs was upregulated in comparison with healthy skin of non-infected dogs. In addition, there was a lower expression of this gene in normal-looking skin of mild diseased dogs in comparison with more severe affected dogs. As for the other cytokines, this finding may be the result of a lack or less prominent microscopic inflammatory lesions in normal-looking skin of mild diseased dogs when compared with dogs with moderate to severe leishmaniosis (chapter 4 and Ordeix et al., 2017). In fact, this transcript was positively correlated with UPC ratio, total protein, beta and gamma globulins, specific antibody levels and parasite density, whereas negative correlation with

hemoglobin was detected. Therefore, based on our results it would seem that PD-L1 upregulation in normal-looking skin of dogs with leishmaniosis is associated with disease severity, suggesting that in the skin this ligand could be a marker of T cell exhaustion in early stages of skin inflammation in more severe affected dogs (Esch et al., 2013).

In this study, numerous significant positive correlations were found between the immune genes studied. It was not unexpected as TLRs communicate with themselves, other PRRs and cytokines with respect to pathogen recognition (Kawasaki et al., 2014; Tartey et al., 2017). Remarkably, more correlations between the immune genes transcripts were found in normal-looking than in clinically-lesioned skin from dogs with CanL, suggesting a more orchestrated immune response in early stages of the inflammatory process induced by the parasite. TLR2, TLR7, IL-10 and IFN-γ were positively correlated among them in normal-looking skin. TLR4 was the receptor that showed less correlations as only a strong positive correlation among this receptor and TLR2 was revealed in clinically-lesioned and normal-looking skin. This correlation between both receptors has been also observed in LSA stimulated blood in an unpublished study (Montserrat-Sangrà et al., 2018b). Correlation between IL-10 and IFN-γ has been evaluated in several studies. However, many of these investigations were performed on peripheral blood samples (Solano-Gallego et al., 2016; Rodríguez-Cortés et al., 2016). Usually a low or absent IFN-γ production has been found in tandem with increased production of IL-10 in human visceral leishmaniosis or studies reported in dogs with CanL (Boggiatto et al., 2010; do Nascimento et al., 2013; Scott and Novais, 2016). However, we report here a positive correlation among IL-10 and IFN-γ in either clinically-lesioned and normal-looking skin of diseased dogs. This result agrees with a previous study in which IFN-γ producers dogs secreted higher levels of IL-10 in LSA stimulated blood when compared with IFN-γ non-producers (Solano-Gallego et al., 2016b). Moreover, there was a positive correlation among blood concentration of IFN-γ and IL-10 transcript in clinically-lesioned skin, whereas, surprisingly, this correlation was negative in normal-looking skin. Suggesting that the proinflammatory (IFN-γ) / anti-inflammatory (IL-10) dichotomy is important only during the early stages of skin inflammation.

Dogs evaluated in this study were classified in stage I-mild, II-moderate and III-severe disease based on a well stablished clinical staging (Solano-Gallego et al., 2009; Solano-Gallego et al., 2017). The analysis of the clinicopathological parameters confirmed that dogs classified in stage I (group A) were in a less severe disease state as they significantly had less altered the clinicopathological parameters associated with disease severity than dogs with moderated and

severe disease (group B). Although urea was unexpectedly higher in dogs from stage I when compared with more diseased dogs, renal parameters did not differentiate dogs considered in the present study, probably due to the fact that dogs in stage IV were not enrolled in this study. In fact, obtaining skin biopsies from very severe diseased dogs with a poor prognosis might be difficult due to owners' decline. In addition, dogs from stage I presented lower *Leishmania*-specific antibody levels and skin parasite load either in clinically-lesioned skin and normal-looking skin (chapter 4 and Ordeix et al., 2017). It has been previously demonstrated that dogs with stage I and mild disease present blood IFN-γ production significantly higher than dogs with more severe disease (Montserrat-Sangrà et al., 2018). However, this difference could not be demonstrated in the present study, probably due to a lower number of dogs included herein.

Conclusions

This study demonstrated, for the first time, different expression profile of immune genes in clinically-lesioned and normal-looking skin from dogs with CanL. Moreover, differences among mild and more severe affected dogs were revealed. Clinically-lesioned skin from mild affected dogs were characterized by a significantly of TLR2 and IL-10 and downregulation of TLR7 and a trend for an upregulation of TLR4 and IFN-γ when compared with more severe affected dogs. On the other hand, normal-looking skin of mild affected dogs was characterized by a downregulation of TLR7, IFN-γ and PD-L1 and a trend for downregulation of TLR2 and IL-10 when compared with more severe affected dogs. In this study, TLR7 and PD-L1 transcripts were determined, for the first time, in canine skin of dogs with leishmaniosis and they appear to be associated with disease severity while IL-10 does not. Altogether, the results of the present study provide further insights into the role of these immune genes in clinical canine leishmaniosis.

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CHAPTER 7

Histological and immunological description of the leishmanin skin test in Ibizan hounds

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Abstract

The leishmanin skin test (LST), a delayed-type hypersensitivity (DTH) reaction to *Leishmania infantum*, can specifically identify dogs that have made a cell-mediated immune response to *L. infantum* infection. The Ibizan hound appears to be more resistant to *L. infantum* infection than other breeds of dog. The aim of this study was to describe the histological and immunohistochemical changes induced by the LST in Ibizan hounds living in an area highly endemic for leishmaniosis. The majority of dogs were apparently healthy, lacked serum antibody to *L. infantum* and blood parasitaemia, but had marked specific interferon gamma production after *in-vitro* blood stimulation with *L. infantum*. Leishmanin (3×10^8 killed promastigotes of *L. infantum*/ml) was injected intradermally and biopsy samples were obtained from a positive reaction at 72 h from nine Ibizan hounds. A moderate to intense, perivascular to interstitial dermatitis and panniculitis characterized the inflammatory response at the injection site. In addition, three samples had diffuse inflammation in the deep dermis and panniculus. Oedema and necrosis were present in the deep dermis and panniculus. Congestion and haemorrhage were observed in five biopsies. T lymphocytes (CD3+) and large mononuclear cells (lysozyme -) were the most prevalent cells. CD3+ cells were significantly more numerous than CD20+ B cells and lysozyme+ cells. B cells were sparsely distributed, especially in the deep dermis and panniculus. Rare neutrophils and macrophages (lysozyme+) were observed with few eosinophils. Toll-like receptor (TLR)-2 protein was expressed in large mononuclear cells mainly located in the superficial dermis. *Leishmania* immunohistochemistry was negative and quantitative polymerase chain reaction was positive in all cases. The intradermal injection of killed *L. infantum* promastigotes in Ibizan hounds causes similar histological and immunohistochemical findings to those described for human subjects and are indicative of a DTH response. Moreover, TLR2 protein is expressed in inflammatory cells similar to findings in clinically affected skin biopsy samples.

Keywords: dog; *Leishmania infantum;* leishmanin skin test; Toll-like receptor 2

Introduction

Canine leishmaniosis (CanL) is a zoonotic vectorborne disease caused by *Leishmania infantum* in the Mediterranean basin. It has a range of disease manifestation in dogs ranging from mild to overt severe fatal disease (Baneth et al., 2008; Solano-Gallego et al., 2009). In addition, subclinical infections are common (Baneth et al., 2008). The presence or absence of disease and its clinical variability is determined by the host's immune response (Solano-Gallego et al., 2009). In fact, both innate and adaptive immune responses play a role in the outcome of *Leishmania* infection. However, only the adaptive immune response has been extensively investigated in dogs (Hosein et al., 2017). The balance between the protective T-helper 1 (Th1) cellular response and the humoral immune response mediated by T-helper 2 (Th2) cells determines the clinical manifestation of the infection. A predominantly Th1 immune response is characterized by production of cytokines such as interleukin (IL)-2, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which induce anti- *Leishmania* activity by apoptosis of parasites in macrophages via nitric oxide and reactive oxygen species metabolism and is therefore capable of controlling infection and progression of disease. On the other hand, Th2 cells induce IL-4, IL-5, IL-10 and transforming growth factor beta (TGF-β) and correlate with antibody production and disease progression (Hosein et al., 2017).

The humoral immune response is detected by means of serological methods including quantitative techniques such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), and qualitative techniques such as rapid tests (Solano-Gallego et al., 2014). Unfortunately, there are few and poorly standardized assays to evaluate *Leishmania*-specific cellular immune responses. These include the leishmanin skin test (LST) or Montenegro's skin test and cytokine production measurement, such as detection of IFN-γ, in lymphocyte proliferation assays or in stimulated whole blood from dogs (Cardoso et al., 1998; Solano-Gallego et al., 2001; Fernández-Bellon et al., 2005; Strauss-Ayali et al., 2005; Rodríguez-Cortés et al., 2010; Solano-Gallego et al., 2016b). The LST consists of the intradermal inoculation of *Leishmania* antigen and the elicitation of a delayed-type hypersensitivity (DTH) reaction in a previously infected dog (Solano-Gallego et al., 2000; Solano-Gallego et al., 2001). Positive response to the intradermal injection of *Leishmania* antigen is widely used as a clinical indicator for evidence for the presence of a parasite-specific cellular immune response in infected dogs. Clinically, a positive LST is associated with absence of disease or mild clinical disease and therefore a good clinical outcome (Solano-Gallego et al., 2000; Ordeix et al., 2005;

Lombardo et al., 2014). However, dogs with moderate disease, before and after treatment, and vaccinated dogs may have a positive reaction as well (Ferrer et al., 2003; Bourdoiseau et al., 2009). By contrast, the response is low or absent in non-infected dogs or dogs with severe disease (Ferrer et al., 2003). Although this test has been proposed by some authors to be a predictor of a good clinical outcome of the infection (Solano-Gallego et al., 2000; Ordeix et al., 2005), little is known regarding the histopathological or immunological reaction in resistant dogs.

Although the innate immune response has been poorly studied in leishmaniosis, it is well established that it instructs the development of long lasting pathogen-specific adaptive immune responses (Hosein et al., 2017). In this sense, Toll-like receptors (TLRs), one of the most important pattern recognition receptor families, are important in the early host defence against the pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Kumar et al., 2009). Although the role of TLRs in the pathogenesis of CanL has not been fully addressed, it would seem that there is an association between TLR2 and the pathogenesis of cutaneous lesions in CanL. In fact, it has been revealed recently that there is lower expression of TLR2 in skin biopsy samples from dogs with mild disease (i.e. papular dermatitis) compared with dogs with moderate or severe disease (chapter 3 and Esteve et al., 2015). Moreover, TLR2 upregulation in blood and skin seems to be associated with disease progression in dogs (Hosein et al., 2015; Montserrat-Sangrà et al., 2016) and reduction in TLR2 transcription has been described with treatment and clinical improvement (Montserrat-Sangrà et al., 2016).

The Ibizan hound has been reported to be resistant to *Leishmania* infection (Solano-Gallego et al., 2000). In fact, this dog breed rarely manifests clinical disease and mounts significant cellular response to the infection demonstrated by a high prevalence of positive LST reactions as well as a potent *Leishmania*-specific IFN-γ production and low or no humoral response when compared with other breeds from the same geographical area (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017).

The literature regarding the histopathological study of positive reactions to intradermal injection of *Leishmania* antigen in dogs is scarce (Genaro et al., 1992; Tafuri et al., 1993). Therefore, the aim of this study was to investigate the histological and immunological changes induced by the LST in Ibizan hounds living in an area highly endemic for leishmaniosis.

Materials and methods

Dogs

Nine Ibizan hounds, living in an area highly endemic for leishmaniosis (Island of Mallorca, Spain) with a positive LST were enrolled in this study (Table 7-1). Briefly, two males and seven females, with a median age of 16 months (range 6-84 months) were included. Three dogs had mild clinical signs suggestive of leishmaniosis characterized by a persistent papulocrusting dermatitis on the inner aspect of pinnae (Figure 7-1) in the absence of haematological and biochemical abnormalities including a normal serum electrophoresis. All but one dog had negative results by quantitative serology for the detection of *L. infantum*-specific antibodies using an in-house ELISA (Solano-Gallego et al., 2014). In addition, blood DNA extraction and *L. infantum* quantitative polymerase chain reaction (qPCR) were performed with an absolute quantification as previously described (Solano-Gallego et al., 2016a), with negative results in five dogs and low-positive results in four dogs.

Table 7-1. Signalment and clinicopathological data of nine Ibizan hounds included in this study

Dog ID	Sex (M/F)	Age (m)	Physical examination	ELISA	*Leishmania* qPCR (blood)	IFN-γ (pg/ml)	LST (mm)
1	M	60	Fly dermatitis in pinnae	Negative	Negative	1115,6	26x21
2	F	84	Apparently healthy	Negative	Negative	13846,1	19x37
3	M	7	Papular dermatitis in left pinna	Negative	Negative	325,9	19x19
4	F	24	Apparently healthy	Negative	Low positive	ND	19x25
5	F	36	Apparently healthy	Negative	Low positive	ND	25x27
6	F	6	Papular dermatitis in left pinna	Negative	Low positive	1378,98	23x19
7	F	9	Papular dermatitis in pinnae	Low positive	Negative	656,71	22x28
8	F	16	Fly dermatitis in pinnae	Negative	Negative	ND	16x15
9	F	16	Fly dermatitis in pinnae	Negative	Low positive	2279,83	10x10

ID: identification number; M: male; F: female; m: months; ND: not done.

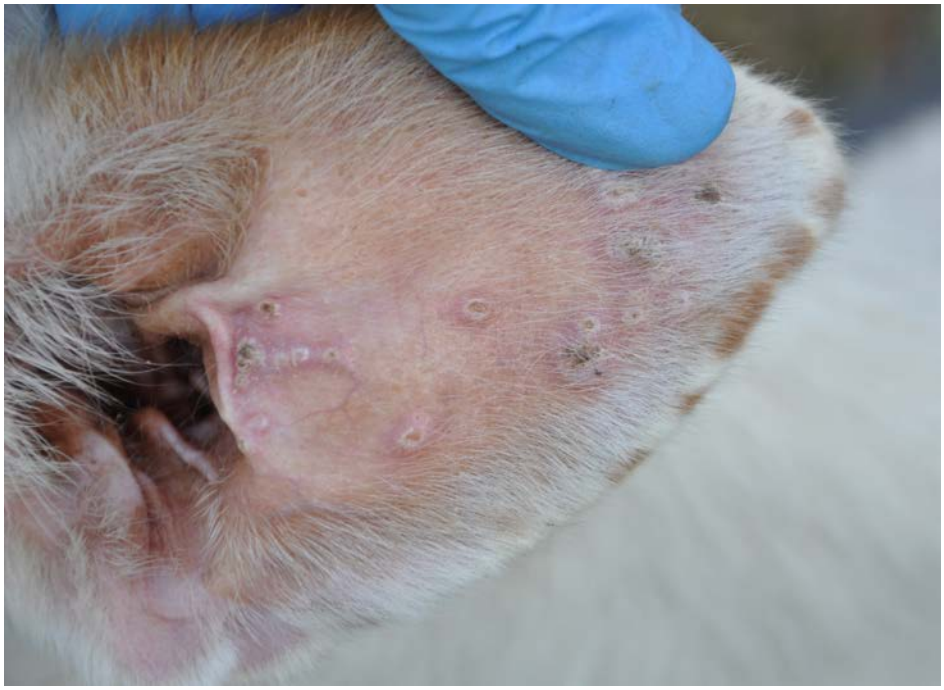

Figure 7-1. Papulocrusting dermatitis on the inner aspect of the pinna in an Ibizan hound living in an area highly endemic for leishmaniosis.

Whole Blood Cytokine Release Assay

Heparinized whole blood cytokine release assay was performed as described previously (Solano-Gallego et al., 2016b). Briefly, three different treatment conditions were established: (1) medium alone; (2) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 mg/ml provided by Dr. C. Riera (LSA 5 mg/ ml, Facultat de Farmacia, Universitat de Barcelona); and (3) medium with the mitogen concanavalin A (ConA, 100 mg Medicago, Uppsala, Sweden) at a concentration of 10 mg/ml. The plates were incubated at 37 C in 5% CO2. Then, blood was centrifuged at 300 g for 10 min and the supernatant was collected and stored at -80º C until used. IFN-γ was measured in supernatants from 5 days after stimulation with ConA and LSA or medium alone as previously described (Solano-Gallego et al., 2016b).

Measurement of Interferon Gamma

Analysis of IFN-γ was performed according to the manufacturer's instructions (DuoSet® ELISA, Development System R&D®, Abingdon, UK) using flat bottomed 96-well cell plates (Costar®, Corning, New York, USA). Slight modifications were made for the IFN-γ ELISA as described elsewhere (Solano-Gallego et al., 2016b). Cytokine concentration for all treatment conditions studied was analyzed after subtracting values obtained for medium alone.

Thereafter, dogs were classified as IFN-γ 'producers' or 'non-producers' as previously described (Solano- Gallego et al., 2016b).

Leishmanin Skin Test

The LST was carried out as described previously using 0.1 ml of an antigen (Solano-Gallego et al., 2000; Solano-Gallego et al., 2001). This antigen consisted of an inactivated suspension of 3 x 10^8 *L. infantum* promastigotes/ml in 0.4% phenol-saline (kindly provided by C. Chicharro, Instituto de Salud Carlos III, Madrid, Spain). The suspension was injected intradermally into the skin of the groin, the skin reactions were read at 72 h and an area of induration and/or erythema >0.5 cm in diameter was considered positive (Figure 7-2).

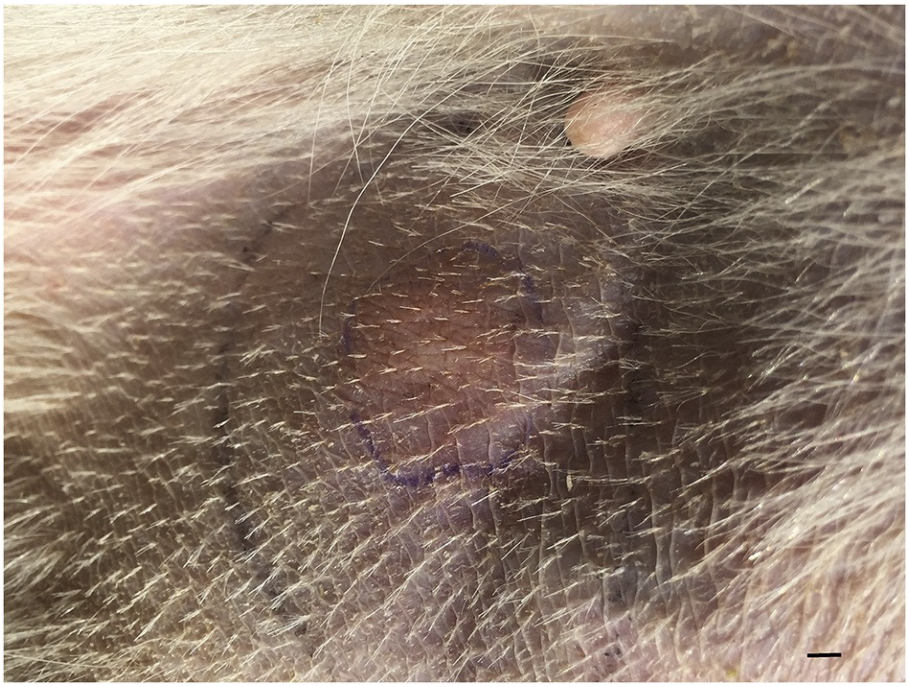

Figure 7-2. Positive skin reaction after intradermal injection of leishmanin in an Ibizan hound characterized by an induration and erythematous area >0.5 cm in diameter. Bar, 1 cm.

Biopsy Samples

Dogs were sedated with a combination of 0.2 ml of medetomidine (Domtor®, Pfizer, Madrid, Spain), 0.2 ml of butorphanol (Torbugesic Vet 10 mg/ml, Zoetis Spain, S.L., Madrid, Spain) and 0.3 ml of al- phaxalone (Alfaxan 10 mg/ml®, Jurox Limited Microbial Developments, Malvern, UK). One biopsy sample was taken from a LST positive reaction at 72 h using a 6 mm disposable punch (Kruuse®, Everest Tecnología Veterinaria, Molins de Rei, Spain). The biopsied material was cut into two halves. One half was fixed in 10% neutral

buffered formalin and the other was submerged in RNA later (RNAlater® Stabilization Solution, Ambion, Inc., Austin, Texas, USA) and kept at -80ºC until used.

Staining and Immunohistochemistry

Sections prepared from paraffin wax-embedded skin (4 mm) were stained with haematoxylin and eosin (HE) to study the morphology of the inflammatory reactions. Macrophage and lymphocyte subpopulations, as well as immunohistochemistry (IHC) for the detection of *Leishmania* antigens, were evaluated using a standard protocol with an AutostainerPlus® (Dako, Glostrup, Denmark). Briefly, slides were dewaxed in xylene and the tissue was rehydrated using graded alcohols. The potassium permanganate/oxalic acid melanin-bleaching technique was used before introducing the sections into the autostainer. Antigen retrieval was performed by means of incubation with PT Link® (Dako) at pH 6.0 at 98ºC for 20 min and proteinase K (Dako) for CD3/ CD20 and lysozyme IHC, respectively. Endogenous peroxidase was blocked with H2O2 3% in distilled water for 30 min. Sections were then incubated with the primary antibody. Thereafter, sections were incubated for 40 min at room temperature (RT) with the Dako EnVision+ System. The chromogen was 3,30-diaminobenzidine (DAB, Sigma-Aldrich Química, Madrid, Spain) with incubation for 2 min. Sections were counterstained with haematoxylin. Primary antibodies included polyclonal rabbit anti-human CD3, CD20 and lysozyme (Dako) and polyclonal rabbit antibody to *L. infantum* (kindly provided by the Instituto de Salud Carlos III, Madrid, Spain).

IHC for the detection of TLR2 was carried out as follows. Briefly, after dewaxing in xylene and tissue rehydration using graded alcohols, endogenous peroxidase was blocked with H_2O_2 3% in methanol for 15 min in a dark environment. Antigen retrieval was performed with citrate buffer (10 mmol/l, pH 6.0) for 20 min. After washing, the slides were treated with 1% Triton X-100 for 5 min and washed again. Blocking of the non-specific binding sites was made with bovine serum albumin 2% (Sigma-Aldrich) for 1 h. Sections were then incubated overnight at RT with the polyclonal goat anti-human TLR2 (1 in 100 dilution, Abcam, Cambridge, UK). One slide for each sample was incubated without primary antibody and constituted a negative control. Biotinylated secondary polyclonal goat anti-rabbit antibody (1 in 200 dilution, Dako) was applied for 1 h at RT followed by an avidin-horseradish peroxidase (Thermo Scientific, Rockford, Illinois, USA) reagent for 1h at RT. The chromogen was DAB. Tissues were counterstained with haematoxylin, dehydrated, cleared and mounted. Lymph nodes were used as a positive control. Moreover, to validate the expression of TLR2 in skin lesions from dogs

with leishmaniosis, three skin biopsy samples from dogs with stage I leishmaniosis (i.e. papular dermatitis) and three skin biopsy samples from skin lesions from dogs with stages II-III leishmaniosis (i.e. exfoliative or ulcerative dermatitis) were used (chapter 3 and Esteve et al., 2015).

Evaluation of Tissue Sections

The distribution pattern of the infiltrate (i.e. perivascular to interstitial or nodular to diffuse with or without granuloma formation); the type of the infiltrate (i.e. small and large mononuclear cells, plasma cells, neutrophils and eosinophils); the degree (i.e. none, mild, moderate or severe) of cellular infiltration in the dermis and panniculus and the presence of other alterations such as haemorrhage, necrosis, oedema and congestion were evaluated.

Quantification of CD3, CD20 and lysozyme-expressing cell populations was carried out by counting labelled cells in 12 fields (three different fields from the superficial, mid and deep dermis and panniculus) using digital images captured at x600. TLR2 scoring was performed by estimating the number of positively labelled cells in six fields at x600.

Deoxyribonucleic acid Extraction Extraction and Leishmania Quantitative Polymerase Chain Reaction

RNA was isolated from skin biopsy samples using the RiboPure® Kit (Ambion, Austin, Texas, USA) and stored at -80ºC until used. The DNA was purified from the interphase and organic phase generated from the RNA purification process by means of the QIAamp DNA Mini Kit® (Qiagen, Manchester, UK) following the manufacturer's instructions with slight modifications. Briefly, 20 ml of proteinase K solution were added in all samples and 200 ml of tissue sample were used for all the cases. The other steps were performed as per the protocol. A fragment of spleen from a clinically healthy non-infected dog was used as a control for DNA contamination during the DNA extraction (chapter 4 and Ordeix et al., 2017).

qPCR was performed with a relative quantification as previously described with minor modifications (Solano-Gallego et al., 2016). Briefly, the PCR mix reaction was prepared with 4 ml of DNA, 10 ml of master mix (TaqMan® Fast Advanced Master Mix, Thermo Fisher Scientific, Waltham, MA USA), 1 ml of *Leishmania* primers and probes (Custom TaqMan® Gene Expression Assay, Thermo Fisher Scientific, Waltham, MA USA) or 1 ml of control assay primers and probes (Eukaryotic 18S rRNA endogenous control; VIC!/MGB Probe, Primer

Limited, Thermo Fisher Scientific, Waltham, MA USA) and 5 ml of H2O. In order to verify that the PCR was done successfully, a positive control for *Leishmania* and a negative control from a non-infected clinically healthy dog were included in the plate. PCR was carried out in a QuantStudio Flex® 7 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA USA). The thermal cycling profile consisted of 2 min at 50ºC in order to activate the amperase enzyme and afterwards, a total of 40 cycles were carried out. Each cycle consisted of 20 sec at 95ºC followed by 40 cycles of 1 sec at 95ºC and 20 sec at 60ºC.

To compensate for variations in total DNA input, mean values of cycle threshold (CT) from duplicate determinations from the *Leishmania* and canine 18SePCR were taken for the calculation of the delta CT (difference of expression between *Leishmania* CT and 18S CT) (chapter 4 and Ordeix et al., 2017).

Statistical analysis

Statistical analyses were performed using the IBM SPSS 22.0 programme for Windows software (SPSS Inc., Armonk, New York, USA). Standard descriptive statistics (mean, standard deviation [SD] and median) were calculated for quantitative variables. A non-parametric Wilcoxon Signed Ranks test was used to compare the IHC data. The non-parametric ManneWhitney test was used to compare expression of TLR2 between different stages of disease and the LST. Differences were considered significant at $p < 0.05$.

Results

Parasite-Specific Interferon Gamma Production

Cytokine analysis was performed in the whole blood in six out of nine Ibizan hounds. All of the dogs tested produced IFN-γ after LSA stimulation. The mean and SD for IFN-γ concentrations were 3,267.2 ± 5,225.9 pg/ml.

Histological Study

The inflammatory response in skin biopsy samples from the LST reactions was characterized by a moderate to intense, perivascular to interstitial dermatitis with and without panniculitis. This infiltrate was located in the superficial and mid dermis in all of the samples and in the deep dermis and panniculus in six samples (Figure 7-3). In addition, three samples

had a diffuse pattern in the deep dermis and panniculus. There was no evidence of granuloma formation in any of the dogs studied. Oedema and necrosis were present in the deep dermis and panniculus of all the samples. Congestion and haemorrhage were observed in five samples (Figure 7-4). Small and large mononuclear cells were the predominant inflammatory cells with few interspersed neutrophils and eosinophils (Figure 7-5).

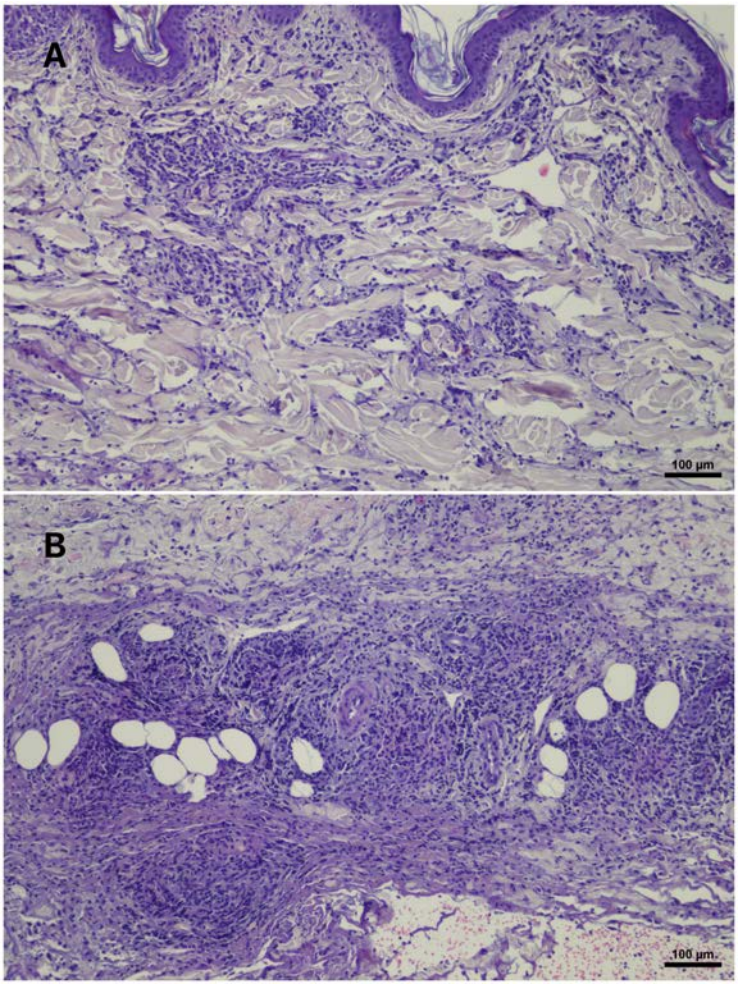

Figure 7-3. Histological appearance of a LST reaction in an Ibizan hound. Note the moderate perivascular to interstitial inflammatory infiltrate in the superficial and mid dermis (A) and in the deep dermis and panniculus (B). HE.

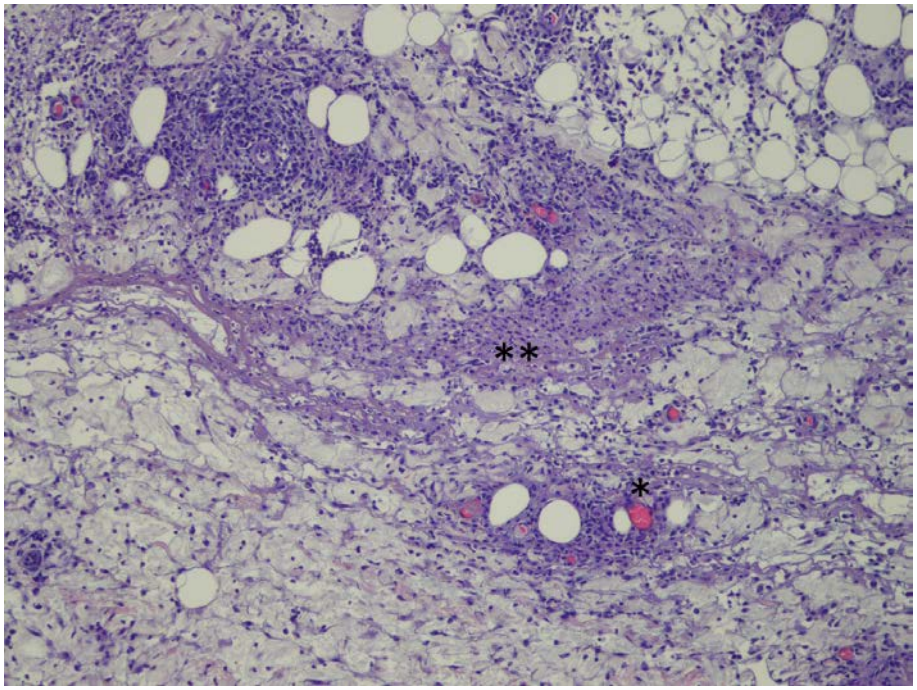

Figure 7-4. Congestion (*), edema and necrosis (**) in the deep dermis and panniculus of a LST reaction in an Ibizan hound. HE.

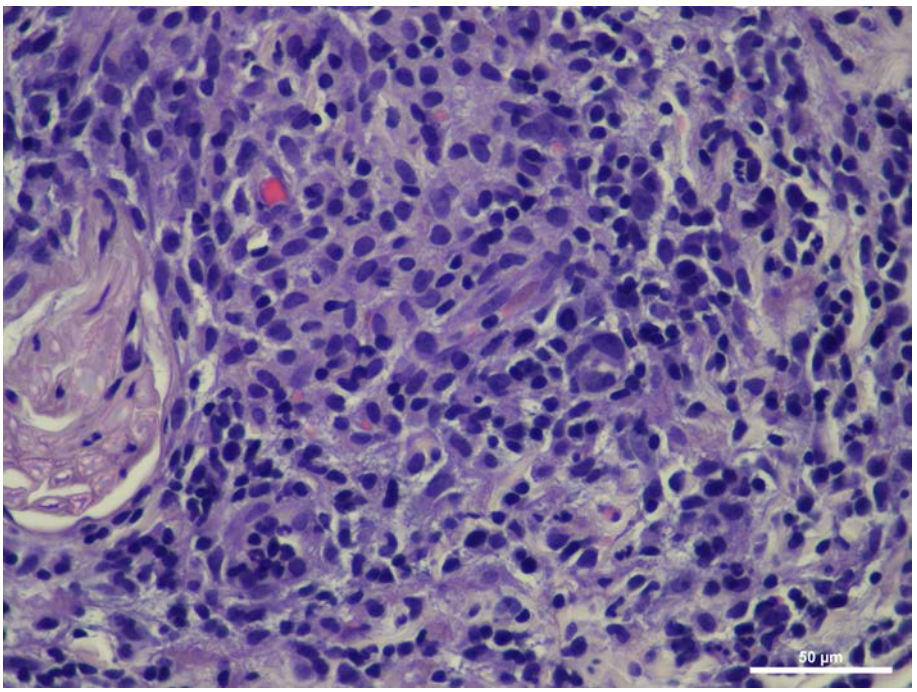

Figure 7-5. Small and large mononuclear cells present as the predominant inflammatory cells with few interspersed neutrophils and eosinophils observed in an HE-stained section of a LST reaction in an Ibizan hound.

Immunohistochemistry

The number of labelled cells and their distribution is shown in Table 7-2. T lymphocytes (CD3+) and large mononuclear cells (lysozyme -) were significantly more prevalent than B cells or lysozyme+ cells (Figure 7-6A). These cells were similarly distributed in all layers of the skin. B lymphocytes (CD20+) were sparsely distributed, but were detected especially in the deep dermis and panniculus (Figure 7-6B). Rare neutrophils and macrophages, which are lysozyme+ cells, were observed and similarly distributed in all the layers of the skin (Figure 7-6C). *Leishmania*-specific IHC was negative in all cases.

TLR2 protein was expressed in large mononuclear cells mainly located in the superficial dermis of LST reactions (Figure 7-7). Although TLR2 expression in LSTs (1.4 ± 1.0 cells per x600 field) was lower than that observed in skin lesions from stage I (2.7 ± 1.1) or II-III (2.5 ± 1.5) diseased dogs, this difference was not significant ($p = 0.100$).

Table 7-2. Immunological results of CD3, CD20 and lysozyme+ cell counts in LST biopsies from Ibizan hounds.

	CD3 (mean +/-SD)	***CD20 (mean +/-SD)***	***Lysozyme (mean +/-SD)***
Superficial dermis	94±45	25±12[c]	35±20
Mid dermis	94±37	31±16[d]	35±18
Deep dermis and panniculus	111±18	50±12[c,d]	40±17
Total	100±34[a,b]	35±17 [a]	37±18[b]

[a]Wilcoxon signed rank test, p = 0.018; [b]Wilcoxon signed rank test, p = 0.018; [c]Wilcoxon signed rank test, p = 0.008. dWilcoxon signed rank test, p = 0.021.

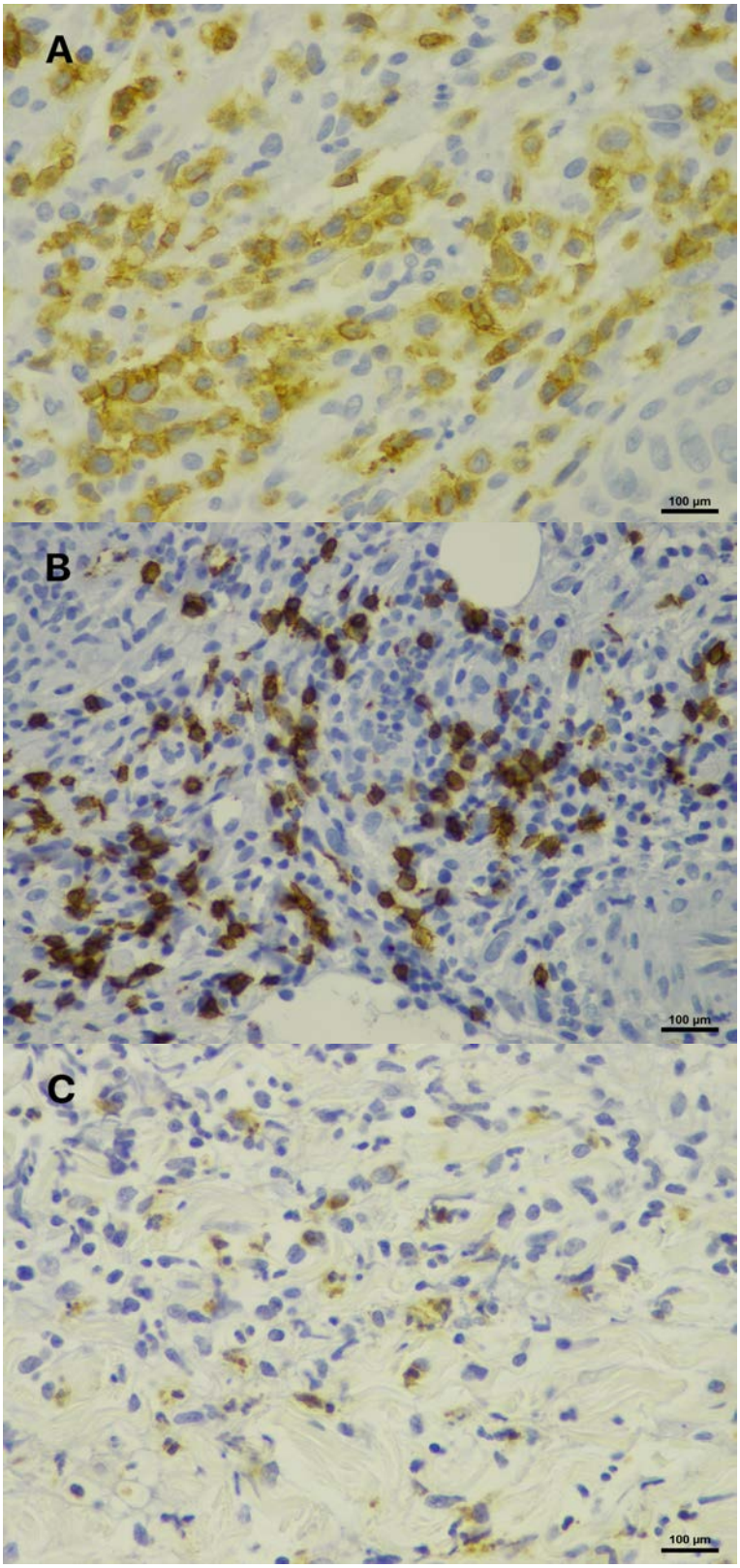
A
100 µm
B
100 µm
C
100 µm

Figure 7-6. Representative photomicrographs of immunohistochemical labelling for CD3 (A), CD20 (B) and lysozyme (C) in a LST reaction in Ibizan hounds. (A) CD3+ cells are distributed in all layers of the skin. (B) CD20+ cells are sparsely distributed and especially detected in the deep dermis and panniculus. (C) Lysozyme+ cells compatible with neutrophils and macrophages are rare and similarly distributed in all layers of the skin.

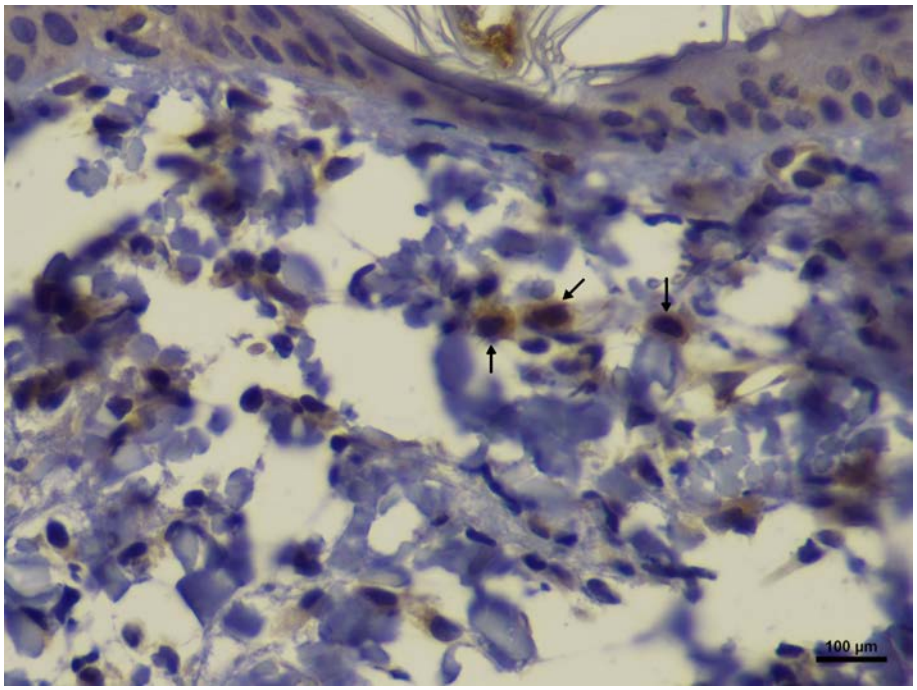

Figure 7-7. TLR2-specific labelling from a LST reaction in an Ibizan hound. TLR2 protein is sparsely expressed in large mononuclear cells mainly located in the superficial dermis.

Quantitative Polymerase Chain Reaction in Leishmanin Skin Test Skin Biopsy Samples

Leishmania qPCR was positive in all samples 72 h after antigen injection. The median delta CT value was 1.26 (range 1.97-6.16).

Discussion

In the present study we examined the histological and immunological characteristics of LST reactions obtained in Ibizan hounds living in an area highly endemic for leishmaniosis. The scientific literature regarding the histological characteristics of the LST in dogs and man is limited (Mayrink et al., 1989; Genaro et al., 1992; Tafuri et al., 1993; Guaríun et al., 2006). Moreover, immunohistochemical analysis has only been performed in a study that aimed to describe alterations in dendritic cell morphology in LST reactions in dogs (Sacchi et al., 2006). Nonetheless, the present histological study described similar results to those of Tafuri et al. (1993) in dogs living in an endemic area and by Mayrink et al. (1989) for positive reactions obtained from people with asymptomatic infection, cutaneous leishmaniosis or from vaccinated patients. All LST reactions in Ibizan hounds showed similar immunohistochemical results and the small differences noted were probably related to individual differences among immune responses. Histologically, these samples were characterized by an infiltration of mainly mononuclear cells at the site of antigen administration. In our study, the most prevalent cells were CD3+ T cells and large mononuclear cells, presumably dendritic cells (lysozyme- cells). This is not surprising, taking into account that the characteristic morphology of a DTH reaction involves infiltration of T cells, particularly CD4+ T cells (Abbas et al., 2015). The characteristic DTH response typically evolves within 24-48 h (Abbas et al., 2015). However, in experimentally and naturally infected dogs, the histological and clinical inflammatory process induced by the intradermal administration of *Leishmania* antigen reaches its maximum 72 h after administration of the antigen (Genaro et al., 1992; Solano-Gallego et al., 2000). Consequently, the time selected for skin biopsy sampling of LST reactions in our study was 72 h.

Although DTH has traditionally been considered to be a CD4+ Th1-mediated injurious reaction, other Th lymphocytes such as Th2 or Th17 cells may contribute to a lesser extent to the inflammation (Abbas et al., 2015). However, and similar to what was described in man, neutrophils and eosinophils were scarce in the LST reactions from the present dogs, suggesting that involvement of other T cells such as Th2 or Th17 cells was less likely. On the other hand, CD8+ T cells may contribute to some DTH reactions (Abbas et al., 2015). In fact, a positive association between the presence of CD8+ cytotoxic T cells and macrophages has been shown in the Montenegro skin reaction of asymptomatic human residents of endemic areas (Guarín et al., 2006). Moreover, this cytotoxic response has been related to the tissue damage and necrosis observed in the skin test biopsy samples of 3 out of 11 individuals with asymptomatic infection (Guarín et al., 2006). Similarly, necrosis was present in the deep dermis and panniculus of all the

samples from the present dogs. Therefore, this finding might suggest a contribution of a cytotoxic T-cell-mediated response in LST reactions in resistant dogs. One limitation of the present study is that other markers, such as those for CD4 and CD8, were not determined in order to further distinguish the infiltrating cell populations.

CD20+ B cells were the third most prevalent type of cells, especially detected in the deep dermis and panniculus. This observation differed from Montenegro skin testing in man, where occasional B cells were described in positive reactions from resistant individuals (Guarín et al., 2006).

This study demonstrated for the first time TLR2 expression mainly in superficial large mononuclear cells from skin biopsy samples of LST reactions. There was a trend for a lower expression in LST reactions compared with cutaneous lesions from dogs with clinical leishmaniosis, although there were no significant differences. This difference in TLR2 expression might be partially explained by the type of cellular infiltrate, being more macrophage-like in clinically affected skin than in the LST reactions, and by the duration of the tissue reaction, being more chronic in clinically affected skin than in LST reactions. In fact, TLR2 upregulation in the skin seems to be associated with disease progression in dogs (Hosein et al., 2017). Therefore, the injurious inflammatory reaction resulting from *Leishmania* antigen might be too short to favour TLR2 expression as it occurs in clinically affected skin.

As expected, *Leishmania*-specific IHC was negative in all cases due to the absence of amastigotes in the inflammatory infiltrate. On the other hand, *Leishmania* DNA was variably present among the dogs. Although data regarding the kinetics of the parasite load after LST are lacking, this result suggests that the *Leishmania* DNA from killed promastigotes is present at the site of inoculation at least 72 h after injection.

In man, the Montenegro skin testing reaction has been suggested as a surrogate of the early response to infection (Guarín et al., 2006). In fact, patients with acute lesions had similar proportions of CD4+ and CD8+ T lymphocytes and macrophages both in lesions and in Montenegro skin test response biopsy samples (Guarín et al., 2006). Unfortunately, to the best of the authors' knowledge, there is no clinical, histological or immunological characterization of acute and chronic cutaneous lesions in naturally infected dogs. CanL is considered a chronic disease and most dogs exhibit clinical signs only years after being in an area of endemicity (Solano-Gallego et al., 2009). However, papular dermatitis due to *L. infantum* is a cutaneous manifestation of *L. infantum* infection diagnosed classically in young animals, generally under 1

year of age, with a protective immune response that has been clinically and experimentally associated with sand fly bite sites (Ordeix et al., 2005, chapter 3 and Esteve et al., 2015; Aslan et al., 2016; chapter 4 and Ordeix et al., 2017). It would be of relevance to study whether LST reactions in dogs might mimic the early inflammation and immune response to *Leishmania* parasites in dogs with protective immune responses.

Ibizan hounds living in an area highly endemic for leishmaniosis show a predominant *L. infantum*-specific cellular immunity demonstrated by means of the LST (Solano-Gallego et al., 2000). In the present study, those dogs with positive LST reactions showed a marked IFN-γ response to LSA stimulation of blood. This feature, together with a clinically healthy or mild disease status, seronegativity and negative parasitaemia in most of the dogs, further support the concept that Ibizan hounds are a 'resistant' breed to *L. infantum* due to a protective immune response (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017).

In conclusion, the intradermal injection of killed *L. infantum* promastigotes in Ibizan hounds causes similar histological and immunohistochemical findings to those described for man and they are indicative of a DTH response. Moreover, TLR2 protein is expressed in inflammatory cells similar to its expression in clinically affected skin.

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Conflict of Interest Statement

The authors declare that they have no competing interests related to the publication of this article.

Supplementary data

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CHAPTER 8

Toll-like receptors 2, 4, and 7, interferon-gamma, interleukin 10 and programmed death ligand 1 transcripts in leishmanin skin test positive reactions of Ibizan hound dogs

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Short Communication

Abstract

Leishmanin skin test (LST) is an *in vivo* technique commonly used to evaluate *Leishmania*-specific cellular immune response in dogs. However, information regarding the local immune response in LST positive reactions is scarce. We examined the pattern of TLR2, TLR4, TLR7, IL-10, IFN-γ and PD-L1 gene expression in LST positive reactions and paired normal-looking skin of nine infected Ibizan hound dogs. Normal skin from ten seronegative dogs from non-endemic area was analyzed as a negative control. Immune genes expressions were examined by qPCR analysis. LST positive reactions presented significant upregulation of TLR4, IL-10, IFN-γ and PD-L1 and downregulation of TLR7 when compared with normal skin of control dogs. Moreover, a trend for TLR2 upregulation was observed. All transcripts but TLR7 were higher in LST positive reaction than in paired normal-looking skin. Immune genes' expression profile in LST positive reactions was similar to that previously observed in clinically-lesioned skin of mild diseased dogs with papular dermatitis.

Keywords *Leishmania infantum*, dog, delayed type hypersensitivity reaction, cytokine, toll-like receptors, gene expression.

Introduction

The outcome of *Leishmania* infection in dogs is the result of a complex immune response mounted by the host against the parasite (Solano-Gallego et al., 2009; Papadogiannakis and Koutinas, 2015). Effective T helper 1 (Th1) cellular immunity, with the activation of macrophages by IFN-γ and TNF-α is associated with the elimination of intracellular amastigotes and the control of disease progression (Solano-Gallego et al., 2009; Papadogiannakis and Koutinas, 2015). There are few and poorly standardized assays to evaluate *Leishmania*-specific cellular immune responses in dogs. One of these tests is the leishmanin skin test (LST) or Montenegro's skin test (Cardoso et al., 1998; Solano-Gallego et al., 2000; Solano-Gallego et al., 2001; Fernández-Bellon et al., 2005). The LST consists of the intradermal inoculation of *Leishmania* antigen and the elicitation of a delayed type hypersensitivity (DTH) reaction in a previously infected dog (Solano-Gallego et al., 2000; Solano-Gallego et al., 2001). Positive LST reactions in dogs are associated with mild or absence of disease (Solano-Gallego et al., 2000; Ordeix et al., 2005; Lombardo et al., 2014). On the contrary, the response is low or absent in non-infected dogs or dogs with severe disease (Ferrer et al., 2003).

Ibizan hound dogs are considered resistant dogs to *Leishmania* infection as they rarely manifest clinical disease and mount significant cellular immune response to the infection demonstrated by a high prevalence of positive LST reactions as well as a potent *Leishmania* specific IFN-γ production and low or null humoral response when compared with other breeds from the same geographical area (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017; chapter 5). The immunohistological features of LST positive reactions in Ibizan hound dogs have been recently described (chapter 7 and Ordeix et al., 2018). LST positive reactions 72 hours after intradermal injection of *Leishmania* antigen were characterized by histological changes indicative of a DTH reaction similar to those findings described for human subjects (Guarí et al., 2006; chapter 7 and Ordeix et al., 2018). A moderate to intense, perivascular to interstitial dermatitis with and without panniculitis was observed. Rarely a diffuse pattern in the deep dermis and panniculus was also observed in few samples. There was no evidence of granuloma formation in any of the dogs studied (chapter 7 and Ordeix et al., 2018). CD3+ T-cells were the more prominent cellular type on the dermal infiltrate. Further immunophenotyping of CD3+ T lymphocytes was not performed, however marked necrosis present in all cases suggested the contribution of cytotoxic T-cell mediated response in LST positive reactions in resistant dogs as it is described for human beings (Guarín et al., 2006, chapter 7 and Ordeix et al., 2018). Moreover, TLR2 protein expression was evident by immunohistochemistry in dermal mononuclear cells (chapter 7 and Ordeix et al., 2018). Although LST is a commonly used assay to evaluate cell-mediated immune response in dogs with

leishmaniosis, to the best of the authors' knowledge, studies aimed to evaluated local immune response have not been previously published.

The objective of the present study was to describe the pattern of expression of TLR2, TLR4 and TLR7, IFN-γ, Il-10 and PD-L1 in the LST positive reactions in Ibizan hound dogs living in a highly endemic area of leishmaniosis.

Materials and methods

Nine Ibizan hound dogs, living in the Island of Mallorca, Spain, with a positive LST were enrolled in this study. These dogs were the same than those previously enrolled in a published study aimed to evaluate the histological and immunological changes induced by the intradermal inoculation of *Leishmania* antigen in resistant dogs (chapter 7 and Ordeix et al., 2018). Signalment, clinicopathological, immunological and parasitological data of these dogs are summarized in table 8-1. Briefly, these dogs were clinically healthy, except for three dogs that presented a papulo-crusting dermatitis on the inner aspect of pinnae suggestive of a mild form of leishmaniosis, in the absence of clinicopathological abnormalities (Solano-Gallego et al., 2009). These dogs were infected by *L. infantum* as all showed positive LST reactions (Solano-Gallego et al., 2000). Moreover, strong specific IFN-γ production in stimulated cultured blood performed as described in chapter 7 (Solano-Gallego et al., 2016b) was detected in all dogs where it was evaluated (6 out total 9 dogs) In addition, blood *Leishmania* qPCR carried out as previously described (Solano-Gallego et al., 2016) was negative and positive in five and four dogs, respectively. All dogs were serologically negative but one (low positive) based on an in house ELISA (Solano-Gallego et al., 2016). Two skin samples were obtained in each Ibizan hound dog. One from an LST positive reaction at 72 hours (Solano-Gallego et al., 2000) and one from normal-looking skin. Skin biopsies of LST positive reactions were cut into two halves. One half was fixed in 10% formalin for the aforementioned histoimmunological study (chapter 7 and Ordeix et al., 2018) and the other half and the skin biopsy from normal-looking skin were submerged in RNA later (RNA*later*® Stabilization Solution, Ambion, Inc., Austin, Texas) and kept at -80º until RNA extraction and cDNA synthesis were performed following previously described protocol (chapter 6). Moreover, *Leishmania* qPCR was performed in skin samples from Ibizan hound dogs with a relative quantification as previously described (chapter 4 and Ordeix et al., 2017). A control group with 10 samples of normal-looking skin from healthy seronegative dogs from a non-endemic area used in previously described study reported in chapter 6 was included. Immune genes' expressions were determined by qPCR as formerly described in chapter 6. The mean number of relative

quantifications of the transcripts from LST positive reactions, normal-looking skin of Ibizan hound dogs and controls were compared by the nonparametric Mann–Whitney U test. Whereas, related variables were compared by the nonparametric Wilcoxon signed-rank test. A probability of similarity of < 0.05 ($p < 0.05$) was considered to indicate a significant difference.

Results

The expression of the immune genes studied in LST positive reactions and healthy skin from Ibizan hound and control dogs are described in table 8-2. Concisely, TLR4 ($p = 0.02$), IL-10 ($p = 0.001$), IFN-γ ($p = 0.0002$) and PD-L1 ($p = 0.01$) transcripts were significantly higher in LST positive reactions than in normal skin from control dogs. Although TLR2 relative quantification was higher in LST positive reactions compared with normal skin of control dogs, this difference was not statistically significant ($p = 0.08$).

Relative quantification of TLR7 was lower in LST positive reactions than in normal skin from control dogs ($p = 0.0003$). Only TLR4 ($p = 0.003$) and IL-10 ($p = 0.01$) transcripts were significantly higher in normal-looking skin from Ibizan hound dogs than in normal skin from control dogs. There was a trend for a higher upregulation of IFN-γ and PD-L1, whereas TLR7 ($p = 0.00001$) was downregulated in normal-looking skin of Ibizan hounds compared with normal skin from control dogs. All the transcripts of the immune genes studied were higher in LST positive reactions from Ibizan hound dogs than in paired normal-looking skin. This difference was significant for TLR4 ($p = 0.0006$), IFN-γ ($p = 0.004$) and PD-L1 ($p = 0.04$), whereas it was a trend for a higher relative quantification of TLR2 and IL-10.

Discussion

To the best of the authors' knowledge, no studies have evaluated immune genes' transcripts in the LST positive reactions of resistant dogs. Moreover, similar studies have not been performed in human or experimental animal models of leishmaniasis. Remarkably, we compared gene transcription in LST positive reactions with normal-looking skin of the same dogs. LST positive reactions were characterized by upregulation of all immune genes studied but TLR7, which resulted downregulated when compared with normal-looking skin of control dogs. Although not statistically significant, it was a tendency for a higher TLR2 overexpression in LST positive reactions in the present study in comparison with healthy skin from control dogs.

Table 8-1. Signalment, clinicopathological, immunological and parasitological data of Ibizan hounds.

Parameters		***Ibizan hound dogs (n = 9)***
Sex		2 males and 7 females
Age		16 months [6-84]
UPC		ND
Creatinine (mg/dL)		1±0.1
Urea (mg/dL)		33.8±7.4
Total protein (g/dL)		6.3±0.3
Albumin (g/dL)		3.5±0.3
Beta globulin (g/dL)		1.3±0.1
Gamma globulin (g/dL)		0.5±0.1
Albumin/globulin ratio		1.2±0.2
Hematocrit (%)		49.3±5.9
Hemoglobin (g/dL)		16.1±1.8
***Leishmania infantum* specific antibody levels (ELISA units)**		18.3±10.3
Blood *L. infantum* specific IFN-γ (pg/mL)		3486±5291.1
Blood parasite load (parasites/mL)		0.9±2.2
Skin parasite load (dCt)	*LST*	3.8±2.6[a]
	Normal-looking	8.9±1.9[a]

UPC: urinary protein/creatinine ratio; ND: not done; ELISA: enzyme linked immunosorbent assay; dCt: delta-cycle threshold (low or negative values of dCt represented high parasite density). [a]Wilcoxon Signed Ranks Test Z = -3.0267, p = 0.002

This result agrees with those obtain in the previously published immunohistopathological study, in which a similar TLR2 expression determined by immunohistochemistry in LST positive reactions was observed in comparison with healthy skin (chapter 7 and Ordeix et al., 2018).

As expected all immune genes' transcripts were higher in LST positive reactions than in paired normal-looking skin of Ibizan hound dogs. In fact, it is assumed that LST positive reactions presented a more prominent microscopic inflammatory lesions than normal-looking skin as documented for mild affected dogs (chapter 4 and Ordeix et al., 2017). Moreover, parasite DNA was predictably more present in LST positive reactions than in normal-looking skin of Ibizan hound dogs in the current study. Therefore, it would be possible that upregulation of immune genes in LST positive skin reactions compared with paired normal-looking skin were the result of a more severe inflammation as the result of a response to the presence of *Leishmania* antigen and of danger associated molecular patterns (DAMPs) which are the components of damaged or apoptotic cells that act as endogenous stress signals (Vidya et al., 2017). TLR7 showed less markedly differences among LST positive reactions and normal-looking skin of Ibizan hound dogs as in both cases relative quantification of this transcript was lower than in normal skin of control dogs. That was an interesting finding and in agreement with our previously study that associated TLR7 upregulation with moderate to severe disease (chapter 6). A pathogenic role of this receptor in visceral leishmaniosis due to *L. donovani* in mice has been recently suggested (Silva-Barrios et al., 2016; Fabié et al., 2018). In this model of *Leishmania* infection, an innate activation of B cells through endosomal TLRs, such as TLR7, induced cytokine (INF type I and IL-10) and endosomal TLR expression, followed by disease exacerbation and hypergammaglobulinemia (Silva-Barrios et al., 2016). Moreover, local tissue damage mediated by persistent inflammation leads to suppression of protective T cell responses during chronic visceral leishmaniosis due to *L. donovani* in mice via TLR7 signaling (Fabié et al., 2018). Therefore, lack of TLR7 overexpression in LST positive reactions and normal-looking skin point out a protective immune response in resistant Ibizan hound dogs.

Interestingly, immune genes' expression profile observed in LST positive reaction was comparable with that observed in clinically-lesioned skin of dogs with stage I leishmaniosis and papular dermatitis. Our group has previously documented a significant upregulation of TLR2, TLR4, IFN-γ and PD-L1 and a downregulation of TLR7 in clinically-lesioned skin of stage I and mild disease when compared with normal skin from the same group of control dogs studied herein (chapter 6). Actually, Montenegro skin testing reaction in human beings has been suggested as a surrogate of the early response to infection (Guarín et al., 2006). Papular dermatitis is a mild cutaneous manifestation of *L. infantum* infection classically diagnosed in young animals, generally

under one year of age, which has been clinically and experimentally associated with sand fly bite sites (Aslan et al., 2016). Dissimilarities between transcripts in LST positive reactions and clinically-lesioned skin of stage I and mild diseased dogs were the lack of a significant increase in TLR2 gene expression and the upregulation of IL-10 transcript in LST positive reactions in comparison with normal skin from control dogs. In the study presented herein, although not statistically significant there was a trend for an increase in TLR2 transcript in LST positive reactions when compared with control dogs. The low number of cases included in the present study might be related with that discrepancy. On the other hand, the type of cellular infiltrate, being more macrophagic and with granuloma formation in clinically-lesioned skin of dogs with stage I leishmaniosis and papular dermatitis than in LST positive reactions, where predominant lymphocytic infiltrate was observed may account for this discrepancy as well (chapter 4 and Ordeix et al., 2017; chapter 7 and Ordeix et al., 2018). As TLR2 upregulation in the skin seems to be associated with disease progression in dogs (Hosein et al., 2015), the injurious inflammatory reaction resulting from *Leishmania* antigen might be too short to favor TLR2 expression as it occurs in chronic clinically-lesioned skin (chapter 6). IL-10 was significantly upregulated in LST positive reactions when compared with normal skin from control dogs, whereas in clinically-lesioned skin of dogs with papular dermatitis a trend for an upregulation of IL-10 when compared with normal skin of the same control dogs was documented, although this difference was not statistically significant (chapter 6). The fact that IL-10 is significantly upregulated in LST positive reactions 72 hours after intradermal injection of *Leishmania* antigen and not in more chronic clinically-lesioned skin might suggest an IL-10 overexpression early on the course of inflammation induced by the parasite. In fact, this cytokine is a vital immunoregulator that modulates immunopathology and tissue damage following increased production of inflammatory cytokines, especially IFN-γ (Maspi et al., 2016).

Normal-looking skin of Ibizan hound dogs presented a similar immune genes' expression profile to LST positive reactions, when compared with normal skin of control dogs. Both TLR4 and IL-10 were significantly upregulated, whereas TLR7 was significantly downregulated. In addition, there was a trend for a major expression of IFN-γ and PD-L1. One limitation of this study is that histological examination of normal-looking skin of Ibizan hound and control dogs was not performed. Therefore, it was not possible to associate or not the immune genes' expression to an already present microscopic inflammation in a normal-looking skin (chapter 4 and Ordeix et al., 2017). Noteworthy, TLR2 expression in normal-looking skin of infected Ibizan hound dogs was similar to normal skin from control dogs, contrary to the situation in LST positive reactions where an upregulation, although not statistically significant, was observed. This result again reinforces the knowledge that TLR2 overexpression is associated with the inflammatory process

(Hosein et al., 2015) and probably denotes a lack or less prominent microscopic inflammatory lesions in normal-looking skin of infected Ibizan hound dogs.

Our results describe, for the first time, the pattern of immune genes (TLR2, TLR4, TLR7, IL-10, IFN-γ and PD-L1) expression in the skin of LST positive reactions and paired normal-looking skin of Ibizan hound dogs infected by *L. infantum*. Our data provide additional support for the important role of TLRs in canine leishmaniosis.

Table 8-2. Immune genes' transcripts in leishmanin skin test (LSTs) positive reactions and normal-looking skin from Ibizan Hound and control dogs.

Target genes	***Mean and standard deviations of transcripts***		
	Ibizan hounds (n = 9)		**Controls (n = 10)**
	LST positive reaction	*Normal-looking skin*	*Normal-looking skin*
TLR2	9.3±6.6	0.9±1.3	0.8±0.7
TLR4	122.8±125.8[a,i]	2.4±6.2[f,i]	1.6±1.8[a,f]
TLR7	0.5±1.4[b]	0.0±0.0[g]	13.9±14.4[b,g]
IL-10	10.7±4.3[c,j]	4.9±1.6[h,j]	0.5±0.6[c,h]
IFN-γ	279.8±216.2[d,k]	4.3±7.2[k]	1.5±2.2[d]
PD-L1	20.9±11.3[e,l]	4.8±3[l]	0.6±0.4[e]

[a]Mann-Whitney U-test, Z = -2.3242, p = 0.02; [b]Mann-Whitney U-test, Z = -3.59585, p = 0.0003; [c]Mann-Whitney U-test, Z = -3.17203, p = 0.001; [d]Mann-Whitney U-test, Z = -3.74211, p = 0.0002; [e]Mann-Whitney U-test, Z = -2.5727, p = 0.01; [f]Mann-Whitney U-test, Z = 2.96072, p = 0.003; [g]Mann-Whitney U-test, Z = -4.30255, p =< 0.00001; [h]Mann-Whitney U-test, Z =-2.5727, p = 0.01; [i]Wilcoxon Signed Ranks Test Z = -3.4078, p = 0.0006; [j]Wilcoxon Signed Ranks Test Z = -1.938, p = 0.052; [k]Wilcoxon Signed Ranks Test Z = -2.8961, p = 0.004; [l]Wilcoxon Signed Ranks Test Z = -2.0447, p = 0.04

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CHAPTER 9
DISCUSSION

Leishmaniosis is one canine infectious disease that probably has been the focus of many studies and doctoral thesis, especially in countries where this disease is endemic and of great clinic importance. One of the aspects that have driven major attention is the wide immunological spectrum observed in dogs with leishmaniosis, which similar to human beings, could dictate the wide range of clinical manifestations associated with this disease (Solano-Gallego et al., 2009; Scott and Novais, 2016).

As pointed out previously, cutaneous manifestations are the most common clinical presentation of CanL (Saridomichelakis and Koutinas, 2014) and several dermatological problems are diagnosed in dogs (Ordeix and Fondati, 2013). The spectrum of cutaneous manifestations ranges from a mild cutaneous presentation such as a persistent papular dermatitis that may self-heal (Ordeix et al., 2005; Bottero et al., 2006) to a more severe dermatological condition such as generalized exfoliative or ulcerative mucocutaneous dermatitis associated with systemic clinical manifestations and devastating without treatment (Ordeix and Fondati, 2013). Our understanding of how host immune response might modulate this clinical spectrum in dogs is limited. It is commonly stated that each presentation might reflect a different host–parasite relationship (Koutinas and Koutinas, 2014). However, evidence supporting this premise is scarce and mainly limited to one cutaneous manifestation of CanL, i.e papular dermatitis. In fact, information regarding this clinical condition is only based on case series with few dogs (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014). Larger studies with a cohort of dogs with papular dermatitis are lacking. In addition, there are not studies aimed to compare this dermatological manifestation of CanL with other cutaneous manifestations.

More than 10 years ago, when the current PhD student was performing the dermatology veterinary residency, together with the present thesis director and resident's mentors published for the first-time scientific evidence that papular dermatitis due to *L. infantum* infection was associated with a good clinical outcome and specific cell-mediated immune response (Ordeix et al., 2005). That was the begging of a story that, far from ending, has resulted in the execution of this doctoral thesis contributing to increase the knowledge of the immunological features that are at the basis for that premise. All the studies that form this doctoral thesis are aimed to demonstrate or corroborate clinical, pathological and immunological factors that make papular dermatitis a specific clinicopathological condition within the wide spectrum of clinical CanL. All those features, in fact, point out papular dermatitis as an entity associated with an immune response able to protect affected dogs against parasite proliferation and disease progression in comparison with dogs with more severe cutaneous manifestations.

New insights on the clinical aspects of papular dermatitis

Papular dermatitis is one cutaneous manifestation of CanL than in endemic areas is considered a typical form of the disease (Ordeix and Fondati, 2013). This clinical manifestation is rarely documented in non-endemic areas of CanL. In fact, it was not diagnosed in any dog in a retrospective study regarding dermatological presentations in 100 dogs with CanL performed in a non-endemic area for the disease (Perego et al., 2014). Although it is considered a relatively uncommon cutaneous manifestation of CanL, its exact prevalence has not been previously studied (Saridomichelakis and Koutinas, 2014).

Clinically, papular dermatitis due to *L. infantum* in dogs is very characteristic. Dogs examined in the various studies carried out in this doctoral thesis showed a papular dermatitis with very well-preserved clinical features (chapters 4, 5 and 7). These lesions were commonly presented as unique or multiple painless, persistent, erythematous papules, which may eventually coalesce into small plaques. They rapidly became ulcerated from their center and covered by a crust tightly adhered to the base. The sloping firm margins with a prominent central crater lent a "volcanic" appearance to the umbilicated ulcer, which is the most distinctive feature of this cutaneous manifestation of leishmaniosis in dogs (Ordeix and Fondati, 2013) and similar to localized cutaneous leishmaniosis in human beings (Malek et al., 2012; McGwire and Satoskar, 2014). See chapters 1 and 5 for clinical pictures. Rarely, this cutaneous manifestation is presented in combination with other cutaneous conditions secondary to leishmaniosis. However, dogs with leishmaniosis and papular dermatitis are considered mild affected dogs with stage I leishmaniosis only when this clinical problem is the unique clinical manifestation, present negative or low antibody levels, and absence of clinicopathological alterations (Solano-Gallego et al., 2009).

Similar to what has been reported previously, we consistently described papular lesions on sparsely haired skin such as the inner aspect of pinnae, bridge of the nose, lips, eyelids and abdominal skin of short haired-dogs (chapters 3, 4, 5 and 7) (Ordeix et al., 2005; Bottero et al., 2006). In human localized cutaneous leishmaniosis lesions appear at or near the site of sand fly bites (Malek et al., 2012; McGwire and Satoskar, 2014). These are usually found on uncovered areas of the body such as the face, forearms and lower legs. Therefore, it is suggested that, as in human beings, these lesions represent the site of parasite inoculation and multiplication. In fact, the results of the study performed by Aslan and collaborators supports our hypothesis (Aslan et al., 2016). In that study, Beagles dogs developed papular lesions on the site of multiple experimental vector bites three months after infection with *L. infantum* (Aslan et al., 2016). Characteristically, Ibizan hound dogs developed papular dermatitis only on the inner aspect of their pinnae (chapter 5). It is possible that the erected and hairless pinnae of these dogs, with a large contact surface, predisposed them

to a major contact with sand-flies and the development of papular dermatitis in that area. It is probable that the term "chancre", used in an experimental infection study in dogs to describe "leishmanial-like lesions" at the sites of injection of promastigotes was actually describing papular dermatitis (Killick-Kendrick et al., 1994). These lesions were typically about 1 cm, crusted, red and dry, with a swelling of the surrounding skin. They appeared 6-14 weeks after inoculation and healed spontaneously three to four months later (Killick-Kendrick et al., 1994). Unfortunately, that clinical description was not accompanied by any picture, however, the authors description closely resembled papular dermatitis.

Papular dermatitis has been more typically documented in young dogs (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2017). In that sense, the results obtained in the present studies have accurately confirmed those observations. Median age of dogs with papular dermatitis included in both descriptive studies was 12 and 10 months, and significantly lower than in dogs with more severe disease (chapters 3 and 4). Moreover, when prevalence of papular dermatitis was assessed in dogs living in a highly endemic area for *L. infantum* infection, dogs predisposed to develop this dermatological pattern were in fact young Ibizan hound dogs, usually less than one-year old (chapter 5). CanL is a chronic disease, and most dogs exhibit clinical signs only years after being in an area of endemicity (Oliva et al., 2006). Therefore, the association of papular dermatitis with a young age, suggest that this is a clinical problem observed after the first contacts with *Leishmania*. In fact, it has been demonstrated in Beagle dogs that a similar pattern of lesions is developed in the site of parasite inoculation 6-14 weeks after an experimental infection (Killick-Kendrick et al., 1994; Aslan et al., 2016).

Although to determine the time of onset of clinical lesions was not the objective of any of the studies that form this doctoral thesis, it was observed that papules appeared mainly in autumn or winter. A seasonality for this clinical condition has been previously suggested (Lombardo et al., 2014).

Finally, from a clinical point of view, it is interesting to remark that as previously published (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2017), dogs with papular dermatitis described in chapters 4, 5 and 6 did not present clinicopathological alterations indicative of disease severity at the time of diagnosis. Traditionally, CanL result in clinicopathological alterations in sick dogs. About 70-80% of diseased dogs present normochromic, normocytic, non-regenerative anemia, increased total protein and globulin (mainly beta- and gamma-globulins) concentrations, decreased albumin and albumin/globulin ratio (Noli and Saridomichelakis, 2014). In fact, dogs with moderate and severe disease included in the studies report in chapters 4 and 6 presented significantly higher values of total protein and beta- and gamma-globulins and lower values of

albumin/globulin ratio, hematocrit and hemoglobin. Therefore, this clinical particularity denotes control of disease progression in dogs affected with papular dermatitis as the sole clinical manifestation.

Papular dermatitis is more frequently observed in resistant dogs

Results from our studies strongly pointed out that papular dermatitis reflects a distinctive immune response, as suggested by initial case series (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014). Based on these thesis descriptive studies, the immunologic profile of dogs affected with this mild condition is compatible with a characteristic innate response and strong T cell-mediated response (chapters 5, 6 and 7). Moreover, the systemic immunological features observed in dogs with papular dermatitis in the present studies parallel those evidenced previously in Ibizan hound dogs, which rarely develop illness related with *L. infantum* infection and is considered to be an excellent canine breed model for resistance (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017b). Those systemic immunological features of Ibizan hound dogs common with dogs with papular dermatitis are:

- Lack or low humoral immune response (Solano-Gallego et al., 2000; Martínez-Orellana et al., 2017b)
- Strong cellular immune response determined by LST and IFN-γ production in stimulated blood (Solano-Gallego et al., 2000; Solano-Gallego et al., 2005; Martínez-Orellana et al., 2017b)

Therefore, we hypothesized that the prevalence of papular dermatitis should be superior in Ibizan hound dogs than in a group dogs of mixed breeds. This is the first study addressing the prevalence of one particular cutaneous manifestation of CanL in a determined population of dogs. We demonstrated that in a highly endemic area for *Leishmania* infection (80.6% rate of infection; chapter 5), papular dermatitis indicative of CanL was more frequently diagnosed in Ibizan hound dogs than in dogs belonging to other breeds (chapter 5). Unfortunately, one limitation of that study is that etiological diagnosis of papular dermatitis was addresses only in the 58% of Ibizan hound dogs whereas clinical diagnosis was made in the rest. As previously reported (Solano-Gallego et al., 2000; Solano-Gallego et al., 2005; Martínez-Orellana et al., 2017b), Ibizan hound dogs evaluated in our study were mostly seronegative, IFN-γ producers with a higher IFN-γ production than dogs from other breeds, and LST responders. Therefore, and as hypothesized, the immunologic background of resistant Ibizan hound dogs predisposes them to the development of papular dermatitis as the unique clinical sign of *Leishmania* infection.

Our findings in short:

- *Papular dermatitis has very well-preserved clinical characteristics being the "volcanic" appearance the most distinctive feature.*
- *Typically, papular dermatitis is distributed on sparsely haired skin in young dogs.*
- *Characteristically, papular dermatitis is not associated to clinicopathological abnormalities indicative of disease severity.*
- *Papular dermatitis indicative of CanL is more frequently diagnosed in Ibizan hound dogs than in dogs belonging to other breeds.*

Further investigations:

- *To develop a model of papular dermatitis caused by natural infection with L. infantum in order to evaluate the time of onset, appearance and evolution of clinical lesions as well as for performing further studies on the immunopathogenensis of CanL.*
- *To perform follow-up studies of a cohort of dogs with papular dermatitis and stage I leishmaniosis without treatment in order to determine the long-term outcome (progression of disease).*

New insights on the direct diagnosis of papular dermatitis

As stated above, clinically papular dermatitis is highly suggestive of CanL in an endemic area for this disease and very few other conditions could induce similar lesions. Differential diagnosis such as mosquito bite reactions or other granulomatous diseases, infectious (canine leproid granuloma) or sterile (pyogranuloma/granuloma syndrome or reactive cutaneous histiocytosis) should be considered individually (Ordeix and Fondati, 2013). Preferably, presumptive clinical diagnosis should be confirmed by additional diagnostic tests. Cytological examination is a simple, rapid, reliable and inexpensive method that directly may reveal *Leishmania* amastigotes in a mixed inflammation (mixture of neutrophils and macrophages, admixed with small lymphocytes, occasional intermediate-sized lymphocytes and/or plasma cells) (chapter 5; Lombardo et al., 2014). Interestingly, eosinophils were not observed in any of the cutaneous smears (n=17) obtained from dogs with papular dermatitis reported in chapter 5. However, the parasite may not be detected in all cases as reported in chapters 4 and 5. In fact, as discussed later, parasite load on clinically-lesioned skin of these dogs might be too low to be detected only by microscopical examination of stained smears. Therefore, immunologic and molecular biologic methods should be further used to determine amastigotes or its DNA on lesioned skin. Interestingly, we used qPCR methods on stained smears, which had been resulted negative to microscopical examination, with promising results (Lima et al., 2017). In our study, 81.8% of dogs with negative cytology presented a positive qPCR from stained smears (chapter 5). In other patients, microscopic examination of

routine stained skin biopsies with or without *Leishmania*-specific IHC may help in increasing sensibility. However, skin biopsies of papules distributed at difficult sites such as inner pinnae, lips, nose or eyelids may be challenging to perform.

In the present thesis, the histological presentation of papular dermatitis has been extensively investigated. Based on our results, it can be concluded that papular lesions present an homogeneous histological pattern. Papular dermatitis is more frequently characterized by a nodular to diffuse pyogranulomatous dermatitis and granuloma formation than other cutaneous manifestations of CanL such as ulcerative or exfoliative lesions in more diseased dogs (chapters 3 and 4). In human and experimental murine leishmaniosis, granuloma formation is the most important inflammatory process in the skin of subjects that control *Leishmania* infection (Lemos de Souza et al., 2000; Tuon et al., 2010). Moreover, granuloma formation with few parasites is also a common finding in self-limiting cutaneous lesions in horses (Solano-Gallego et al., 2003). This histological finding suggests an adequate Th1 adaptive immune response and an attempt of the immune system to eliminate parasites. Results obtained in the studies presented in chapters 3 and 4 agree with this premise. A trend of lower parasite load in skin lesions of dogs with papular dermatitis was found when compared with other cutaneous manifestations of CanL such as ulcerative or exfoliative lesions in more diseased dogs determined by means of *Leishmania*-specific IHC (chapter 3). This finding was later confirmed in the larger descriptive study by means of molecular analysis (chapter 4). Therefore, granuloma formation with low parasite load in clinically-lesioned skin of dogs with papular dermatitis, denotes control of parasite proliferation in this group of dogs with CanL. It is possible that in acute papules parasite load were higher than in chronic lesions, where a reduction in the number of parasitized macrophages concomitant with the appearance granulomas might preclude lesion resolution.

As reported previously (Solano-Gallego et al., 2004), we also demonstrated that normal-looking skin of dogs with leishmaniosis is frequently affected by microscopical lesions (56%) and harbors the parasite (72% by qPCR) (chapter 4). Interestingly, we originally found that normal-looking skin of dogs with papular dermatitis was less frequently inflamed (27.3%) and presented a lower parasite density, evaluated by means of qPCR, than normal-looking skin of more severe sick dogs (chapter 4). This is an interesting finding that, again, reinforce the idea that dogs with papular dermatitis and stage I leishmaniosis do not commonly show dissemination of the infection as has been suggested previously (Lombardo et al., 2014).

Our findings in short:

- *Cytologically, papular dermatits is characterized by a mixed inflammation without eosinophils with frequently absence of leishmanial amastigotes.*
- *Leishmania DNA is mainly detected by qPCR on stained smears when compared with cytological examination alone.*
- *Histologically, papular dermatitis is characterized by a nodular to diffuse pyogranulomatous inflammation and granuloma formation with low parasite load.*
- *Normal-looking skin of dogs with papular dermatitis is less frequently inflamed and present a lower parasite density than normal-looking skin of more severe sick dogs.*

Further investigations:

- *To develop a model of papular dermatitis caused by natural infection with L. infantum in order to evaluate the parasitological kinetics on papular lesions during follow up as well as other diagnostic parameters such as serology.*
- *To study parasite persistence on the skin previously afflicted after resolution of papular dermatitis.*

Systemic immune response in dogs with papular dermatitis

Systemic immune responses in dogs with papular dermatitis were evaluated previously in several case series and one descriptive study (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014). In those studies, negative or low antibody levels, evaluated by means of specific ELISA or IFAT (Ordeix et al., 2005; Bottero et al., 2006; Lombardo et al., 2014), and specific cell-mediated immune response, studied by means of LST (Ordeix et al., 2005; Lombardo et al., 2014) was commonly described. The present studies of this thesis have corroborated those observations (chapters 4, 5 and 6).

Humoral immune response

Dogs affected by papular dermatitis and stage I leishmaniosis are more frequently serologically negative than more severe dogs as demonstrated in the large descriptive study of this doctoral thesis (chapter 4). Moreover, dogs affected by this clinical mild condition have significantly lower levels of *Leishmania* antibodies than dogs with more severe cutaneous manifestations (chapter 4). Comparable results have been obtained in a study aimed to compare systemic immune response in dogs with different clinical stages of leishmaniosis (Montserrat-Sangrà et al., 2018). CanL is often associated with an exaggerated humoral immune response. Because *Leishmania* parasites are killed by IFNγ-activated macrophages and are not neutralized by antibodies, individuals with strong humoral response are unable to control the parasite load (Hosein et al., 2017). In fact, a marked

association was seen between antibody levels, tissue parasite density and the clinical status of the dogs (Reis et al., 2006). On the other hand, low antibody levels are characteristic of subclinical infections (Hosein et al., 2017). Therefore, lack or low humoral immune response in patients with papular dermatitis and stage I leishmaniosis may be indicative of a polarized specific cellular immune response, which might confer protection against disease progression.

T cell-mediated immune response

Classical clinical CanL is associated with the absence of DTH to *Leishmania* antigens, whereas a high proportion of *Leishmania* infected but healthy dogs present a positive LST reactions (Cardoso et al., 1998; Solano-Gallego et al., 2000). Dogs with papular dermatitis frequently show positive reactions to LST (chapters 5 and 7; Ordeix et al., 2005; Lombardo et al., 2014). We performed LST in 22 out of 31 Ibizan hound dogs with papular dermatitis. LST positive reactions were obtained in 20 out 22 dogs (chapter 5). Differences in the intensity of the Montenegro skin test reactions have been demonstrated among human patients with LCL and different treatment's response and among patients with different tegumentary disease severity (Nogueira et al., 2008; Maurer-Cecchini et al., 2009). Although, there is disagreement in the literature concerning the meaning of changes in the intensity of Montenegro skin test positive reactions in human beings, due to no standardized and reliable methodology to measure the intensity of the reaction, it is noteworthy that this assay may evaluate the degree of cell-mediated immunity also in dogs. Unfortunately, LST was not performed in the large descriptive studies presented in this doctoral thesis (chapters 4 and 6). Therefore, higher frequency and/or larger positive LST reactions in mild affected dogs compared with dogs with more severe disease could not be determined. Nonetheless, positive reactions in Ibizan hound dogs were larger than those obtained in clinically-healthy infected dogs belonging to different breeds (chapter 5). Moreover, in that study a slight agreement between LST and papular dermatitis was obtained (chapter 5).

Although LST is a reliable method to detect parasite-specific T cell-meditate immunity, it has been scarcely studied in the dog (Genaro et al., 1992; Tafuri et al., 1993; Sacchi et al., 2006). It has been suggested that Montenegro skin test positive reactions in human beings mimics the microscopic inflammatory reaction that occurs in leishmaniotic lesions (Guarín et al., 2006; Nogueira et al., 2008). Therefore, part of this doctoral thesis was aimed to better characterized LST positive reactions in dogs, particularly in resistant Ibizan hound dogs, in order to identify immunological similarities between this reaction and papular dermatitis in dogs with mild disease. We described immunohistological features similar to those observed in human beings and they were indicative of a DTH (chapter 7). Interestingly, and as in human reactions (Guarín et al., 2006), necrosis was observed, suggestive of a contribution of cytotoxic T cell mediated response (chapter

7). In human beings, granulomas are formed in many positive reactions from patients with ML, which is a clinical form associated to an exaggerated cell mediated immunity, whereas they are rarely seen in positive reactions from individuals with LCL (Nogueira et al., 2008). In our cases granulomatous reaction was not a histological feature of positive LST reactions, suggesting that cell-mediated immunity observed in resistant Ibizan hound dogs is rather protective than pathogenic.

Measurement of *L. infantum* specific IFN-γ production in stimulated blood is another method of assessment of T cell-mediated immunity in CanL (Solano-Gallego et al., 2016; Martínez-Orellana et al., 2017). It has been demonstrated that dogs with papular dermatitis and stage I leishmaniosis are more commonly IFN-γ producers than dogs with more severe disease (Montserrat-Sangrà et al., 2018). In addition, *Leishmania*-specific IFN-γ concentrations were superior in dogs with papular dermatitis than in dogs with clinically more severe cutaneous forms (Montserrat-Sangrà et al., 2018). Although not statistically significant, we observed a trend for a higher IFN-γ production in stimulated blood from dogs with papular dermatitis and mild clinical disease in comparison with dogs with more severe clinical disease (chapter 6). Discrepancy among our study and that of Montserrat-Sangrà and collaborators is probably due to a lower number of dogs included in chapter 6. Noteworthy, IFN-γ production in stimulated blood from dogs with papular dermatitis and mild clinical disease was similar to that detected in a control group of resistant Ibizan hound dogs (chapter 6). In fact, 24 out 29 Ibizan hound dogs with papular dermatitis in which this assay was performed, were identified as an IFN-γ producers. Moreover, in that study a slight agreement between IFN-γ production in stimulated blood and papular dermatitis was obtained (chapter 5).

Considering all together the aforementioned systemic immunological features (i.e low or absent anti-*Leishmania* antibody levels, positive response to LST and high *Leishmania*-specific IFN-γ production in blood), a T-cellular immunity is strongly advocated in dogs with papular dermatitis and stage I leishmaniosis.

Our findings in short:

- *Dogs with papular dermatitis display a low or absent humoral immune response in comparison with more severe dogs.*
- *Dogs with papular dermatitis show a cell-mediated immune response characterized by positive LST reactions and consistent parasite specific blood IFN-γ production.*
- *There is a slight agreement between LST positive reactions, IFN-γ production and papular dermatitis.*

Further investigations:

- *To evaluate if dogs with papular dermatitis which have spontaneously cure present a stronger immune response than clinically-healthy infected dogs.*
- *To assess if dogs with papular dermatitis present higher frequency and/or larger positive LST reactions compared with dogs with more severe disease.*
- *To evaluate if dogs with poor anti-Leishmania treatment response or clinical relapse show different systemic immunological parameters than dogs with favorable outcome.*
- *To investigate a possible exaggerated cell mediated immunity associated with pathology in CanL.*

Skin immune responses in dogs with CanL

As introduced early, immune response in CanL has been mainly investigated in peripheral blood mononuclear cells, lymph nodes, bone marrow and spleen and rarely in the skin of naturally or experimentally infected dogs (Hosein et al., 2017). In addition, research has been mainly focused on adaptive immune response and in particular in T-cell mediated immunity (Hosein et al., 2017). In addition, skin immune response has been mainly determined in normal-looking skin of naturally or experimentally infected dogs (Menezes-Souza et al. 2011; Hosein et al., 2015; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017). Therefore, this doctoral thesis fill considerably gaps of knowledge on local immune response and in particular innate immune response, in the skin of diseased dogs with CanL. Moreover, studies performed on dogs classified based on their clinical status and clinical staging are very limited (Solano-Gallego et al., 2009; Menezes-Souza et al., 2011). This doctoral thesis originally supports, for the first time, a distinctive skin immune response in dogs with mild skin disease in comparison with more severe dogs.

Dogs with papular dermatitis present a distinctive immune genes' expression in the skin

This is for sure the most thought-provoking finding of this doctoral thesis. So far, we have demonstrated the clinical and pathological consequences of a parasite-specific cell-mediated protective immune response in dogs with papular dermatitis and stage I leishmaniosis (chapters 3 and 4). Subsequently, we have demonstrated distinct systemic immune responses in these dogs compared with more severe affected dogs, indicative of a stronger *Leishmania*-specific cellular immunity (chapters 4, 5 and 6). Finally, our results on expression of immune genes in either clinically and normal-looking skin have demonstrated different skin or local immune response in dogs that correlates with their clinical status (chapter 6) (Table 9-1 and 9-2).

Table 9-1. Innate immune genes' expression in papular dermatitis in comparison with more severe diseased dogs.

Clinically-lesioned skin	***Normal-looking skin***
↑ *TLR2*	↓ TLR2*
↑ *TLR4**	= TLR4
↓ *TLR7*	↓ TLR7

* not statistically significant

Table 9-2. Adaptive immune genes' expression in papular dermatitis in comparison with more severe diseased dogs.

Clinically-lesioned skin	***Normal-looking skin***
↑ *IL-10*	↓ IL-10*
↑ *IFNγ**	↓ IFNγ
= *PD-L1*	↓ PD-L1

* not statistically significant

TLRs' expression has been determined in extra-cutaneous organs (blood, liver, spleen, intestine, brain or lymph node) or in normal-looking skin of naturally or experimentally *Leishmania*-infected dogs (Hosein et al., 2015; Montserrat-Sangrà et al., 2016; Pereira-Fonseca et al., 2017; Grano et al., 2018). Therefore, ours studies report for the first-time information on TLRs genes expression in clinically-lesioned skin (chapters 3 and 6). We have observed and increase on TLR2 expression in clinically-lesioned skin when compared with healthy skin of non-infected dogs by means of IHC (chapter 3), which was later confirmed by qPCR (chapter 6). This was not an unexpected finding, as TLR2 overexpression seems to be associated with disease severity and progression in dogs in other tissues (Figueiredo et al., 2013; Melo et al., 2014; Montserrat-Sangrà et al., 2016). Although a lower expression of TLR2 in clinically-lesioned skin of dogs with papular dermatitis than in the skin of more severe affected dogs was initially suggested (chapter 3), our larger descriptive study using molecular analysis, a more accurate and sensitive technique than IHC, clearly contradicts that result (chapter 6). Initial findings were questioned as TLR2 upregulation was, in fact, expected in papular lesions, which were more macrophagic in nature and less lympho-

plasmocytic (chapters 3 and 4). This discrepancy may be justified by the low number of cases included in the study described in chapter 3 Moreover, in that study, cases were retrospectively selected only on the basis of type of cutaneous lesion present and staging of their leishmaniosis was not properly assessed. TLR2 overexpression has been associated with disease severity and progression in dogs (Figueiredo et al., 2013; Melo et al., 2014; Montserrat-Sangrà et al., 2016). However, those studies did not compare among different clinical stages. According with our results, we also demonstrated a negative correlation among TLR2 gene expression in clinically-lesioned skin of diseased dogs and total protein and specific *L. infantum* antibodies (chapter 6). Noteworthy, our results partially disagree with those published previously by Montserrat-Sangrà and collaborators who described a lower TLR2 gene expression in non-stimulated blood from dogs with stage I leishmaniosis than that observed in dogs with more severe disease (Montserrat-Sangrà et al., 2018). That difference was not statistically significant. Moreover, different gene expression of the TLRs may vary according to the organ compartment evaluated (Melo et al., 2014; Grano et al., 2018). However, according with our observations, varied TLR2 gene expression has been documented in different clinical presentations of tegumentary leishmaniasis in human beings (Campos et al., 2018). Mild forms of the disease (i.e LCL and borderline DCL) cause by *L. braziliensis* presented higher TLR2 expression than the severe form ML (Campos et al., 2018).

We detected a lack of TLR2 over expression in normal-looking skin of dogs with CanL when compared with healthy skin of non-infected dogs (chapter 6). It would seem that our results disagree with those previously published also in normal-looking skin (Hosein et al., 2015; Pereira-Fonseca et al., 2017). Hosein and collaborators demonstrated and upregulation of TLR2 up to 6 months after a susceptible experimental infection, with posterior downregulation (Hosein et al., 2015). Whereas, upregulation of TLR2 gene was evidenced in normal-looking skin of dogs naturally affected by CanL (Pereira-Fonseca et al., 2017). However, only more severe affected dogs showed an overexpression of TLR2 in normal-looking skin, and, although not statistically significant, dogs with papular dermatitis showed lower TLR2 gene expression than more severe diseased dogs (chapter 6). This difference may be related to the increased frequency of microscopic inflammatory lesions and higher parasite load in normal-looking skin of more severe than in mild affected dogs (chapter 4). Accordingly, in normal-looking skin, TLR2 expression was associated with clinicopathological parameters indicative of disease severity (chapter 6). Taken all these findings together, it would seem that in more severe affected dogs there is a progressive TLR2 downregulation from earlier stages of inflammation to more chronic dermatitis. This reflection agrees with the observation of Hosein and collaborators who described an upregulation of TLR2

in the skin only in the earlier stages of an experimental infection when compared with the controls (Hosein et al., 2015).

A significant upregulation of TLR4 was observed in diseased skin (chapter 6). This receptor has been scarcely studied up to now in CanL in several tissues (Melo et al., 2014; Hosein et al., 2015; Montserrat-Sangrà et al., 2016; Grano et al., 2018), but, to the best knowledge of the authors, never in lesioned skin of dogs with leishmaniosis. Diverse results have been revealed according to the tissue assessed. However, it would seem that most of previously published data indicate an upregulation of TLR4 in several tissues (Melo et al., 2014; Montserrat-Sangrà et al., 2016; Grano et al., 2018). Although not significant, a higher TLR4 transcription in clinically-lesioned skin of dogs with papular dermatitis than in more severe affected dogs was observed. Moreover, a negative correlation among this transcript and γ globulins was demonstrated for the first time, suggesting it overexpression with less severity (chapter 6). Also, an organ compartmentalization of TLR4 gene expression could be possible as no differences were found in TLR4 relative quantification in non-stimulated blood between mild and more severe affected dogs (Montserrat-Sangrà et al., 2018). Nonetheless, and similar to TLR2, milder forms of human cutaneous leishmaniasis (i.e LCL and borderline DCL) due to *L. braziliensis* are associated with higher expression of TLR4 (Campos et al., 2018).

In agreement with that previously published (Hosein et al., 2015), gene expression of TLR4 in normal-looking skin of diseased dogs were not changed when compared with healthy skin of non-infected dogs (chapter 6). In addition, there were no differences among its expression in normal-looking skin of dogs with different disease severity, which was unexpected given the increased frequency of microscopic inflammatory lesions and higher parasite load in normal-looking skin of more severe affected dogs (chapter 4). All these findings together, might suggest that TLR4 does not play an important role in the early stages of the inflammatory process and that, with disease progression, upregulation of TLR4 would help in controlling parasite proliferation. In fact, TLR4 polymorphisms have been associated with susceptibility to cutaneous leishmaniasis (Ajdary et al., 2011; Ziakas et al., 2013).

A trend for upregulation of TLR7 was observed in diseased skin, whereas its expression was not changed in normal-looking skin of diseased dogs when compared with healthy skin of non-infected dogs (chapter 6). Information regarding TLR7 expression on tissues of dogs with CanL is scarce (Grano et al., 2018) and the role of this receptor has not been anticipated. TLR7 gene expression was significantly lower in both clinically-lesioned and normal-looking skin of dogs with papular dermatitis than in more severe diseased dogs (chapter 6). Moreover, TLR7 overexpression in normal-looking skin was associated with altered parameters suggestive of disease

severity (chapter 6). Based on our results, we suggest for the first time a pathogenic role of this innate receptor in CanL. In fact, there are recent evidences that associated TLR7 activation with disease exacerbation of VL due to *L. donovani* in mice (Silva-Barrios et al., 2016; Fabié et al., 2018). Endosomal TLR7 activation in B cells by *L. donovani* is responsible for disease exacerbation through IL-10 and IFN-I production and for the promotion of hypergammaglobulinemia (Silva-Barrios et al., 2016). Moreover, local tissue damage mediated by persistent inflammation leads to suppression of protective T cell responses during chronic visceral leishmaniosis due to *L. donovani* in mice via signaling of TLR7 by apoptotic cell material, which induces the upregulation and activation of interferon regulatory factor-5 (IRF-5) resulting in upregulation of death receptor 5 and caspase 8 and cell death of protective CD4 T cells (Fabié et al., 2018).

Our results further support a concomitant expression of mixed cytokines (pro-inflammatory and regulatory) in either clinically and normal-looking skin of dogs with leishmaniosis (Brachelente et al., 2005; Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016). Therefore, it would seem that an absolute polarized profile is not necessary to incline the immune system toward either a pathogenic or protective response.

As stated above, studies performed regarding cytokines on clinically-lesioned skin are very limited (Brachelente et al., 2005). Noteworthy, IL-10 gene expression was studied in clinically-lesioned skin for the first time (chapter 6). As expected, it was increased, however, even if it is an immunoregulatory cytokine, we found a positive relationship of this cytokine expression with parameters associated to disease control (chapter 6). IL-10 has multiple roles in immunopathology (Day, 2011). It is an immunosuppressive cytokine produced by different T-cells subsets, those promoting cell-mediated immunity, humoral immunity and suppressive response. Therefore, the roles of IL-10 in CanL remain uncertain as well as if this cytokine can be a marker of disease susceptibility as reported in mice and humans (Belkaid et al., 2002; Nylén et al., 2007). In fact, we found significant higher IL-10 gene expression in papular dermatitis than in the skin lesions of more severe affected dogs (chapter 6). Therefore, it seems that IL-10 is not a marker of disease severity at least in clinically-lesioned skin as previously observed in IFN-γ whole blood release assays (WBA) (Rodríguez-Cortés et al., 2016; Solano-Gallego et al., 2016). In one study performed in WBA in naturally diseased dogs, IFN-γ producers dogs secreted higher levels of IL-10 in LSA stimulated blood when compared with IFN-γ non-producers (Solano-Gallego et al., 2016). However, polysymptomatic diseased, naturally infected dogs presented an increased IL-10 production by T lymphocytes from blood along with increased blood parasite burden (Boggiatto et al., 2010). As previously described (Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017), we detected an upregulation of IL-10 in normal-looking skin of dogs

with moderate leishmaniosis (chapter 6). Moreover, in those studies, and in agreement with the present findings (chapter 6), a correlation with parasite density was concluded in normal-looking skin (Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016; Pereira-Fonseca et al., 2017). In addition, we found an association with other parameters related to disease severity (chapter 6). Interestingly, we detected a lower relative quantification of IL-10 gene in normal-looking skin of mild affected dogs (chapter 6). However, this difference was not statistically significant. This may be the consequence of a lower inflammatory process present in those dogs when compared with more severe affected dogs (chapter 4). It has been demonstrated that experimentally infected dogs develop a sharp increase of IL-10 gene expression in normal-looking skin only 6 months post-infection (Rodríguez-Cortés et al., 2016). Therefore, low IL-10 response in normal-looking skin of mild affected dogs might be also the consequence of an early inflammatory stage post infection, as suggested also for the young age of these patients (chapters 3, 4 and 5). Taken all results about IL-10 gene expression on skin of dogs with different disease severities together, it would seem that IL-10 has a dual pattern of expression in CanL, at least on the skin. Higher levels of IL-10 gene expression would be an immunologic parameter marker of disease severity in normal-looking skin whereas in clinically-lesioned skin not.

As published previously, we found an increase in IFN-γ gene quantification in clinically-lesioned skin of diseased dogs (Brachelente et al., 2005). Although not statistically significant, higher IFN-γ gene expression was observed in clinically-lesioned skin of mild affected dogs when compared with more severe affected dogs. IFN-γ is a protective Th-1 associated cytokine, which increases the leishmanicidal activity of macrophages (Papadogiannakis and Koutinas, 2015; Hosein et al., 2017). Therefore, it is plausible that overexpression of this pro-inflammatory cytokine in mild affected cases may be the result of more granuloma formation in papular lesions with consequent lower parasite density as demonstrated by our studies (chapters 3 and 4). IFN-γ gene expression in normal-looking skin of dogs with leishmaniosis found in our study agrees with that previously observed (chapter 6; Menezes-Souza et al., 2011; Rodríguez-Cortés et al., 2016). Relative quantification of IFN-γ in normal-looking skin from mild affected dogs was significantly lower than in normal-looking skin from more diseased dogs. The lower inflammation observed microscopically in normal-looking skin from mild affected dogs may account for this finding (chapter 4). This result is in line with the results of a previous study on normal-looking skin from natural infected dogs that demonstrated increased IFN-γ expression in symptomatic dogs in comparison with asymptomatic dogs (Menezes-Souza et al., 2011). High expression of IFN-γ in normal-looking skin was associated with pathological findings indicative of disease severity, high specific antibody levels and high parasite density. Our results are in accordance with the

observations of Rodríguez-Cortés and collaborators that reported a positive correlation in the normal-looking skin among IFN-γ, parasite load and antibody levels (Rodríguez-Cortés et al., 2016). Therefore, this pro-inflammatory environment observed in normal-looking skin of more severe affected dogs is not enough to confer protection, as has already been suggested (Rodríguez-Cortés et al., 2016).

For the first time, we reported an increase of PD-L1 in clinically-lesioned and normal-looking skin of dogs with leishmaniosis (chapter 6). This protein is related to decreased T-cell mediated immunity due to T-cell exhaustion via its union with PD-1 on T-cells surface (Esch et al., 2013). As suggested in human leishmaniasis, expression of PD-L1 might represent a mechanism that parasites exploit to avoid the immune response (Barroso et al., 2018). However, similar expression in clinically-lesioned skin was observed among different disease stages (chapter 6). This was an unexpected finding as higher T-cell apoptosis was hypothesized in clinically-lesioned skin of more severe affected dogs. It would be interesting to evaluate if a large number of studied dogs would change this finding. On the other hand, it is possible that factors other than PD-L1 exist as a cause of suppression of Th1 cell effector function as already previously suggested (Silva et al., 2013; Perosso et al., 2014). On the other hand, a lower PD-L1 gene expression was determined in normal-looking skin from dogs with papular dermatitis than in more severe affected dogs (chapter 6). This was an expected finding, considered the lower inflammatory process observed in normal-looking skin of mild affected dogs (chapter 4). In accordance, positive correlations of this transcript with clinicopathological parameters associated with disease severity, antibody levels and parasite density were detected in normal-looking skin (chapter 6). Therefore, we point out PD-L1 as an immunological marker for disease severity only in normal-looking skin.

Skin immune response in dogs with papular dermatitis is similar to LST positive reactions in resistant dogs

As mentioned previously (subsection *T cell-mediated immune response* from this discussion section), Montenegro skin test positive reactions in human beings have been advocated as surrogate of the early response to infection (Guarín et al., 2006). In fact, in a comparative histopathologic study of biopsies of lesions of cutaneous leishmaniasis with differing time of evolution and their respective Montenegro skin test, the proportions of the different cell populations were similar only in acute lesions (≤ 1 month) and their Montenegro skin testing. Therefore, if cutaneous lesions in leishmaniosis and the Montenegro skin test share similar cellular responses, the skin test could be a surrogate of acute cutaneous lesions for the study of the *in situ* events occurring during the early stages of cutaneous leishmaniasis in human beings (Guarín et al., 2006). Consequently, we

investigated this possibility also in CanL. In other words, could LST positive reaction be a surrogate of acute cutaneous lesions in dogs?

Cutaneous lesions in CanL have a variable duration, and, to the best of the author knowledge, comparative studies regarding immunohistological features of acute and chronic lesions in dogs naturally infected are lacking. Papular dermatitis is one clinical manifestation classically diagnosed in young animals, generally under one year of age. It is likely hat this is a clinical problem observed after the first contacts with *L. infantum* in an immunocompetent dog. Moreover, similar lesions develop at the site of parasite inoculation 6-14 weeks after an experimental infection in canines (Killick-Kendrick et al., 1994; Aslan et al., 2016). Therefore, we hypothesised that local immune response in papular lesions in dogs with stage I leishmaniosis might be similar to that observed in LST positive reactions from resistant dogs such as the Ibizan hounds.

Description of the histological characteristics of the LST in Ibizan hound dogs showed that tissue response in both LST positive reactions and papular lesions were different (chapters 4 and 7). In fact, granuloma formation, which characterised papular dermatitis, was lacking in LST positive reactions (chapter 7). Nonetheless, immune genes' expression profile observed in LST positive reaction was comparable with that observed in papular lesions (chapters 6 and 8). In fact, when these two groups were compared with normal-looking skin from the same group of control dogs, both showed increase transcription of TLR4, IFN-γ and PD-L1 and lower expression of TLR7. Although TLR2 was not significantly increased in LST positive reactions, this transcript was increased as described in papular dermatitis. Finally, IL-10 was significantly upregulated in LST positive reactions, whereas it was a trend for increased IL-10 transcripts in papular dermatitis. Therefore, it would seem that initial events of the immune response to *Leishmania* are an overexpression of innate receptors (TLR2 and TLR4) probably secondary to the inflammatory process. The lack of a granulomatous inflammatory process would account for the lack of a significant increase of TLR2 in LST positive reactions when compared with healthy skin of control dogs. Interestingly, and as observed for papular dermatitis, LST positive reactions were associated with a lower expression of TLR7 when compared with healthy skin of control dogs. TLR7 has been associated with exacerbation of VL in a mice model of *L. donovani* infection, as previously discussed (Silva-Barrios et al., 2016; Fabié et al., 2018). Moreover, an association of TLR7 with disease severity in CanL was demonstrated in chapter 6. LST positive reactions showed a mixed pattern of Th1/Th2 cytokines as demonstrated in clinically-lesioned skin. Interestingly, it would seem that in the initial events of the immune response there is an increased expression of IL-10. This cytokine is a regulatory cytokine that modulates immunopathology and tissue damage

following increased production of inflammatory cytokines, especially IFN-γ as observed in LST positive reactions (Maspi et al., 2016).

On the other hand, normal-looking skin of dogs with papular dermatitis and stage I leishmaniosis and normal-looking skin of resistant Ibizan hound dogs showed different pattern of immune genes' expression in comparison with normal skin of control dogs (chapter 6). Interestingly, while dogs with papular dermatitis showed only a lower expression of TLR7 in comparison with normal skin of control dogs, Ibizan hounds presented significantly higher TLR4 and IL-10 transcripts and lower TLR7 transcript than healthy skin from control dogs. Moreover, a trend for higher upregulation of IFN-γ and PD-L1 was observed in normal-looking skin of Ibizan hound dogs. Unfortunately, histological examination of normal-looking skin of Ibizan hound was not performed. Therefore, it was not possible to associate or not the immune genes' expressions to an already present microscopic inflammation secondary to *Leishmania* infection in a normal-looking skin (chapter 4). However, parasite density in normal-looking skin from both groups was similar (chapter 6). Therefore, a major transcription of immune genes secondary to a major presence of parasite would be improbable. Moreover, although skin samples were from sites of normal appearing skin, other vector borne diseases and ectoparasitosis, such as fleas, ticks or flies,that could activate the immune system were not excluded in Ibizan hound dogs (Baxarias et al., 2018).

Our findings in short:

- *Dogs with papular dermatitis present a distinctive innate immune genes' expression in clinically-lesioned skin in comparison with more severe dogs characterized by increased TLR2 and TLR4 expression and decreased TLR7 expression.*
- *Dogs with papular dermatitis present a distinctive adaptive immune genes' expression in clinically-lesioned skin in comparison with more severe dogs characterized by increased IL-10 and IFN-γ expression.*
- *Dogs with papular dermatitis present a distinctive innate immune genes' expression in normal-looking skin in comparison with more severe dogs characterized by decreased TLR2 and TLR7 expression.*
- *Dogs with papular dermatitis present a distinctive adaptive immune genes' expression in normal-looking skin in comparison with more severe dogs characterized by decreased IL-10, IFN-γ and PD-L1 expression.*
- *TLR7 upregulation in the skin is associated with disease severity.*

- *IL-10 and PD-L1 appear to be an immunological marker for disease severity only in normal-looking skin.*
- *Skin immune response in clinically-lesioned skin of dogs with papular dermatitis is similar to LST positive reactions in resistant dogs.*

Further investigations:

- *To evaluate if dogs with poor anti-Leishmania treatment response or clinical relapse show different skin immunological parameters than dogs with favorable outcome.*
- *To study the role of IRF-5 and interferons type I in CanL.*
- *To further assess the role of PD-1 and its ligands as well as other mechanisms of apoptosis in CanL.*
- *To investigate the use of PD-1 blockade in clinical severe disease in CanL.*

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CONCLUSIONS

1. Papular dermatitis as stage I leishmaniosis present an homogeneous clinical pattern characterized by papules, with the "volcanic" appearance, on sparsely haired skin in young dogs in the absence of other clinicopathological abnormalities.
2. Papular dermatitis as stage I leishmaniosis appears to be a frequent cutaneous manifestation encountered in young Ibizan hounds.
3. Papular dermatitis as stage I leishmaniosis appears to present a characteristic histological pattern when compared with more severe clinical manifestations.
    a. It is characterized by a nodular to diffuse pyogranulomatous dermatitis with granuloma formation and low parasite density.
    b. Perivascular to interstitial lymphoplasmacytic dermatitis characterized lesioned skin of more severe afflicted dogs presenting exfoliative or ulcerative dermatitis.
4. Normal-looking skin of dogs with papular dermatitis and stage I leishmaniosis is less frequently inflamed and have lower parasite density than dogs with more severe disease.
5. Dogs with papular dermatitis and stage I leishmaniosis present a distinctive adaptive immune response characterized by lower or absent humoral immune response, positive reactions to LST and more frequent and higher production of IFN-γ in blood than dogs with more severe disease.
6. Dogs with papular dermatitis and stage I leishmaniosis present a distinctive innate and adaptive immune response in clinically-lesioned and normal-looking skin when compare with dogs with more severe disease.
7. LST positive reactions from Ibizan hounds have similar local immune response to papular lesions.