Universitat Autònoma de Barcelona

VITAMIN D AND CANINE LEISHMANIASIS

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VITAMIN D AND CANINE LEISHMANIASIS

by Clara Martori Muntsant

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In witness whereof, we hereby sign this document,

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Fem constar que la tesis titulada "Vitamin D and canine Leishmaniasis" presentada per la **CLARA MARTORI MUNTSANT** per optar al Grau de Doctor ha estat realitzada sota la nostra supervisió i autoritzem la seva presentació per realitzar la seva defensa oral.

I, perquè així consti, signem aquest document,

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PhD, MSc, DVM, BSc

Doctoral Advisor / Director de tesi

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DVM, PhD

Doctoral Advisor / Directora de tesis

Als meus pares,

A l'Aida

Un somni estrany

En un somni estrany
el món era un lloc millor

Ningú plorava mai d'impotència allà
Tothom tenia una opció
i no hi havia mala fe enlloc
I amb cultura per a tothom
s'havien acabat
els problemes per gènere,
origen o orientació
i ja cadascú podia creure
i estimar i ser o sentir-se el que volgués
i els diners i el poder i la violència
no eren mai l'objectiu enlloc
era preciós·

Col·laborant, no competint
i la natura sagrada
tothom cuidava el seu entorn
i a ningú li tocava
tenir fred, gana o por
i era un món preciós
i era de totxs·

I allà les úniques tristeses
eren per la mort i l'amor
Allà les úniques tristeses
eren per l'amor i la mort

En un somni estrany
el món era un lloc millor
molt millor·

Pau Vallvé

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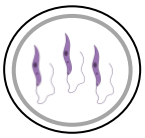
ACRONYMS

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3 (also known as 1,25-dihidroxicolecalcifer or calcitriol)
25(OH)D	25-hydroxyvitamin D
$25(OH)D_3$	25-hydroxyvitamin D_3 (also known as 25-hidroxycholecalciferol, calcifediol or calcidiol)
Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
AmB	Amphotericin B
APCs	Antigen presenting cells
CAMP	Cathelicidin antimicrobial peptide
CanL	Canine leishmaniasis
CBD	Canine β-defensin
CL	Cutaneous leishmaniosis
ConA	Concanavalin A
CpG	Cytosine-phosphate-guanosine
CTLA	Crude Total *Leishmania* Antigen
CYP24A1	1,25-hydroxyvitamin D_3 24-hydroxylase
CYP27B1	25-hydroxyvitamin D_3 1-α-hydroxylase
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	ELISA units
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
Ig	Immunoglobulin

IL	Interleukin
IM	Intramuscular
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal
KMPII	Kinetoplastid Membrane Protein-11
LACK	*Leishmania* homologue of receptors for activated C kinase
LSA	*Leishmania* soluble antigen
LST	*Leishmanin* skin test
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
NK	Natural killer cells
NO	Nitric oxide
NP	Nanoparticle
OD	Optical density
PAPLE22	Potentially Aggravating Protein of *Leishmania infantum*
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSS	Physiological salt solution
qPCR	Quantitative polymerase chain reaction
r	Recombinant
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription PCR
SNP	Single nucleotide polymorphism
Th	T *helper* lymphocyte
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

Treg	Regulatory T cells
TRYP	Tryparedoxin peroxidase
VDR	Vitamin D Receptor
VitD	Vitamin D
VL	Visceral leishmaniasis
WHO	World Health Organization
ZVL	Zoonotic visceral leishmaniasis



ABSTRACT

Leishmaniasis are a group of neglected vector-borne diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania*. The disease is considered endemic in tropical and subtropical regions of the Palearctic and Neotropic ecozones, and in ecoregions around the Mediterranean Basin. Human visceral leishmaniasis (VL) can be fatal if left untreated, resulting in 26 000–65 000 deaths per year. Canids are the main reservoir and hosts of *L. infantum*, the causative agent of zoonotic VL in the Mediterranean Basin. Only in western Mediterranean countries, there is an estimate of 2.5 million infected dogs. The mechanisms that regulate the outcome of the infection are undisclosed, although it is well known that immune system plays a key role in leishmaniasis disease control. Several studies have shown that vitamin D plays an important role on immune response. Furthermore, the relationship between vitamin D deficiency and the risk of suffering from a plethora of health disorders, including infectious diseases has been described. Considering all this, we aimed to study if vitamin D have a relevant contribution in canine leishmaniasis (CanL).

In the first study, we measured vitamin D concentration in serum samples from a cohort of healthy and ill dogs from a highly endemic area and we have also studied the relationship of vitamin D concentration with parasitological and immunological parameters. The sick dogs presented significantly lower ($P < 0.001$) vitamin D levels (19.6 ng/mL) than their non-infected (31.8 ng/mL) and the asymptomatic (29.6 ng/mL) counterparts. In addition, vitamin D deficiency correlated with several parameters linked to leishmaniasis progression: clinico-pathological score, serology, and parasite burden in blood. However, there was no correlation between vitamin D levels and the *Leishmania*-specific cellular immune response. Moreover, both the leishmanin skin test and the IFN-γ levels displayed negative correlations with serological, parasitological and clinical signs. We also aimed to investigate whether genetic variation within the vitamin D receptor (VDR) gene locus is associated with the progression of CanL, but the allelic frequencies of the four single nucleotide polymorphisms (SNPs) found were not statistically different between groups. Nevertheless, if vitamin D deficiency is a risk factor to develop clinical leishmaniasis remained to be answered. It was also unknown if vitamin D participates in *Leishmania* control.

Afterwards, in the second study, we analysed retrospectively vitamin D concentration in serum samples from a cohort of healthy dogs collected in different periods of the year. The results showed that there is not a seasonal variation of vitamin D concentration in dogs. We also included dogs with clinical leishmaniasis and non-infected healthy dogs, in which we measured vitamin D levels at the beginning of the study, when all dogs were negative for serology and qPCR, and 1 year later. Whereas non-infected dogs showed no changes in vitamin D levels

along the study, those developing clinical leishmaniasis showed a significant vitamin D reduction at the end of the study (35.4 %). When we compared vitamin D concentration between the two groups at the beginning of the study, no differences were detected (43.6 [38–59] ng/mL, *P* = 0.962). Therefore, our results showed that vitamin D concentration is not a risk factor for developing canine leishmaniasis, but it diminishes with the onset of clinical disease. An *in vitro* model using a canine macrophage cell line proved that adding active vitamin D ($1,25(OH)_2D_3$) leads to a significant reduction in *L. infantum* load (31.4 %). Analyzing expression of genes related to vitamin D pathway on primary canine monocytes, we showed that *CBD103* expression was significantly enhanced after active vitamin D addition. The *in vitro* results corroborated the hypothesis that vitamin D plays a role in parasitic control and pointed out that it regulates infection through *CBD103* expression. These results opened the possibility for studies testing vitamin D as an adjuvant in leishmaniasis therapy or prophylaxis.

In the last chapter of this work, we studied the suitability of vitamin D as an adjuvant to enhance the effect of a prophylactic DNA vaccine against VL. Vitamin D is known for playing an immunomodulatory role by activating innate immune system and modulating the adaptive immune response. In our study, BALB/c mice were treated with vitamin D concomitantly with a DNA vaccine consisting in four plasmids carrying the *Leishmania* genes *LACK*, *TRYP*, *PAPLE22* and *KMPII* encapsulated in liposomes. Two weeks after vaccination, the animals were infected intraperitoneally with *L. infantum* parasites. Parasite load was measured in target tissues and immune response was evaluated before challenge and six weeks post-infection by determining anti-*Leishmania* specific antibodies in combination with cytokine expression analysis and determining percentage of $CD4^+$ and $CD8^+$ T cells. The results showed that our DNA vaccine did not significantly reduce parasite load in liver nor spleen, but vitamin D coadministration showed a tendency to diminish parasite load in target organs. The study of cell response in splenocytes suggested that higher levels of $CD4^+$ and $CD8^+$ T cells may be responsible for the partial protection mediated by the DNA vaccine with vitamin D as enhancer.

INTRODUCTION

1. LEISHMANIASIS IMPORTANCE

Leishmaniases are a group of neglected disease caused by protozoan parasites of the genus *Leishmania*, transmitted by infected female sandflies. Leishmaniases are the third most important human disease among those transmitted by vectors, after malaria and lymphatic filariasis. It is one of the main infectious diseases affecting the poorest population, people living mainly in rural and suburban areas. They are a major, although grossly underestimated, health problems with over 350 million people being at risk in 88 endemic countries in tropical and subtropical biogeographic zones. WHO estimates 700 000–1 million new cases occur each year, of which between 50 000 and 90 000 are of visceral leishmaniasis (VL), the most serious and frequently fatal form if left untreated. Annual mortality from leishmaniases is estimated at between 26 000 and 65 000 deaths (World Health Organization, 2020). However, the actual incidence of the disease could be even higher, as only one third of affected countries are required to officially report cases of leishmaniasis, so a substantial portion of cases are never recorded (Hotez et al., 2008).

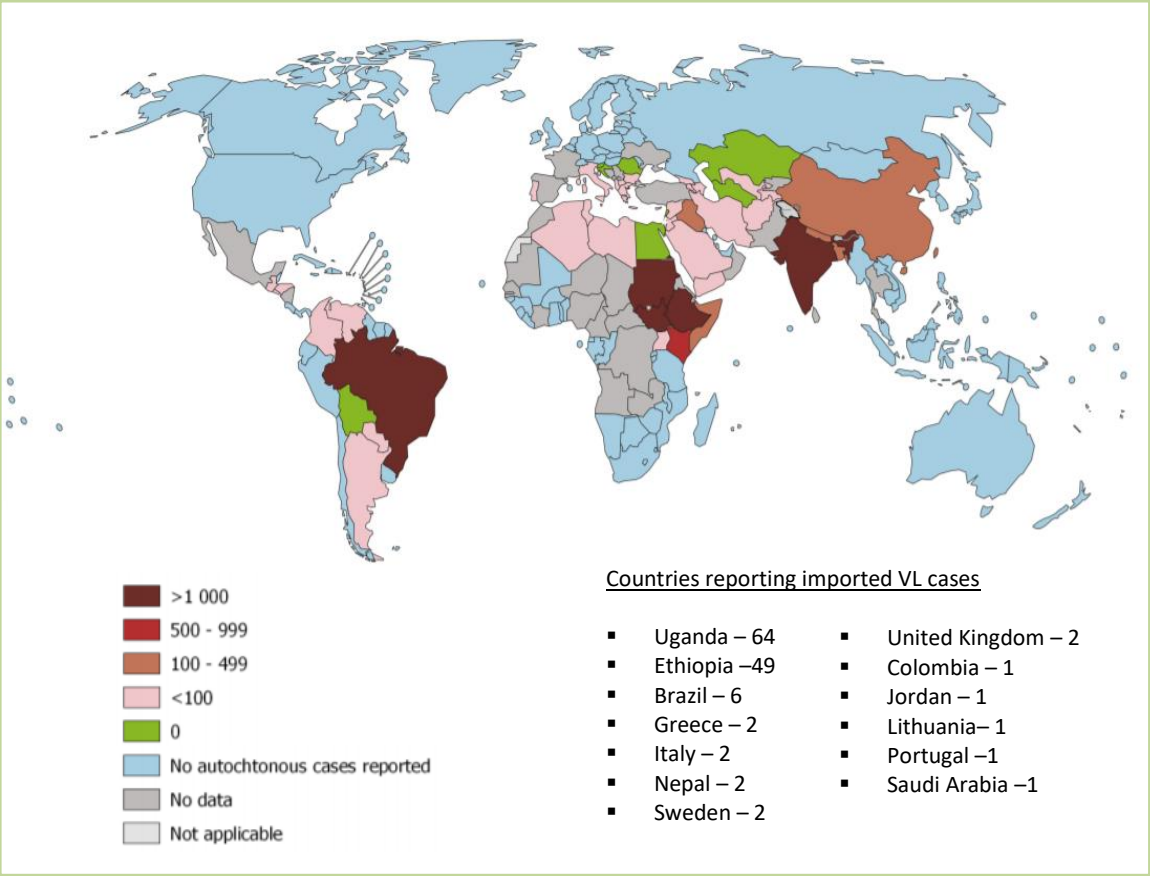


Figure 1. Status of endemicity of visceral leishmaniasis worldwide (2018). Figure adapted from World Health Organization (2020).

95 % of new cases of cutaneous leishmaniasis occur in the Americas, the Mediterranean Basin, the Middle East and Central Asia. Instead, most cases of visceral leishmaniasis occur in Brazil, East Africa and in India. More than 95 % of VL new cases reported to WHO occurred in 10 countries: Bangladesh, Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, Sudan and South Sudan (World Health Organization, 2020).

The disease effects of the poor populations on earth. Since, factors such as poor housing conditions and malnutrition are associated with an increased risk of leishmaniasis. Lack of waste management or open sewerage may increase sandflies breeding and resting sites, as well as their access to humans, while diets lacking protein-energy, iron, vitamin A and zinc increase the risk that an infection will progress to a full-blown disease (Alvar et al., 2006a). Other important risk factors are environmental changes —such as deforestation or dam construction—and climate change (World Health Organization, 2017). For example, small fluctuation in temperature could have profound effect on the developmental cycle of *Leishmania* promastigotes in sandflies, allowing transmission of the parasite in areas not previously endemic for the disease (González et al., 2010).

Special consideration should be given to people co-infected with *Leishmania* and HIV, as in these patients both pathogens are mutually reinforcing: while HIV promotes the ineffectiveness of parasite treatment, *Leishmania* accelerates the onset of AIDS (Alvar et al., 2008). Antiretroviral treatment reduces the progression of the disease, delays relapses and increases the survival of coinfected patients. However, high rates of *Leishmania*-HIV coinfection are still reported from Brazil, Ethiopia and the state of Bihar in India (World Health Organization, 2020) where it could be determined that people who have been infected with *Leishmania* and HIV have much higher rates of relapse and mortality with respect to patients without HIV infection (Burza et al., 2014).

To the spread of the parasite caused by climate change, migration and globalization, we must add the high incidence of concomitant infections, the ineffectiveness of treatments, the emergence of drug resistance and the absence of a vaccine. The combination of all these factors lead to the number of patients with leishmaniasis continues to increase alarmingly (Ghorbani and Farhoudi, 2018; Shaw, 2007; Sundar, 2001).

2. ETIOLOGY AND CLINICAL FORMS

Protozoan parasites of the genus *Leishmania* belonging to the Trypanosomatidae family are responsible for provoking leishmaniosis. So far, 53 *Leishmania* species have been described,

and 20 of them are known to be pathogenic to humans (Akhoundi et al., 2016) leading to skin, mucocutaneous, or visceral disease.

Table 1. Scientific classification of *Leishmania* parasites.

LEISHMANIA	
Kingdom	Protista
Phylum	Euglenozoa
Class	Kinetoplastida
Order	Trypanosomatida
Family	Trypanosomatidae
Genus	*Leishmania*
Species	*infantum* (*chagasi*), *panamensis*, *donovani*, *major*, *amzonensis*, etc.

Cutaneous leishmaniasis (CL) is the most common clinical form. CL is mainly caused by *L. major*, *L. aethiopica* and *L. tropica* and has an estimated incidence of 0.7–1.2 million cases, mostly in Afghanistan, Iran, Syria, Algeria, Brazil and Colombia (World Health Organization, 2020). Localized CL causes injuries to the face, arms, and legs, and although it heals spontaneously, it can leave permanent marks on the skin and cause severe disability. Diffuse CL produces lepromatous lesions and is associated with vigorous immune states against *Leishmania*; it does not heal spontaneously and ends up leading to chronification as the treatment is not very effective. In contrast, disseminated CL produces a large number of acneiform lesions widespread through the body, but usually responds well to treatment (World Health Organization, 2016a).

Mucocutaneous leishmaniasis (MCL) is mainly associated with *L. brazilensis* infections and has a low incidence, with 90 % of cases concentrated in South America, particularly in Bolivia, Brazil and Peru (World Health Organization, 2020). MCL begins with a skin lesion that after a few months or even years ends up destroying the membranes of the nose, pharynx or larynx leading to deforming lesions (Reithinger et al., 2007).

Visceral leishmaniasis (VL), also known as kala-azar, is the most serious form of the disease with a fatal outcome if left untreated. It is characterized by episodes of fever, weight loss, hepatosplenomegaly, and anemia. In some patients, after treatment and subsequent recovery of VL, a chronic skin form called post-kala-azar leishmaniasis appears (Zijlstra et al., 2003). VL is caused by *L. donovani* and *L. infantum*. It seems that *L. donovani* is mostly transmitted

anthroponotically, from human to human, in the subtropical areas of Africa and Asia. In contrast, *L. infantum* is distributed in ecozones of South America and the Mediterranean Basin and follows a zoonotic cycle, where the dog plays an important role as a domestic and peri-domestic reservoir, thus increasing the risk of infection in these areas. susceptible human population (Boelaert et al., 2009; Organization, 2016; World Health Organization, 2016b).

Figure 2. Image of leishmaniasis patients. A hand with skin lesion caused by *Leishmania* (left), a patient with mucocutaneous lesion (center) and a child with visceral leishmaniosis (right). Excerpt from Public Health Image Library.

3. THE VECTOR AND TRANSMISSION CYCLE OF LEISHMANIA

Leishmaniases are mainly transmitted by infected female sandflies that act as vectors (Diptera, Psychodidae). There are more than 800 species of sandflies from which 90 belong to either the *Phlebotomus* genus (in the Afrotropical, Palearctic and Indomalayan ecozones) or the *Lutzomyia* genus (in the Neotropical ecozone), which are able to mediate infection to humans (Akhoundi et al., 2016). In the Mediterranean Basin, *P. Perniciousu*s and *P. Ariasis*are responsible for *L. Infantum* infection (Ready, 2014).

Figure 3. Phlebotomus papatasi (left) and Lutzomyia longipalpis (right).

Extracted from doi: 10.1371/image.ppat.v05.i08.

The life cycle of *Leishmania* begins when a parasitized female phlebotomine ingests blood from a vertebrate host and simultaneously inoculates metacyclic promastigotes. In the vertebrate host, *Leishmania* is an obligate intracellular parasite, with the macrophage being the main host cell. The host macrophages phagocyte the parasite, which remains in

endophagic vesicles, which fuse with lysosomes to become a parasitophorous vacuole, where *Leishmania* is adapted to grow at low pH (McConville et al., 2007; Naderer and McConville, 2008). In the parasitophorous vacuole, metacyclic promastigotes are transformed into amastigotes, which have a high replicative rate. The multiplication of the parasites leads to the rupture of the macrophage, thus releasing the parasites that will be phagocytosed by other host cells. When a new sandfly ingests blood from the host, the parasitized macrophages will be destroyed in the digestive tract of the insect releasing amastigotes, which will develop a flagellum and will be transformed again into procyclic promastigotes. These parasites can divide in the gut of the invertebrate host. From there, migration starts to the anterior midgut, where parasite maturation is induced (Gossage et al., 2003) and metacyclic promastigotes will be ready to be regurgitated during the next blood meal, completing the cycle

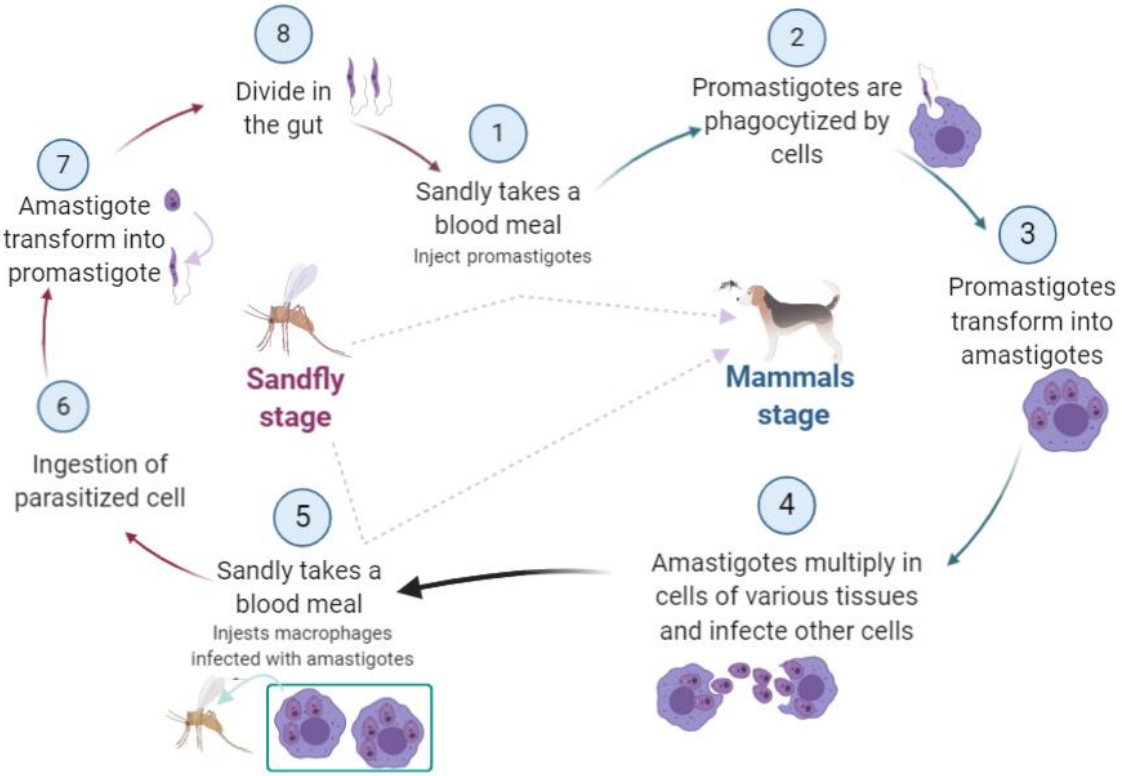


Figure 4. Life cycle of *Leishmania* spp parasites. (Figure created with BioRender.com).

Leishmania infections due to laboratory accidents (Herwaldt, 2001), organ transplants (Singh and Sehgal, 2010; Sirvent-von Bueltzingsloewen et al., 2004), vertical transmission (Boggiatto et al., 2011; Meinecke et al., 1999) and by sharing contaminated syringes (Cruz et al., 2002; Kaushik et al., 2011) have also been described.

Humans become infected accidentally and do not act as reservoir hosts for *L. infantum* (Moreno and Alvar, 2002). VL caused by *L. infantum* follows a zoonotic cycle where the dog is

the main domestic and peri-domestic reservoir and plays a central role in the transmission cycle of the parasite to humans, increasing the risk of infection in the susceptible human population (Boelaert et al., 2009; Moreno and Alvar, 2002; Solano-Gallego et al., 2009). Among dogs, the parasite is transmitted from an infected animal to an uninfected animal by a phlebotomus bite, although direct, dog-to-dog transmission, and blood transfusion pathways have been described (Owens et al., 2001).

It is known that in addition to the dog, wild canids such as wolfs, foxes, jackals (Alvar et al., 2004), cats (Bezerra et al., 2019b; Mancianti, 2004), horses (Solano-Gallego et al., 2003a), rabbits and mice (Risueño et al., 2018) can be also infected by *L. infantum*. The species exposed to this zoonosis could be part of the epidemiological chain of transmission of VL. For example, the largest outbreak of leishmaniasis ever detected in Spain occurred between 2010 and 2012 in Fuenlabrada, with more than 400 people infected (Arce et al., 2013). Molecular characterization of isolates obtained from infected sandflies after xenodiagnoses showed that hares (*Lepus granatensis*) had been infected by *L. Infantum* and they were identified as the reservoir implicated in Fuenlabrada outbreak (Molina et al., 2012).

4. CANINE LEISHMANIASIS

The dog is the main vertebrate host of the parasite and acts as a reservoir in zoonotic visceral leishmaniasis (ZVL) (Gramiccia and Gradoni, 2005; Moreno and Alvar, 2002; Solano-Gallego et al., 2009). It plays a key role in controlling the transmission of this disease, as a reduction in dog incidence decreases the prevalence of VL in humans (Porrozzi et al., 2007).

Cases of canine leishmaniasis (CanL) occur mostly in Central and South America (especially Brazil), in Asia, and in Mediterranean coastal countries. However, cases have been reported in non-endemic areas, such as northern Europe (Baldelli et al., 2001; Capelli et al., 2004; Dereure et al., 2009) and North America (Enserink, 2000). In some cases, dogs can be infected during travel to endemic areas (Maia and Cardoso, 2015; Mettler et al., 2005a; Shaw et al., 2009) or by the emergence of an autochthonous endemic focus (Anderson et al., 1980; Schantz et al., 2005). Aspects such as globalization, mass migration and, especially, climate change could be exacerbating the spread of the disease (González et al., 2010; Marcondes and Day, 2019; Short et al., 2017). All of this would explain why new cases of CanL have recently been reported each year in areas traditionally considered non-endemic where it was believed that the vector did not exist (Mihalca et al., 2019; Palatnik-De-Sousa and Day, 2011).

Table 2. Geographical distribution of *Leishmania* species infecting dogs and their vector sandfly species.
Reviewed by Solano-Gallego et al., 2009.

Leishmania species	Geographical Distribution	Sandflies vectors
L. infantum	Mediterranean Basin Middle East	*P. perniciosus, P. ariasi, etc.* *P. perfiliewi, P. neglectus,* *P. langeroni, P. tobbi. etc.*
	Southern Asia, Iran, Armenia, Afghanistan	*P. kandelakii*
	Central Asia, China	*P. chinensis, P. alexandri*
L. chagasi	Central and South America	*Lu. longipalpis, Lu. evvansi,* *Lu. olmeca olmeca*
L. donovani	East Africa	*P. orientalis, P. martini*
L. tropica	Nord Africa	*P. sergenti, P. arabicus*
L. braziliensis	Central to South America	*Lu. amazonensis, Lu. migonei,* *Lu. panamensis, Lu. paraensis,* *Lu. complexus, Lu. pessoai, etc.*
L. peruviana	Peruvian Andes	*Lu. noguchii, Lu. Pescei*
L. panamensis	Central America	*Lu. shannoni, Lu. ovallesi, etc*

In the Mediterranean Basin, the parasite is distributed in endemic foci. The prevalence of infection in dogs estimated only in basis of serology resulting in rates between 2 % and 50 % (Amusategui et al., 2004; Cardoso et al., 2004; Manzillo et al., 2018; Mattin et al., 2014; Miró et al., 2012; Papadopoulou et al., 2005; Sideris et al., 1999; Solano-Gallego et al., 2011). A recent study shows that seroprevalence in the province of Girona, in Catalonia, was 19.5 % (Velez et al., 2019).

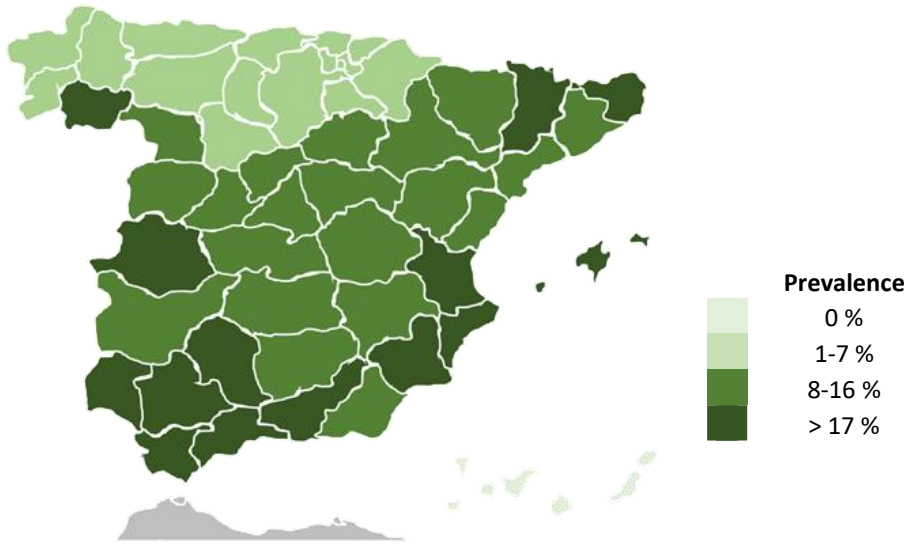


Figure 5. Distribution of canine *L. infantum* infection cases in Spain according seroprevalence data extracted from Gálvez et al., 2020

However, inclusion of *Leishmania*-specific cellular response and parasite detection increase considerably the estimation of the infection rate. Thus, the infection rates determined by PCR and serology can reach 70 %–90 % in endemic areas, as described in the Balearic Islands (Solano-Gallego et al., 2001a), in the Marseille area in France (Berrahal et al., 1996) and in Greece (Leontides et al., 2002). Regarding incidence, it is estimated that approximately 2 %–10 % of dogs become infected in each period of transmission in rural or semi-rural areas (Amela et al., 1995; Paradies et al., 2006). For instance, in a study conducted in Spain an incidence of 2.52 % was observed considering new cases of CanL diagnosed within 12 months (Mattin et al., 2014).

Even so, most of *L. infantum* infected dogs are resistant and coexist with the pathogen in a subclinical way, remaining clinically healthy for years (Acedo Sánchez et al., 1996; Fisa et al., 1999; Jones et al., 1998; Natami et al., 2000; Solano-Gallego et al., 2009). Only 5 %–20 % of cases develop symptoms and can be considered patients with leishmaniasis (Cardoso et al., 2004; Carrillo and Moreno, 2009; Fisa et al., 1999; Manzillo et al., 2018), which can end up with a fatal outcome if left untreated (Alvar et al., 2004). In the Girona area, for example, it was determined that 6.8 % of seropositive dogs are symptomatic (Velez et al., 2019).

Age is a risk factor for the development of CanL, showing a bimodal distribution of prevalence, with the highest risk ages 3–4 years and 7–8 years (Amela et al., 1995). There is no unanimous opinion on the influence of sex on the development of the disease. Some studies suggest that there would be no differences in risk between sexes (Abranches et al., 1991a; Solano-Gallego et al., 2006), while others point to a higher prevalence in males (Dantas-Torres and Brandão-Filho, 2006; Zaffaroni et al., 1999). Regarding breed, purebred dogs are more likely to develop CanL than crossbreed, and short-haired dogs have a higher incidence than long-haired dogs (França-Silva et al., 2003). The breeds in which a higher incidence is described are: boxer, cocker, rottweiler, doberman and German shepherd (Abranches et al., 1991a; França-Silva et al., 2003; Miranda et al., 2008). In contrast, the Ibizan hound could be resistant to CanL (Solano-Gallego et al., 2000). These differences may be due, in part, to the genetic basis of the animals. There are some associations between polymorphisms in the genes *SLC11A1*, *MHC* and *cBD1*, and susceptibility to the disease (Quinnell et al., 2003; Sanchez-Robert et al., 2008; Da Silva et al., 2017). Besides, a study pinpointed chromosomic regions related to the cell-mediated response that potentially affect the clinical complexity and the *L. infantum* replication in dogs (Batista et al., 2016).

5. PATHOGENESIS AND SYMPTOMATOLOGY OF CanL

Leishmaniasis is a systemic disease that can show a wide range of clinical signs and the time of onset of symptoms is very variable. Affected dogs, 5 %–20 % of *Leishmania*-infected, can present a range of symptoms from mild popular dermatitis to severe glomerulonephritis (Nieto et al., 1992; Ordeix et al., 2005) and disease can manifest after several months of infection or within a few years (Oliva et al., 2006; Slappendel, 1988).

The *Leishmania* parasite is inoculated into the skin of the host, where it invades macrophages. From there, it is first transported to the lymph nodes and spleen. Then, it spreads to the liver and kidney and finally to the skin, bone marrow, eyes, joints, digestive tract, respiratory system and sexual organs (Alvar et al., 2004; Miró et al., 2008). Lesions that occur due to infection depend on several pathogenic mechanisms involved in the response to this parasitosis (Solbach and Laskay, 2000). One of the most important pathogenic mechanisms is the generation of granulomatous reactions, responsible for skin, hepatic and enteric lesions. Granulomas are composed mainly of parasitized macrophages and to a lesser extent of lymphocytes and plasma cells (Rallis et al., 2005; Sanchez et al., 2004; Tafuri et al., 2001). Lesions can also be caused by the production of circulating immunocomplexes, which are deposited on the wall of blood vessels and lead to develop vasculitis, glomerulonephritis, uveitis, meningitis, and arthritis (Brandonisio et al., 1990; Ferrer, 1992; Nieto et al., 1992; Viñuelas et al., 2001).

Lymph nodes of infected dogs have hyperplasia, due to increased size and number of lymphoid follicles and hyperplasia of modular macrophages (Keenan et al., 1984; Lima et al., 2004). Spleen is a key point of immune system with the parasite interaction. During CanL, splenomegaly occurs accompanied by an increase in plasma cells and parasitized macrophages (Natami et al., 2000; Santana et al., 2008). Regarding the liver, infected dogs may show hepatomegaly caused by congestion and by chronic hepatitis (Giunchetti et al., 2008). As the infection progresses there is a degradation of hepatocytes that leads to a loss of liver function (González et al., 1988). Concerning kidney, injuries are attributed to the deposit of immunocomplexes (Benderitter et al., 1988; Poli et al., 1991), but it has also been suggested that $CD4^+$ T lymphocytes may play a role in renal pathogenesis (Costa et al., 2000). Kidney deterioration leading to renal failure is the most common cause of death in dogs with leishmaniasis (Benderitter et al., 1988; Ferrer, 1992; Poli et al., 1991). Skin lesions are the most common clinical sign, with exfoliative, nodular, ulcerative or postulate lesions (Baneth et al., 2008; Meléndez-Lazo et al., 2018; Solano-Gallego et al., 2011). Exfoliative injuries are

associated with a certain control of parasitism and nodular ones with an uncontrollability of the infection (Fondevila et al., 1997). Infected dogs with a patent disease show a large number of parasites on the skin (Giunchetti et al., 2006; Verçosa et al., 2008).Dogs with mild disease and papular dermatitis present lower specific antibody levels and blood parasitaemia than dogs with more several disease (Montserrat-Sangrà et al., 2018). When the disease becomes chronic, there is a decrease in type I collagen and an increase in type III collagen, causing changes in extracellular matrix due to the progressive destruction of tissue (Giunchetti et al., 2006).

6. DIAGNOSIS

Effective diagnosis is key to detect the infection and be able to act quickly to control the disease. Estimating the prevalence and incidence of CanL are important epidemiological parameters for transmission control and depends on the true identification of infected dogs.

6.1. Clinicopathological diagnosis

The first approach to the presumptive diagnosis of CanL is based on the clinical presentations, together with the result of the clinicopathological data of the animal. This type of diagnosis can be difficult because it is a multisystemic disease with very variable clinical signs and times of onset. Manifestations of the disease may become evident from a few months to more than 7 years after infection (Sideris et al., 1999; Slappendel, 1988). In addition, infected dogs can present from mild dermatitis to severe glomerulonephritis without a pathognomonic sign(Solano-Gallego et al., 2011). For this reason, the diagnosis of leishmaniasis should consider the clinicopathological data and those obtained with other more specific diagnostic techniques together. In this way, different parameters can be combined to give an overall score. A staging system based on clinical signs, clinicopathological abnormalities and serological status was proposed by LeishVet group to establish cu-off points and divide the disease into different stages (mild, moderate, severe and very severe disease)(Solano-Gallego et al., 2011).

6.2. Parasitological diagnosis

Cytology and histopathology allow to diagnose *Leishmania* infection by detecting the parasite in blood or tissue samples. They are a relatively fast method of diagnosis, although of generally low sensitivity (30 %–70 %)(Bourdoiseau et al., 1997a; Moreira et al., 2007; Xavier et al., 2006).

A sensitive method for diagnosing infection is polymerase chain reaction (PCR) to detect DNA from the parasite (Ashford et al., 1995). It is common to amplify kinetoplast DNA fragments as

they appear repeatedly in multiple copies in the parasite, thus increases the sensitivity of the technique (Lachaud et al., 2002; Moreira et al., 2007). In addition, PCR has the advantage that it can be realized in a great variety of samples; lymph nodes, bone marrow and skin are tissues in which a high sensitivity is obtained (Maia et al., 2009; Solano-Gallego et al., 2001a). Detection in blood by PCR is suitable only for dogs with clinical signs, because in clinically healthy dogs the sensitivity is very low in this kind of sample (Manna et al., 2004; Rodríguez-Cortés et al., 2007a). Currently, real-time quantitative PCR (qPCR) technique allows for quantifying the relative number of parasites in relation to a standard curve, and allows for better sensitivity than traditional PCR, especially in blood samples. It is considered the most reliable method and allows the detection of values of less than 1 parasite per mL, saving time and reducing the risk of contamination (Carson et al., 2010; Francino et al., 2006; Hernández et al., 2015; Rodríguez-Cortés et al., 2007a).

Recently, a new method of nucleic acid amplification has been developed, the LAMP technique (loop-mediated isothermal amplification). Unlike PCR, it uses polymerases capable of amplifying DNA at constant temperatures. Although it is not commonly used for the diagnosis of CanL yet, it is a promising technique because it is simpler and faster than PCR. Furthermore, it has been shown to have high sensitivity (90.7 %–100 %) and specificity (97.7 %–100 %) in blood samples from VL patients(Ibarra-Meneses et al., 2021; Khan et al., 2012; Mukhtar et al., 2018; Verma et al., 2013). In China, the use of LAMP in conjunctival samples identified 61 % of infected dogs, more than PCR (58.6 %), and only one sample from the control group was positive for the LAMP test and PCR (97 % specificity) (Gao et al., 2015).

6.3. Humoral immunological diagnosis

Detection of serum antibodies produced during infection has been used routinely as a diagnostic marker (Maia and Campino, 2008), despite being less specific than parasitological methods because it may present cross-reactions and the cut-off choice is not always obvious (Rodríguez-Cortés et al., 2010).

The most common sample for antibody detection is serum, where specific anti-*Leishmania* antibody concentrations correlate with parasitaemia and the clinical status of the animals (Reis et al., 2006a; Rodríguez-Cortés et al., 2007a). Less invasive samples such as urine can be used but the sensitivity is lower (Solano-Gallego et al., 2003b; Todolí et al., 2009). It should be noted that the presence of antibodies does not imply the presence of active disease, so it is advisable to evaluate serology in conjunction with the clinical symptoms (Fisa et al., 1999; Nieto et al., 1999). It is important to note that not all infected animals have antibodies (Alvar et al., 2004).

The most used serological techniques are direct agglutination test (DAT), immunofluorescence antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA). DAT is simple, cheap and reliable (Adams et al., 2012; Sousa et al., 2011). It has a sensitivity and specificity of 91 %–100 % and 72 %–100 %, respectively, but it has long incubation periods, requires a certain level of expertise and the reading is subjective (Adams et al., 2012; Gómez-Ochoa et al., 2009; Oliveira et al., 2016). IFAT has traditionally been considered the reference method (or *gold standard*), but has the limitation of cross-reactions with other pathogens (Paltrinieri et al., 2016; Solano-Gallego et al., 2014), subjectivity and is less sensitive in identifying asymptomatic dogs (Mettler et al., 2005b). It has been proved that the most appropriate test to identify individuals infected with *Leishmania* is the ELISA (Rodríguez-Cortés et al., 2010), which can be easily automated, objective, and allows the use of multiple antigen combinations, thus increasing the sensitivity and specificity of the method (Santarém et al., 2010). Other serological methods of diagnosis are the Western-Blot (Fernández-Pérez et al., 1999; Marín et al., 2007), dot-ELISA (Mancianti et al., 1996), immunochromatographic tests or dipsticks (da Costa et al., 2003), and immunodiffusion (Bernadina et al., 1997).

Conventional ELISA tests use crude antigen preparations, either whole promastigotes or their soluble extracts. The use of crude total antigen of the parasite (CTLA) is limited by its composition, which is highly variable, and by the difficulty of producing large amounts of antigen. In order to obtain a more standardizable, specific and accurate serodiagnosis tool, different methods based on recombinant proteins have been developed, with sensibility values of 95 %–100 %, for example, using rK39 (Porrozzi et al., 2007; Rosati et al., 2003; Scalone et al., 2002), although its sensitivity decreases when detecting asymptomatic individuals (Teixeira et al., 2019). New designs of multi-epitope chimeric proteins improve the detection of asymptomatic infected dogs (Faria et al., 2015).

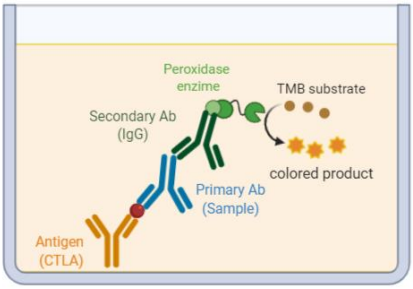


Figure 6. Scheme of ELISA technique operating. (Figure created with BioRender.com).

Flow cytometry, a global trend in the development of high-performance diagnostic methods, may also be useful for humoral diagnosis of CanL. It is a fast and accurate technique that allows the evaluation of particles in a fluid, such as antibodies against superficial *Leishmania*

antigens (Maia and Campino, 2008; Silvestre et al., 2008). It has proven to be a viable tool for serodiagnosis, sensitive and specific, and useful to detect both dogs with clinical leishmaniasis and asymptomatic (Andrade et al., 2009; Ker et al., 2019; Sousa et al., 2011). The combination of different antigen systems allows to test antibodies against multiple recombinant antigens from a single serum sample (Ker et al., 2019).

6.4. Cellular immunological diagnosis

L. infantum-infected dogs develop a specific cellular response. Detecting this response in epidemiological studies allows the detection of a very high percentage of infected dogs that do not show clinical signs and that would have been underestimated in serological tests (Solano-Gallego et al., 2000). Several *in vitro* tests are currently being performed, as well as the intradermal reaction or leishmanin *in vivo* (LST) test.

The LST is based on the intradermal inoculation of an inactivated promastigotes solution, that after a period of 48–72 hours will result in type IV DTH (delayed-type hypersensitivity reaction) in individuals who have had contact with the parasite and exhibit an adequate cellular immune response. A reaction greater than 5 mm is considered positive (Cardoso et al., 1998). Although the test has traditionally been performed with the entire phenolized parasite, it has also been performed with recombinant proteins (Todolí et al., 2010).

To assess the *in vitro* cellular response, lymphoproliferation assay can be performed, which consist in culturing peripheral blood mononuclear cells (PBMCs) with parasite antigens to assess their blastogenic capacity. It can be measured by radioactive, immunoenzymatic and fluorocytometric methods (Fernández-Bellon et al., 2005; Pinelli et al., 1994a). This technique has a sensitivity of only 60 %–75 % and a specificity of 83 % (Quinnell et al., 2001a). Another *in vitro* assay is IFN-γ detection from lymphocyte supernatants stimulated with *Leishmania* antigen. Currently, commercial kits are available to detect IFN-γ by ELISA technique (Fernández-Bellon et al., 2005). Flow cytometry is also used to evaluate and characterize $CD4^+$ and $CD8^+$ T lymphocyte populations associated with CanL (Alves de Pinho et al., 2020; Papadogiannakis et al., 2010; Rosypal et al., 2005).

Nowadays, these tests are only performed on an experimental level in immunoepidemiological studies.The LST test is the one that could be most easily applied as a diagnostic method because it is easier to perform and more sensitive (Fernández-Bellon et al., 2005). However, since the aforementioned tests evaluate different aspects of the cellular immune response, it would be best to perform them together (Rodríguez-Cortés et al., 2007b).

7. THE IMMUNE RESPONSE AGAINST *LEISHMANIA*

7.1. The innate immune response

Once inoculated into the skin, *Leishmania* promastigotes interact with resident dendritic cells, macrophages and γδ T lymphocytes. These cell populations recognize pathogen-associated molecular patterns (PAMPs) through toll-like receptors (TLR) (Janeway and Medzhitov, 2002; Tuon et al., 2008). Then, these activated cells produce a cascade of chemokines that induce the recruitment of neutrophils, macrophages, and natural killer (NK) cells to the site of infection (Teixeira et al., 2006) and will initiate a cascade of innate immune response to fight against parasites.

Toll-type receptors recognize PAMPs structures and activate the immune response machinery (Chauhan et al., 2017; Kawai and Akira, 2010). *Leishmania*'s lipophosphoglucan (LPG) induce TLR2 expression and enhances TNF-α and IFN-γ production (Becker et al., 2003). TLR2 has been also related with nitric oxide (NO) production (Chandel et al., 2014; Kavoosi et al., 2010). TLR4 has been shown to be protective in *L. major* infection (Antoniazi et al., 2004; Kropf et al., 2004) and is linked to reactive oxygen species (ROS) production (Faria et al., 2011). The few studies performed in dogs show that TLR2 is increased in dogs with severe disease (Montserrat-Sangrà et al., 2016, 2018) and that TLR4 and TLR7 agonists increase TNF-α production (Martínez-Orellana et al., 2018), suggesting that may play an active role in the inflammatory response.

Leishmania is an obligated intracellular parasite with macrophages being the main host cell. Other phagocytic cells, such as neutrophils and dendritic cells (DCs), are also susceptible of being infected, but there is no evidence of replication in other cell types than macrophages (Peters and Sacks, 2006). Neutrophils are the first cell type recruited at the site of infection but their role in the pathogenesis of *Leishmania* is complex and not entirely clear (Guimarães-Costa et al., 2009; Peters et al., 2008). Macrophage invasion is crucial for parasite survival. Those that fail to invade these target cells will be quickly destroyed by the complement system, neutrophils, and NK cells. Infected neutrophils can destroy parasites or be phagocytosed by macrophages, also serving as a gateway to this target cell (De Almeida et al., 2003; van Zandbergen et al., 2004).

Macrophages come from monocytes, which begin their process of formation in the bone marrow from myeloid stem cells under the influence of stimuli. Monocytes enter the bloodstream where they circulate for hours before crossing the walls of capillaries of

connective tissue and transforming into macrophages. The main function of macrophages is to ingest and destroy pathogens (Duque and Descoteaux, 2014). Once in the macrophage, the parasites are encapsulated in a phagosome, which after maturation and fusion with lysosomes and endosomes forms a parasitophore vacuole (Antoine et al., 1998). Phagocytosis produces a respiratory outbreak that activates the production of ROS that help fight the parasite, along with NO, the main leishmanicidal molecule. NO is generated by inducible nitric oxide synthase (iNOS) after activation of cells by interferon-ɣ (IFN-γ) (Novais et al., 2014; Scott and Novais, 2016). *Leishmania* is well adapted to the host cell and seeks to survive by developing various mechanisms of evasion of the immune response, which lead to impaired macrophage activity to destroy parasites (Burchmore and Barrett, 2001; Gupta et al., 2013; Sacks and Sher, 2002). When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as TNF-α, IL1, IL6, and IL12 that help to stimulate the acute phase response and to catalyse inflammation (Duque and Descoteaux, 2014).

Dendritic cells activated through TLRs produce the pro-inflammatory cytokine IL12, which activates and induces the differentiation of different types of immune cells such as $CD4^+$ and $CD8^+$ T cells and NK (Schleicher et al., 2007). NK cells also play a key role in the early stages of infection, due to their cytotoxic activity and because they are an early source of IFN-γ, needed to activate the response of type 1 T helper lymphocytes (Th) in lymph nodes (Laskay et al., 1993; Martín-Fontecha et al., 2004; Müller et al., 2001). Simultaneously, infected dendritic cells migrate to regional lymphatic tissue, where they will initiate the adaptive immune response by activating immature collaborating T lymphocytes (E Sousa, 2004; Scott and Novais, 2016).

7.2. The adaptive immune response

In the most studied model of leishmaniosis, the mouse infected with *L. major*, has been described and accepted for many years an existence of a dichotomy in Th lymphocytes, Th1 vs Th2, associated respectively with resistance or susceptibility to disease (Mosmann et al., 1986). In this widely studied model, the immune response is characterized by the production of Th1 $CD4^+$ in presence of IL12 secreted by antigen presenting cells (APCs). These Th1 cells collaborate by secreting the pro-inflammatory cytokines IFN-γ and TNF-α, responsible for the classical activation of macrophages (M1) (Kaye and Scott, 2011). These M1 macrophages are proinflammatory and microbicidal and are capable of producing nitric oxide through an induction of iNOS2 (Bogdan et al., 1990a; Kaye and Scott, 2011; Locati et al., 2020). In contrast, Th2 lymphocytes lead to cytokines IL4, IL5, IL10 and IL13 production that promote the

immunoglobulins (Ig) production by B cells and entails an alternative activation of macrophages towards M2 (Dayakar et al., 2019), which are anti-inflammatory and immunosuppressive (Locati et al., 2020).

This model is, however, a simplification of all the immune processes that really happen, both in people and in dogs. Although current knowledge suggests that protective responses are associated with the activation of a specific cellular immunity and a Th1 pro-inflammatory response (Carrillo and Moreno, 2009), the mechanisms that regulates the outcome of infection are not yet completely defined. Because susceptible individuals develop the disease progressively by increasing parasitic loads despite a strong production of Th1-derived cytokines, it is believed that the inability to control the parasite in tissues would not be due to the lack of Th1 response, but by an inhibition of this response (Corrêa et al., 2007; Lage et al., 2007; Quinnell et al., 2001b; Strauss-Ayali et al., 2007).

7.2.1. Humoral adaptive immune response

Canine leishmaniasis is characterized by polyclonal B cell activation that results in hypergammaglobulinemia and in production of large amounts of specific antibodies against the parasite (Alvar et al., 2004; Giunchetti et al., 2006; Keenan et al., 1984; Martínez-Moreno et al., 1995; Reis et al., 2006b) which are greater in sick infected dogs over clinically healthy ones (Bourdoiseau et al., 1997b; Leandro et al., 2001; Solano-Gallego et al., 2001b).

Specific antibody concentrations correlate with parasite load and clinical status (Reis et al., 2006b; Rodríguez-Cortés et al., 2007a). The main Ig produced during CanL is specific IgG, which occurs 1 to 4 months after infection (Abranches et al., 1991b; Martínez-Moreno et al., 1995; Quinnell et al., 1997; Riera et al., 1999). It is the most suitable isotype for diagnosis in dogs due to the high correlation between the decrease in anti-*Leishmania* IgG levels and the clinical improvement given by treatment. However, in dogs that have suffered from leishmaniasis, the presence of IgG is maintained for a long time and complete sero-reversal rarely occurs (Koutinas et al., 2001; Riera et al., 1999). Specific anti-*Leishmania* IgA, IgM, and IgE isotypes have also been correlated with disease (Almeida et al., 2005; Iniesta et al., 2005; Rodríguez-Cortés et al., 2007a). In contrast, serum IgD levels do not appear to be a marker of disease (Martínez-Orellana et al., 2019).

7.2.2. Cellular adaptive immune response

In the dog, *Leishmania* infection does not show a very clear Th1/Th2 dichotomy as described in the *L. major* mouse model. A mixed pattern is detected (Quinnell et al., 2001b; Strauss-Ayali et

al., 2007) and probably is the equilibrium between these two responses what will eventually determine the outcome of the disease's evolution (Baneth and Aroch, 2008; Carrillo and Moreno, 2009).

In human VL it is clearly established that the suppressive role is played primarily by the cytokine IL10, which is known to deactivate the mechanisms of parasite destruction of macrophages and promote T cell dysfunction by eliminating the presentation of antigens and downregulate IFN-γ production (Ito et al., 1999), but in CanL no conclusions could be drawn (Quinnell et al., 2001b). Resistance to *L. infantum* infection is associated with an early protective cellular response with specific proliferation of T lymphocytes (Baneth et al., 2008; Cabral et al., 1992; Moreno et al., 1999a; Pinelli et al., 1994a; Rodríguez-Cortés et al., 2007a). As the disease progresses, immunosuppression occurs, which causes the loss of this response given by T cells (Dos-Santos et al., 2008; Martínez-Moreno et al., 1995; Pinelli et al., 1994a). Although the causes of this immunosuppression are not entirely clear, some factors that may be related to it have been described: $CD4^+$ and $CD8^+$ T lymphocytes population (Baneth et al., 2008; Barbiéri, 2006; Pinelli et al., 1994b), decreased expression of co-stimulatory molecules (Pinelli et al., 1999), involvement of Treg lymphocytes (Rodrigues et al., 2009), as well as increased expression of anti-inflammatory cytokines (Carrillo and Moreno, 2009).

$CD4^+$ helper T cells are associated with disease protection (Baneth et al., 2008; Barbiéri, 2006). Resistance to leishmaniasis is associated with the ability of $CD4^+$ T lymphocytes to generate IFN-γ, which will activate the parasitized macrophage to kill intracellular parasites (Heinzel et al., 1991; Liew et al., 1989; Mosmann et al., 1986). Studies in naturally infected dogs showed low $CD4^+$ expression, which correlated with a decrease in T-cell-mediated immune response and with disease progression. When treating CanL there is a recovery of $CD4^+$ T lymphocytes (Guarga et al., 2002; Moreno et al., 1999a). The $CD8^+$ T lymphocyte population is also implicated in *Leishmania* infection, as it participates in the lysis of infected macrophages (Pinelli et al., 1994b). The role of $CD8^+$ T cells in the control of *Leishmania* species causing visceral disease has been demonstrated in mice (Gomes-Pereira et al., 2004; Murray and Hariprashad, 1995; Tsagozis et al., 2003) and in humans (Mary et al., 1999). The generation of $CD8^+$ cytotoxic T cell lines from asymptomatic dogs revealed a direct cytolytic effect on *L. infantum*-infected macrophages that could be an effector mechanism of CanL resistance (Pinelli et al., 1995). $CD8^+$ T cell population is higher in clinically healthy infected dogs than in uninfected and sick dogs. The more parasitic load and symptomatology, the less $CD8^+$ T lymphocytes (Reis et al., 2006c).

Clinically healthy infected dogs or those protected by a vaccination strategy exhibit a Th1 pattern, with IFN-γ, IL2, and TNF-α production (Carrillo et al., 2007, 2008; Chamizo et al., 2005; Lemesre et al., 2005; Pinelli et al., 1994a; Santos-Gomes et al., 2002; Strauss-Ayali et al., 2005). These cytokines, mainly IFN-γ and TNF-α, will stimulate the leishmanicidal activity of macrophages by producing NO, the main molecule secreted by the macrophage with leishmanicidal activity, which will induce apoptosis of amastigotes present into cell (Bogdan et al., 1990b; Holzmuller et al., 2006; Rodrigues et al., 2007). The expression level of the inducible isoform of iNOS and the concentration of NO produced by macrophages correlate with the leishmanicidal capacity of these cells (Panaro et al., 2001; Pinelli et al., 2000; Rodrigues et al., 2007; Zafra et al., 2008). In contrast, sick dogs exhibit a mixed Th1/Th2 pattern and lower IFN-γ and TNF-α production (Carrillo et al., 2008; Pinelli et al., 1994a; Strauss-Ayali et al., 2005).

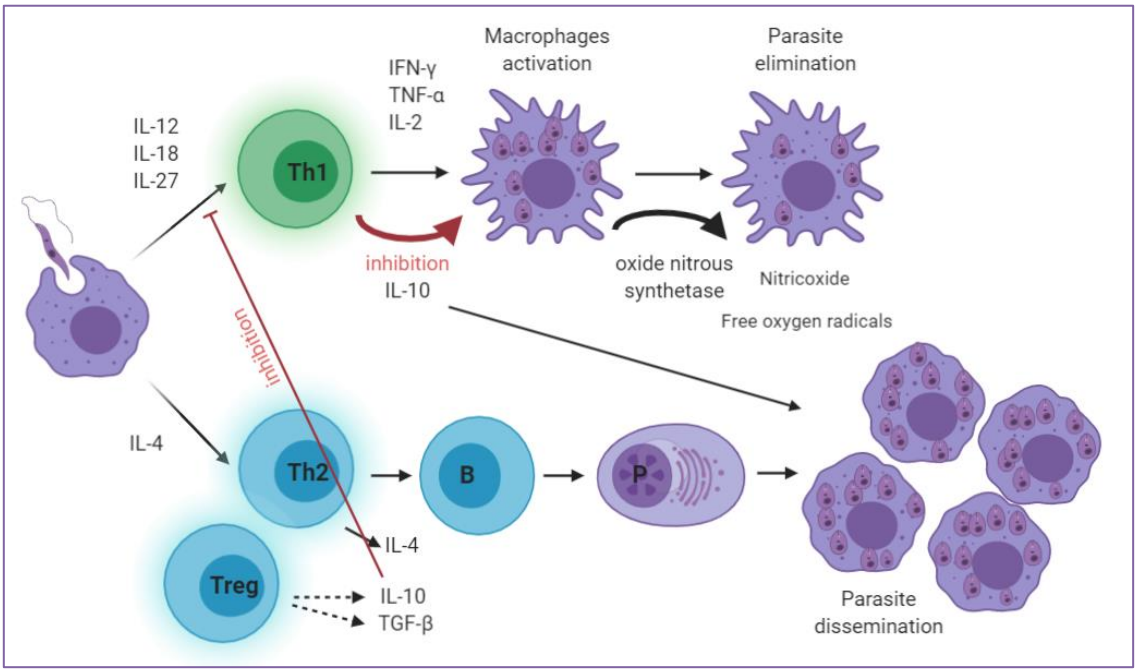


Figure 7. Cellular immune responses in CanL. A strong Th1 response is associated with resistance. IFN-γ, IL2 and TNF-α induce macrophage activation and NO killing of parasites. By contrast, IL10, TGF-β and IL4 are involved in the parasite dissemination associated with increased B-cell activity. Th1 responses are downregulated by IL10 produced by Treg cells. (Figure created with BioRender.com).

Some studies suggest that the immune response to *L. infantum* infection in the dog may be compartmentalized and different depending on the organ (Rodríguez-Cortés et al., 2016; Sanchez et al., 2004). In the spleen and lymph nodes, the cytokines IL10 and TGF-β are responsible for the persistence of the parasite (Alves et al., 2009; Corrêa et al., 2007; Lage et al., 2007). In contrast, in the skin and bone marrow, IL4 is responsible of infection proliferation

(Brachelente et al., 2005; Quinnell et al., 2001b). IL10 could be aggravating the infection in the liver (Corrêa et al., 2007).

Recent studies showed that Th17 cells act synergistically with the Th1 population to control the growth of *L. infantum*. IL17 modulates some key regulatory cytokines, including IL10, and maintains IFN-γ production by Th1 cells at sites of inflammation (Nascimento et al., 2015). The role of the Th2 response during the progression of infection is under discussion, but active disease has been linked to elevated antibody levels and an immune response that inactivates Th2 in the presence of strong inflammatory reaction (Rodríguez-Cortés et al., 2016).

7.3. Biomarkers of CanL resistance and susceptibility

A broad spectrum of immune responses can be found in *L. infantum* infection. The balances established between factors involved in the host immune response are reflected in the spectrum of clinical forms that can be found in infected dogs (Reis et al., 2010). At one pole there are cases with a strong humoral response (high levels of specific antibodies), a little or no cellular response associated with disease, and a wide spread of the parasite to different tissues (high parasitic loads). At the other pole there are animals that remain clinically healthy, which show a potent cellular response with increased levels of $CD4^+$ and $CD8^+$ T cells —producers of IFN-γ, IL2 and TNF-α— and B cell subsets and positive leishmanin skin test. Between the two extremes, however, there is a wide variety of presentations (Baneth et al., 2008; Barbiéri, 2006; Pinelli et al., 1994a; Reis et al., 2010; Solano-Gallego et al., 2009).

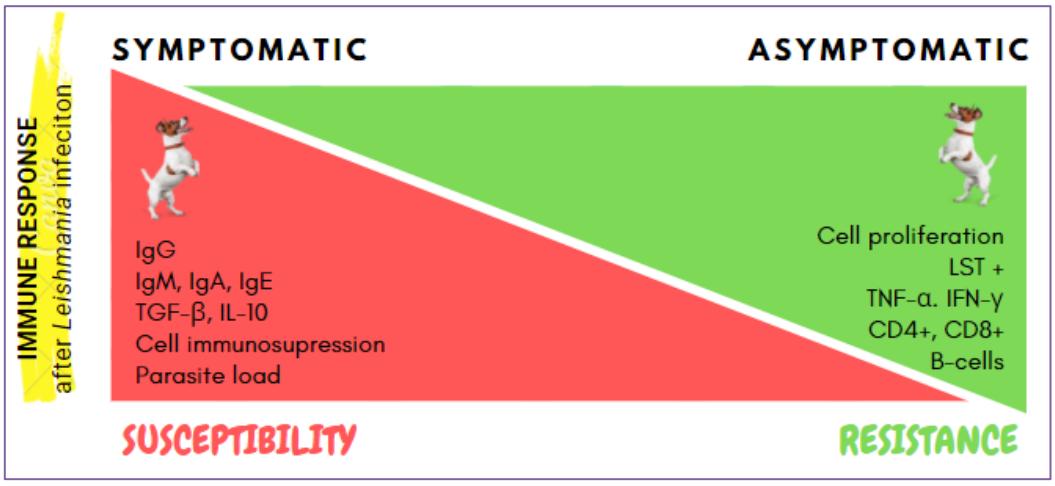


Figure 8. Biomarkers of resistance and susceptibility in CanL.

8. VITAMIN D AND ITS ROLE IN IMMUNE RESPONSE

8.1. Chemistry and biosynthesis of vitamin D

Vitamin D is a generic term used to refer to a group of closely related secosteroids involved in important biological functions that are classified as D2, D3, D4, D5, D6, and D7. The two main forms are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) which differ structurally in the side chain (Norman, 2008). Vitamin D_2 is produced in fungi and yeast by UVB exposure of provitamin ergosterol, while vitamin D_3 is produced in the skin by UVB exposure of provitamin 7-dehydrocholesterol. Therefore, the natural source of vitamin D_2 is limited to the diet, and the only foods with significant vitamin D_2 content are some mushrooms (Mattila et al., 1994; Teichmann et al., 2007). Vitamin D_3 can be produced endogenously in the skin by UVB exposure of 7-dehydrocholesterol or can be obtained directly from a small number of foods. Fish (especially fatty fish such as mackerel, salmon and blue fish), egg yolk and animal liver are vitamin D_3 sources (Lamberg-Allardt, 2006; Lu et al., 2007). Both forms of vitamin D can be obtained, if necessary, through dietary supplements (Rapuri et al., 2004). Some foods, such as milk, margarine, and orange juice may be fortified with vitamin D (Biancuzzo et al., 2010; Holick et al., 1992; Moulas and Vaiou, 2018; Piirainen et al., 2007). Anyway, as natural dietary sources of vitamin D are scarce and low in content, the main source of vitamin D in humans in most countries remains being exposed to solar radiation (Holick, 2017).

Vitamin D_3, also known as cholecalciferol, is the precursor of the steroid hormone calcitriol, also called 1-α,25-dihydroxycholecalciferol or 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). During exposure to UVB radiation, high-energy photons are absorbed in the B-ring 6,7-diene conjugate of 7-dehydrocholesterol (provitamin D_3), resulting in ring opening between C9 and C10 to form previtamin D_3. Previtamin D_3 is biologically inactive and thermodynamically unstable, so is thermally induced into vitamin D_3 (Havinga, 1973). Vitamin D_3 synthesized in the skin diffuses into the blood. Both the vitamin D_3 synthesized in the skin and the one obtained directly from the diet is transported to the liver by the vitamin D binding protein (DBP). Vitamin D can be previously captured by adipose tissue, where it can be stored for months (Jones et al., 1998). Vitamin D_3 activation involves two hydroxylations. This metabolic activation is carried out by specific enzymes of cytochrome P450 (CYP) superfamily. First, it is hydroxylated inthe liver at carbon C-25 by the enzyme 25-hydroxylase (CYP2R1), producing calcifediol, also known as 25-hydroxyvitamin D_3 ($25(OH)D_3$), the circulating form. The activity of 25-hydroxylase is poorly regulated and depends mainly on vitamin D concentration (Jones et al., 1998; Prosser and Jones, 2004). $25(OH)D_3$ is transported to the kidneys where it is hydroxylated at the α position of C-1 by 1α-hydroxylase (CYP27B1) generating $1,25(OH)_2D_3$, the

calciotropic hormone biologically active (Figure 9) (Jones et al., 1998; Prosser and Jones, 2004). This last bioconversion is regulated by serum calcium and phosphorus levels, blood levels of $1,25(OH)_2D_3$, and parathyroid hormone levels (Prosser and Jones, 2004). Inactivation of vitamin D_3 metabolites also occurs by a hydroxylation step, performed by the enzyme 24-hydroxylase (CYP24A1). This enzyme catalyses the active form calcitriol ($1,25(OH)_2D_3$) when it is no longer needed and also, although to a lesser extent, the stored form $25(OH)D_3$, giving rise to the molecules $1,24,25(OH)_3D_3$ and $24,25(OH)_2D_3$ respectively and thus self-regulating the availability of vitamin D in the body (Jones et al., 1998; Kundu et al., 2014).

Vitamin D_2 activating process is very similar to that described for vitamin D_3, but there are evidences that vitamin D_3 is more efficient increasing and maintaining metabolite 25(OH)D serum concentrations than vitamin D_2 (Heaney et al., 2011; Lehmann et al., 2013; Trang et al., 1998; Tripkovic et al., 2012). Therefore, although 25(OH)D includes both $25(OH)D_2$ and $25(OH)D_3$ molecules, the last one is predominant and is the majority form when detecting circulating vitamin D (Lehmann et al., 2013).

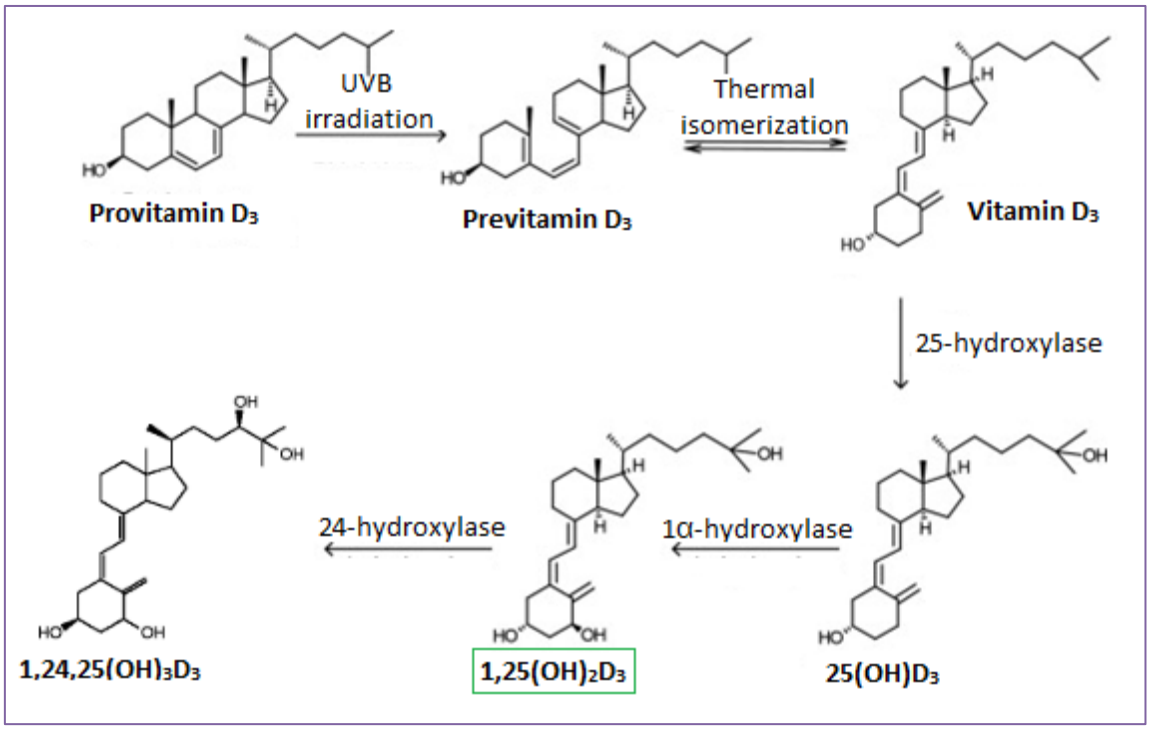


Figure 9. Vitamin D synthesis and activation.

Because hepatic 25-hydroxylase activity is not tightly regulated, any change in skin production or vitamin D intake alters circulating levels of the metabolite. For this reason, circulating 25(OH)D concentration is an accepted biomarker for determining vitamin D status (Lips, 2007). In addition, it is a very stable form due to its strong affinity for DBP.

Vitamin D_3 synthesis in human skin depends on season and latitude. An increase in the sun's zenith angle results in increased absorption of UVB photons by the stratospheric ozone layer. When the zenith angle becomes very oblique, very few UVB photons can penetrate the earth's surface and this results in a low cutaneous production of vitamin D_3 (Holick, 2003). This is the explanation for why during the winter little, if any, vitamin D_3 is produced in the skin of people living at latitudes above and below 35°N and 35°S (Ladizesky et al., 2009; Webb et al., 1988).It has been demonstrated that healthy people living in southern Switzerland have lower levels of 25(OH)D in non-summer seasons (Rabufetti et al., 2019). Pigmentation has also been shown to reduce vitamin D_3production in the skin: black people have lower circulating levels of vitamin D throughout the year (Harris, 2006). This process of vitamin D_3 synthesis in the skin is unclear whether it occurs in the same way in dogs. One study suggested that dogs are not able to properly synthesize vitamin D_3 in the skin and depend mainly on their dietary intake, but there is no evidence from other studies about it (How et al., 1994).

8.2. Biological functions of vitamin D

The active form of vitamin D, $1,25(OH)_2D_3$, has traditionally been known to regulate the metabolism of calcium and phosphorus, being a key element in bone formation (Holick, 2006). Nevertheless $1,25(OH)_2D_3$, has a physiological role beyond skeletal homeostasis. The role of vitamin D in the regulation of the immune response became aware after the discovery of the presence of the activating enzyme CYP27B1 and the vitamin D receptor (VDR) in various types of immune system cells, including macrophages, DCs and even T lymphocytes and B cells (Adams and Gacad, 1985; Chen et al., 2007; Hewison et al., 2003a; Overbergh et al., 2000; Phillip Koeffler et al., 1985; Provvedini et al., 1983). Local production of $1,25(OH)_2D_3$ has autocrine and paracrine properties that help to mediate the effects of vitamin Don immunity (Hewison et al., 2007; Lang et al., 2013). Activation of macrophage TLRs upregulates VDR and CYP27B1 expression (Liu et al., 2006a). The cell can convert the circulating form of vitamin D (25(OH)D) into the active form ($1,25(OH)_2D_3$), which will bind to the nuclear receptor of vitamin D. VDR will act as a transcription factor modulating the expression of many genes in a specific way (Baeke et al., 2010a; Hart et al., 2011).

Some observational, pre-clinical, and clinical studies have shown that low levels of vitamin D increase the risk of developing various diseases, such as cancer (Feldman et al., 2014), diabetes (Takiishi et al., 2010), autoimmune diseases (Baeke et al., 2010a), and infections such as toxoplasmosis (Rajapakse et al., 2007), AIDS (Coussens et al., 2015), malaria (Cusick et al., 2014), influenza (Morris et al., 2016) or COVID-19 (Meltzer et al., 2020). Since Colston *et al.*

and Abe *et al*. showed that calcitriol inhibited melanoma cell growth, a large number of epidemiological studies have been published showing an inverse relationship between cancer incidence and ultraviolet irradiation, vitamin D intake and/or circulating concentration of 25(OH)D (Abe et al., 1981; Colston et al., 1981). Some studies have also examined the association between polymorphisms in the *VDR* gene and the risk of certain types of cancer, showing that certain SNPs in the *VDR* gene are associated with an increased risk of colorectal, prostate and breast cancer (Ahn et al., 2009). The antineoplastic actions of calcitriol are carried out by inhibition of proliferation, induction of apoptosis, stimulation of differentiation, anti-inflammatory effects, inhibition of invasion, metastasis and angiogenesis. (Chung et al., 2009; Koli and Keski-Oja, 2000; Leyssens et al., 2013; Liu et al., 1996; Simboli-Campbell et al., 1997).

The mechanism by which vitamin D modulates immune function has been studied in different contexts and in multiple pathogens. The infectious disease in which the relationship between sunlight and vitamin D deficiency was initially documented was tuberculosis, a model in which the action of vitamin D has also been extensively investigated (Green, 2011; Liu et al., 2006a).

8.3. Vitamin D and immune response

The large number of studies linking vitamin D deficiency or insufficiency to various diseases and aberrant immune responses highlights its physiological importance to the immune system. It is well established that it plays its immunomodulatory role by activating the innate immune system and modulating the adaptive immune response against infection (Jeffery et al., 2009).

8.3.1. Effects on the innate response

Monocytes and macrophages are a key element in the innate immune response to infectious agents, as they immediately detect pathogen-associated molecular patterns (PAMPs) through toll-like receptors (TLRs). It has been seen that in mice with vitamin D deficiency there may be defects in some function of macrophages related to antimicrobial activity, including chemotaxis, phagocytosis, and the production of pro-inflammatory cytokines (Gombart, 2009; Griffin et al., 2003). $1,25(OH)_2D_3$ has effects on monocyte differentiation to macrophages, directing it towards an M1 phenotype with high phagocytic capacity (Martineau et al., 2007; Xu et al., 1993). Several studies suggest that $1,25(OH)_2D_3$ induces the expression of the antimicrobial peptide (AMP) cathelicidin (CAMP) in various cell types, including myeloid cells, keratinocytes, and neutrophils, increasing thus antimicrobial activity (Liu et al., 2007; Yim et al., 2007). The increase in CAMP resulting from the action of vitamin D metabolite is related to immune response mediated by toll-like receptors. TLR1 and TLR2 activation induce the

expression of VDR and 1-α-hydroxylase in human monocytes. For example, after TLR2/1 activation of human macrophages by *Mycobacterium tuberculosis* antigens, the expression of VDR and CYP27B1 is increased (Liu et al., 2006a; Nagy et al., 2012). Then, these cells can convert the circulating metabolite, 25(OH)D, into the active form of vitamin D, $1,25(OH)_2D_3$, which induces CAMP (Hewison et al., 2003b). There are other AMPs involved in the antimicrobial response of vitamin D through human TLRs, such as defensin β-4 (DEFB4). Induction of the *DEFB4* gene requires convergence of the VDR and IL1β pathways (Liu et al., 2009). Vitamin D also moderately increases the expression of defensin β-2 (DEFB2) in some human cell lines (Wang et al., 2004). These AMPs perform various antimicrobial functions by acting directly on bacterial wall (Scott and Hancock, 2000), increasing the formation of ROS (Zughaier et al., 2005), modulating cytokine expression (Jeffery et al., 2009) and inducing autophagy (Yuk et al., 2009).

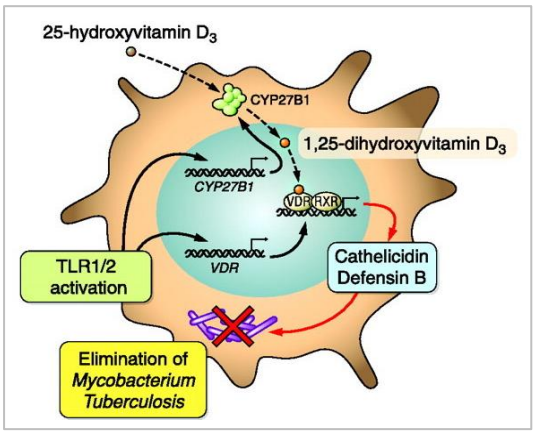


Figure 10. TLR1/2 activation by *M. tuberculosis* regulates VDR and CYP27B1. Accumulation of $1,25(OH)_2D_3$ activates VDR inducing genes of cathelicidin and β-defensin. Figure adapted from Nagy et al. 2012.

The induction of AMPs by the vitamin D pathway explains that UV therapy produced a clinical improvement in tuberculosis patients. However, there could be other mechanisms that would be having a positive effect in the fight against infections. One mechanism that has been studied and continues to be studied is ROS due to its antimicrobial effects. In a human macrophages line, $1,25(OH)_2D_3$ increased iNOS (Rockett et al., 1998). Conversely, Chang et al. observed that PBMCs from tuberculosis patients produced less ON than those from control subjects and they determined that in a mouse cell line (RAW cells) the enzyme iNOS was inhibited in presence of $1,25(OH)_2D_3$ in a dose-dependent manner (Chang et al., 2004). However, exposure of monocytes and macrophages to $1,25(OH)_2D_3$ reduces the stimulatory capacity and the ability to present antigens, with less expression of major histocompatibility complex class II (MHC-II) and stimulatory molecules such as CD40, CD80, and CD86 (Almerighi et al., 2009; Xu et al., 1993). The capacity to respond to PAMPs decreases by downregulating the expression of TLR2 and TLR4, probably providing a negative regulatory mechanism to prevent excessive receptor activation and stop the inflammatory response in post-infection stages (Yuk et al., 2009). Active vitamin D also plays an anti-inflammatory role by down-

regulating the expression of *TNFA*, *IL6*, *IL1*, *IL8*, and *IL12* (D'Ambrosio et al., 1998; Giulietti et al., 2007).

Dendritic cells (DCs), the major antigen presenting cells (APCs), are also an important component of the immune system; they capture, process, and present antigens to T cells. DCs are known to be targets of vitamin D. Activation of VDR signalling pathway inhibits dendritic cell maturation by maintaining an immature phenotype characterized by low expression of MHC-II complex molecules and co-stimulatory molecules (CD40, CD80, and CD86), low expression of other maturation-induced surface markers such as CD38 and CD83, and increased expression of mannose receptors (Berer et al., 2000; Pedersen et al., 2009; Penna and Adorini, 2000). Positive regulation of mannose receptor expression by the hormone $1,25(OH)_2D_3$ is associated with an improvement in endocytosis capacity as this receptor is involved in antigen uptake (Piemonti et al., 2000). Furthermore, $1,25(OH)_2D_3$ also modulates the expression of cytokines and chemokines derived from DCs by inhibiting the production of IL12 and IL23 (which lead to differentiation of naïve T cells into Th1 and Th17, respectively) and increasing the secretion of the anti-inflammatory cytokine IL10 (Pedersen et al., 2009; Penna and Adorini, 2000; Tang et al., 2009). IL10 induction promote Treg cells production and support tolerance to immunity (Penna et al., 2007b, 2007a).

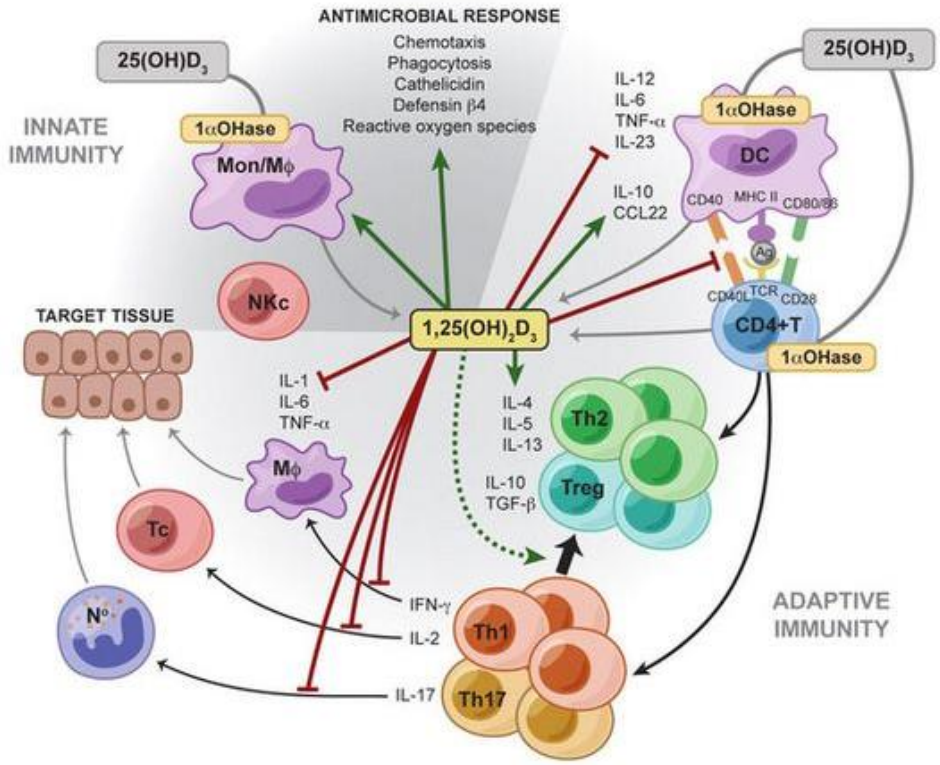


Figure 11. Immunomodulatory effects of $1,25(OH)_2D_3$ (Baeke et al., 2010a).

8.3.2. Effects on the adaptive response

T and B lymphocytes have been shown to express more VDR when they are activated and therefore also regulated, in part by $1,25(OH)_2D_3$ (Baeke et al., 2010b; Provvedini et al., 1983; Veldman et al., 2000). VDR expression levels appear to vary according to the activation stimulus so that there are different results on the effects of vitamin D on T cell proliferation (Baeke et al., 2010b; Correale et al., 2009; Lacey et al., 1987). $1,25(OH)_2D_3$ alters T cell cytokine profiles by inhibiting the production of inflammatory Th1 cytokines (IL2 and IFN-γ) and Th17-derived cytokines (IL17, IL21, and IL22)(Colin et al., 2010; Ikeda et al., 2010; Jeffery et al., 2009; Mahon et al., 2003; Tang et al., 2009). The effect on Th2 response is not so clear but some studies suggest that vitamin D may be promoting the Th2 response by positively regulating the expression of GATA-3 and c-maf specific transcription factors and increasing production of cytokines such as IL4 (Boonstra et al., 2001; Mahon et al., 2003). $1,25(OH)_2D_3$ inhibits B cell proliferation and differentiation and promotes apoptosis. It also inhibits memory B cell generation and IgG and IgM immunoglobulins secretion (Chen et al., 2007; Provvedini et al., 1983). In an *in vitro* study with activated B cell, treatment with $1,25(OH)_2D_3$ significantly increased IL10 production (Heine et al., 2008).

8.3.3. Could vitamin D play a prominent role in CanL?

Although vitamin D has potent immunomodulatory properties that have promoted research on its function in response to infections and its potential use in the prevention and treatment of diseases (Liu et al., 2006a), studies linking vitamin D and leishmaniasis are sparse and contradictory (Bezerra et al., 2019a; Mukhopadhyay et al., 2015; Ramos-Martínez et al., 2013). In addition, studies about vitamin D involvement in immune system have been conducted primarily in human and rodent models, so available information on the effect of vitamin D on canine immune response is limited.

In studies of cutaneous leishmaniasis it was observed that vitamin D deficiency causes an increase in resistance to *L. major* and *L. amazonensis* infection (Bezerra et al., 2019a; Ehrchen et al., 2007; Whitcomb et al., 2012). Instead, mice infected with *L. mexicana* and treated with $1,25(OH)_2D_3$ showed a reduction in lesion size and a decrease in proinflammatory cytokine production (Ramos-Martínez et al., 2013). On the other hand, pre-treatment with vitamin D showed parasitological protection in mice infected with promastigotes of *T. cruzi*, a protozoan that also belongs to Trypanosomatidae family (Silva et al., 1993).

The anti-parasitic activity of vitamin D is supposed to be mediated by CAMP. A study conducted in CAMP knock-out mouse model showed that cathelicidin influences the control of *L. major* infection (Kulkarni et al., 2011). The bovine AMP cathelicidin (BMAP-28) showed activity against *L. major* amastigotes in a human macrophages cell line (Lynn et al., 2011).It has also been determined that the gene of this peptide with anti-*Leishmania* activity (*CAMP*) is a direct target of VDR and is heavily regulated by $1,25(OH)_2D_3$ in human macrophages (Gombart et al., 2005). A study conducted with samples from patients with post-kala-azar dermal leishmaniasis indicates that CAMP increases anti-*Leishmania* activity of macrophages in a VDR-dependent manner (Das et al., 2017). Although it is known that cathelicidin has an immunomodulatory and microbicide role in cutaneous infections, its role in VL remains uncertain.

Although no information is available linking CanL to vitamin D, there are indications that may be playing a role in the canine immune response. As in humans, a relationship between hypovitaminosis D and cancer risk has been described in dogs (Selting et al., 2016; Wakshlag et al., 2011). It has also been shown that dogs with spirocercosis which undergoing neoplastic transformation have lower 25(OH)D levels compared to those without neoplasia and healthy animals (Rosa et al., 2013). Studying biomarkers of babesiosis they found that there was differential expression of proteins that are part of vitamin D pathway metabolism in dogs suffering this parasitosis (Kuleš et al., 2014).

Considering the large differences that may exist in the immune response between species, the pathway and mechanism of action of vitamin D in the response to CanL could be quite different. For example, in bovine PBMCs vitamin D modulates the immune response by increasing NO production (Waters et al., 2003), but cathelicidin genes are not found to be increased in the presence of $1,25(OH)_2D_3$ in bovine monocytes (Nelson et al., 2010). In contrast, β-defensin genes are found to be regulated by vitamin D pathway in bovine macrophages (Merriman et al., 2015). In dogs, β-defensin 103 (cBD103) was shown to have antimicrobial activity against the *Bordetella bronchiseptica* pathogen (Erles and Brownlie, 2010) and was found to be slightly down-regulated in the skin of atopic dogs (van Damme et al., 2009).

9. CONTROL OF ZOONOTIC VISCERAL LEISHMANIASIS

Because there is no effective vaccine against VL, control of the disease in endemic areas is primarily based on the early diagnosis and treatment (Chappuis et al., 2007). Nonetheless,

control of VL must take into account the other components of the parasite's epidemiological cycle: the vector and the reservoir.

9.1. Vector and reservoir control

Avoiding the vector bite and controlling the reservoir is one way to prevent infection. Vector control is a complex task due to the diversity of species and the cost of measurements. Vector control measures are based on the introduction of phlebotomies traps (Reithinger and Davies, 2002), the use of mosquito nets and the fumigation with pyrethroids (Davies et al., 2003). The strategies of using mosquito nets and systematically fumigation protects in the short term but when the measure is abandoned the prevalence of leishmaniasis returns to initial levels (Davies et al., 1994).

Until now, human leishmaniasis control programs in Brazil have focused on the elimination of seropositive dogs (Dantas-Torres et al., 2019). While it is true that reservoir control has proven to be an effective way to reduce the spread of this zoonosis (Palatnik-de-Sousa, 2012), slaughtering *Leishmania* seropositive dogs is a practice that presents an ethical dilemma and has not proven to be entirely effective in reducing the incidence of the disease in humans (Ashford et al., 1998; Courtenay et al., 2002; Dietze et al., 1997; Grimaldi et al., 2012; Moreira et al., 2004; Reithinger and Davies, 2002). Preventive methods, such as the use of insecticides and vaccination of companion dogs, in addition to the treatment of infected animals, are common procedures in veterinary medicine in southern Europe that help to control the reservoir (Otranto and Dantas-Torres, 2013; Podaliri Vulpiani et al., 2011; Vulpiani et al., 2011). Vaccination of dogs also reduces the incidence in humans, but currently the ideal vaccine does not exist (Velez and Gállego, 2020). Pharmacological treatment of infected dogs could temporarily reduce their infectivity, as it decreases the parasitic load (Henao et al., 2004), but the high number of recurrences and some maintenance of post-therapy infectivity make it not a completely feasible control measure (Saridomichelakis et al., 2005).

The most widely used insecticides to prevent vector bites in dogs are pyrethroids, such as permethrin (Mercier et al., 2003; Miró et al., 2007; Molina et al., 2006; Otranto et al., 2007) or deltamethrin (David et al., 2001; Ferroglio et al., 2008; Halbig et al., 2000; Killick-Kendrick et al., 1997). The most common is to use deltamethrin formulated in collars as it is more comfortable and lasts longer (Reithinger et al., 2001), but also can be used topically insecticides. The use of these repellents has been shown to protect dogs from sandflies bites (Fernandez et al., 2018; Maroli et al., 2001) and to reduce the incidence of human VL (Mazloumi Gavgani et al., 2002; Otranto et al., 2007).

9.2. Pharmacological treatments

Currently there are several treatment options available for leishmaniasis. The effectiveness and choice of treatment may depend on the immune response, parasite species, geographic area, or socioeconomic status. Chemotherapeutic compounds are not particularly cheap, can have adverse effects, and there can often be resistance problems (Alvar et al., 2006b; Baneth and Shaw, 2002). Using the same drugs to treat canine and human VL increases the risk of resistance, as it should be keep in mind that CanL therapies do not always achieve absolute elimination of the parasite (Faraut-Gambarelli et al., 1997).

The first-line drugs for VL are pentavalent antimonials (sodium stibogluconate and meglumine antimony). Amphotericin B deoxycholate (AmB) is considered a second-line drug and miltefosine is considered the third-line treatment. In veterinary medicine, CanL treatment is also based on pentavalent antimonials or miltefosine, but often combined with allopurinol. Allopurinol should be given with other drugs for the first month and then continued as the only treatment (Solano-Gallego et al., 2011). Allopurinol is rarely used in human VL therapies and it is therefore a good option for the treatment of CanL, however there are resistances to this compound and it is responsible of relapses after treatment (Yasur-Landau et al., 2016),in addition to its low efficacy when used as monotherapy (Koutinas et al., 2001). The clinical efficacy of antimonials reaches values of 67–100 % but may have adverse effects such as nephrotoxicity and their prolonged use generates resistance (Alvar et al., 2004; Carrió and Portús, 2002; Gramiccia et al., 1992; Noli and Auxilia, 2005). Miltefosine has no renal or hepatic toxicity and has the advantage of being administered orally, but recurrences may occur (Manna et al., 2015; Mateo et al., 2009). The use of AmB is very rare in veterinary medicine, because although it induces clinical improvement in 85–100 % of treated dogs (Noli and Auxilia, 2005), there is a high risk of recurrence (Cortadellas, 2003; Moreno et al., 1999b) and has the drawback of being very nephrotoxic. The liposomal formulation is less toxic than the free form (Lamothe, 2001; Oliva et al., 1995), but it is also not commonly used because it requires intravenous administration and because of the high cost (Alvar et al., 2006b).

9.3. Immunotherapy

Because the outcome of *Leishmania* infection depends largely on the immune response of the host (Hosein et al., 2017; Khadem and Uzonna, 2014; Paltrinieri et al., 2010), activation of the host immune system may play a protective role and promote infection control (Singh and Sundar, 2014; Taslimi et al., 2016). After seeing that immunocompromised VL patients did not respond to drugs (Fernández-Guerrero et al., 1987) and following evidence of the potential of

immunotherapy in clinical practice, numerous studies have been conducted over the past years with the aim of improving the immune response to *Leishmania* infection. Even though immunotherapy does not directly attack the pathogen, as other drugs would, it is an attractive strategy that receives great attention because it is cost-effective, does not generate resistance and reduces side effects (Dayakar et al., 2019; Singh and Sundar, 2014). Among the different immunotherapeutic approaches, research on cytokines, TLR receptor agonists, dietary nucleotide, host defence peptides and the therapeutic use of vaccines stand out.

The P-MAPA protein aggregate, known to activate TLR2 receptors (Fávaro et al., 2012), demonstrated a decrease in parasitic load and clinical improvements in dogs with leishmaniasis (Santiago et al., 2013). The acylated synthetic AMP Oct-CA(1-7)M(2-9) was also shown to be safe and effective in treating CanL (Alberola et al., 2004). Several studies that have evaluated the use of vaccines, marketed or in development, for the clinical treatment of CanL have resulted in a clinical improvement, which is more evident in cases of mild or moderate disease (Borja-Cabrera et al., 2004, 2010; Toepp et al., 2018; Trigo et al., 2010) and, in some cases, it is accompanied by a reduction of the parasitic load (Ferreira et al., 2014; Roatt et al., 2017; Santos et al., 2007; Viana et al., 2018).

There are currently two marketed compounds with immunomodulatory properties to treat CanL: Leisguard® and Impromune® (Baxarias et al., 2019). The first is domperidone, an antagonist of dopamine D2 receptor that can boost the immune response by modulating the effect of prolactin. It enhances the activity of phagocytic cells such as monocytes, macrophages and neutrophils and helps to establish a predominantly Th1 immune response (Gómez-Ochoa et al., 2009; Lladró et al., 2017; Passos et al., 2014; Sabaté et al., 2014). The HMA (Head of Medicine Agencies) especially recommends it as a preventive measure to reduce the risk of developing an active infection in seronegative healthy dogs and also as an improvement treatment in cases of mild disease (Baxarias et al., 2019). Impromune® is part of the group of AHCC compounds (dietary nucleotides and active hexose dietary compound), which have been shown to promote the phagocytic activity of macrophages and T lymphocytes (Van Buren et al., 1985; Jyonouchi et al., 1994, 1996; Navarro et al., 1999). It has also been described to promote NK cell activity, macrophage proliferation, and T lymphocyte differentiation toward Th1 type (Aviles et al., 2008; Lee et al., 2012). Studies performed in dogs show that the combination of meglumine antimoniate with an immuno-modulatory diet achieves an improvement in clinical signs (Cortese et al., 2015) and the same efficacy as when combined with allopurinol (Segarra et al., 2017). It is a good alternative for dogs with

leishmaniasis that have adverse effects to allopurinol, such as urolithiasis or renal mineralization (Koutinas et al., 2001; Segarra et al., 2017).

9.4. Current vaccines

Preventive vaccine for CanL have limited efficacy (Velez and Gállego, 2020) and there are no vaccines marketed for human use. There are two candidates in development to prevent human VL. Both are recombinant protein vaccines: LEISH-F3 and MuLeVaClin (ClinicalTrials.gov 2020; European Commission 2015).

There are currently 3 vaccines marketed to prevent CanL: one in Brazil (Leish-Tec®) and two in Europe (CaniLeish® and LetiFend®). Leish-Tec® is formulated with recombinant protein A2 from *L. donovani* amastigotes and contains saponin as an adjuvant. The license was obtained in Brazil in 2007. CaniLeish® is composed of purified protein extracts secreted by *L. infantum* and it also contains saponin as an adjuvant. It was approved in 2011. LetiFend® is a recombinant vaccine containing a chimeric protein composed of 5 antigenic fragments from 4 different proteins of *L. infantum* (the ribosomal proteins LiP2a, LiP2b and LiP0 and the histone H2A), to which any adjuvant has been added. It was licensed in Europe in February 2016. Until 2014 there had been another licensed vaccine to prevent CanL (Leishmune®), it was a second generation vaccine registered in Brazil in 2004, but it was withdrawn by the Brazilian Ministry of Agriculture for lack of evidence of efficacy in phase III trials (Velez and Gállego, 2020).

After years of using these vaccines to prevent canine leishmaniasis, doubts remain about their efficacy in preventing infection or disease, as well as their potential to reduce risk of transmission. The efficacy of commercialized vaccines differs according to the criteria applied but would be around 58.1–80.8 % in the case of LeishTec® (Grimaldi et al., 2017). The first field study using CaniLeish® showed effectiveness in preventing clinical signs of 68.4 %. It should be noted that antibodies induced against this vaccine have subsequently been detected to cross-react with the commonly used serological test for the diagnosis of natural *L. Infantum* infection (Velez et al., 2020). The overall efficacy of LetiFend® was described as 72 % in the prevention of confirmed cases of leishmaniasis in endemic areas (Fernández Cotrina et al., 2018). But even though the phase III clinical trial included 549 dogs, at the end of the study had developed leishmaniasis only 4.7 % of dogs in the vaccine group (n = 8) and 10.2 % of dogs from control group (n = 19). Despite the low number of infected animals, the differences were statistically significant.

These data show the imminent need to continue developing vaccination strategies against CanL that will improve the results of the vaccines that are available on the market and highlight the urgency of finding elements to enhancing protective effectiveness.

10. DNA VACCINES

Most vaccines available against infectious agents can be grouped into 3 categories: first-generation vaccines (live, attenuated or killed parasite), second-generation vaccines (recombinant protein or purified fractions) and third-generation vaccines (DNA or RNA vaccines).

Third-generation vaccines based on the administration of DNA sequences are a very useful tool in the development of vaccines. This strategy consists of inserting DNA sequences encoding one or more antigens of interest into a vector plasmid. Following DNA vaccine administration, antigen-presenting cells are able to express the protein of interest (Wolff et al., 1990), which will be processed and stimulate the cellular and humoral immune response (Huygen, 2005). In addition, plasmid vectors act as immunological adjuvants due to the presence of unmethylated CpG motifs in prokaryotic DNA. These bacterial sequences trigger the activation of TLR9 in antigen presenting cells (Hemmi et al., 2000) and increase the Th1 response by promoting non-specific IFN-γ production and the $CD4^+$ and $CD8^+$ response (Gurunathan et al., 2000a; Huygen, 2005). In *L. major*-infected BALB/c mice, treatment with CpG oligodeoxynucleotides besides increasing IFN-γ also inhibited IL4, a Th2 cytokine (Zimmermann et al., 1998). The suitability of DNA vaccines is also based on the low cost of production and ease of storage, as they are thermostable vaccines that do not need to be stored at low temperature. In addition, because they do not use pathogens in the inoculum, they are safe non-virulent vaccines (Gurunathan et al., 2000a).

DNA vaccines have been very successful in many pre-clinical studies, showing to be safe and immunogenic in rodent animal models (Feng et al., 2020; Yi et al., 2017). Endogenous production of antigens by vaccine-transfected host cells can elicit strong humoral and cellular immune responses. That is why the idea of using DNA to immunize animals and people immediately gained widespread recognition and quickly led to clinical trials (Villarreal et al., 2013). The first phase I clinical trial, which was performed to evaluate a DNA vaccine, was to combat human immunodeficiency virus (HIV-1) infection (MacGregor et al., 1998). Other DNA-based vaccine trials would soon follow, including vaccine trials against other HIV antigens, hepatitis B and malaria (Boyer et al., 1996; Kwissa et al., 2000; Wang, 1998). These early assays established that DNA vaccines are tolerable in humans and can induce T cell proliferation and

activate cytotoxic T cells. However, the immune responses detected in non-human primates and humans have been shown to be weaker than expected based on pre-clinical data (Catanzaro et al., 2007; Graham et al., 2006; Villarreal et al., 2013). This insufficient immunogenicity in primates, both by intramuscular and intradermal injection, is due to inefficient uptake of plasmid by host cells (Suschak and Schmaljohn, 2019). For this reason, improvements in delivery methods are needed, such as electroporation (Hannaman et al., 2016; Hooper et al., 2014) or use of nanoparticles (Farris et al., 2016; Fotoran et al., 2017; Tian et al., 2018). A large number of clinical trials of DNA vaccines have recently been conducted using different improvement strategies to fight against some cancers and also against various infectious diseases such as Ebola, AIDS and tuberculosis (Elizaga et al., 2018; Harris et al., 2013; Sarwar et al., 2015; Yang et al., 2014).

In relation to leishmaniasis, several DNA vaccines have been tested but in mice and hamster models (Aguilar-Be et al., 2005; Fragaki et al., 2001; Gomes et al., 2007, 2008; Marques-da-Silva et al., 2005; Zanin et al., 2007), being mostly immunogenic and protective with efficiencies of 63 %–99 %. In dogs, only two studies have been published. A multigenic vaccine against CanL was found to be immunogenic in healthy dogs, but its efficacy against infection was not tested (Saldarriaga et al., 2006). The only study that evaluated the efficacy of a DNA vaccine against an experimental infection in dogs did not observe parasitological protection (Rodríguez-Cortés et al., 2007c). Despite the wide variety of possible vaccination strategies and the countless attempts made, the ideal leishmaniasis vaccine has not been achieved.

10.1. Genes used in DNA vaccines: our vaccine approach

Our research group has previously studied the effect of a naked DNA vaccine based on a pVAX plasmid vector carrying the following *Leishmania* genes: Tryparedoxin Peroxidase (*TRYP*), *Leishmania* homolog of receptors for Activated C kinase (*LACK*), Potentially Aggravating Protein of *L. infantum* (*PAPLE22*) and Kinetoplastid Membrane Protein-11 (*KMPII*) (Todolí et al., 2012). In previous studies, these antigens, especially PAPLE22 and TRYP, showed to be recognized by T cells in *Leishmania*-infected dogs (Todolí et al., 2010). It was hoped that the use of a multicomponent DNA vaccine encoding these *Leishmania* antigens improve the spectrum of immune response. The vaccine, however, did not induce a good immunogenic response when administered alone to hamsters, although it showed protection when the prime-boost strategy was used (Todolí et al., 2012). The same DNA vaccine co-administered with the metabolic modulator rapamycin was shown to produce a significant decrease in parasitic load on skin, lymph nodes, and spleen in experimentally infected hamsters (Martínez-Flórez et al., 2020).

We believed it is worth exploring the use of the 4 exposed *Leishmania* antigens in order to obtain an appropriate vaccine for the prevention of leishmaniasis. The effectiveness of DNA vaccines could be improved by using an appropriate adjuvant or a new method of plasmid delivery (Kulkarni et al., 2017; Sin, 2000).

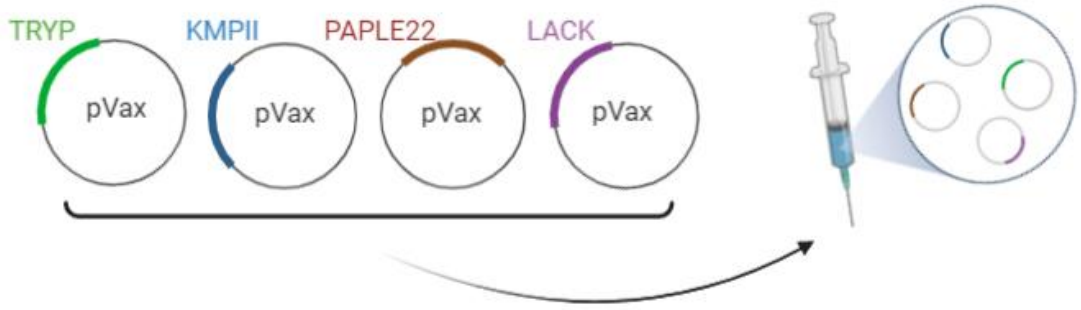


Figure 12. Vaccine designed in our laboratory: *L. infantum TRYP*, *KMPII*, *LACK* and *PAPLE22* gene sequences inserted in pVAX plasmids.

KMPII is a membrane antigen, abundantly present in *Leishmania spp.* (Tolson et al., 1994). It is expressed mainly in the promastigote phase (Berberich et al., 1998; Lynn et al., 2013). It could be involved in parasite mobility, binding to the host cell (Thomas et al., 2000), in membrane stabilization (Jardim et al., 1995) and in resistance to antimonials (El Fadili et al., 2009). Several studies show that it is a potent immunogen to B cell (Berberich et al., 1997; de Carvalho et al., 2003; Jensen et al., 1998; Ramírez et al., 1998; Trujillo et al., 1999) and to T lymphocytes (Basu et al., 2007; Delgado et al., 2003; Jensen et al., 1998; Kemp et al., 1993; Kurtzhals et al., 1994), also playing an important role in infectivity and as a virulence factor (de Mendonça et al., 2015).

LACK is a highly conserved antigen among *Leishmania* parasites (Sinha et al., 2013), and is widely known for being recognized by T cells and promoting non-protective Th2 in the murine model infected with *L. infantum* (Launois et al., 1997; Schilling and Glaichenhaus, 2001). It is located in the cytosol close to the kinetoplast, and possibly interacts with sequences from proteins involved in DNA replication and RNA synthesis (Gonzalez-Aseguinolaza et al., 1999). In addition, LACK plays an essential role for infection viability (Kelly et al., 2003). In human VL patients is able to stimulate B lymphocytes (Maalej et al., 2003) and induces a mixed pattern of Th1/Th2 cytokines in CL patients (Antonelli et al., 2004; Bottrel et al., 2001; Bourreau et al., 2002; Carvalho et al., 2005; Maasho et al., 2001).

TRYP, initially known as TSA protein, due to its resemblance to eukaryotic thiol-specific-antioxidant protein (Webb et al., 1998), is an enzyme with an important role in the protection

of the parasite from oxidative damage by catabolizing hydrogen peroxide (Levick et al., 1998). It is highly conserved across *Leishmania* species. It has been known to participate in parasite resistance to antimonial drugs (Henard et al., 2014; Iyer et al., 2008; Wyllie et al., 2008) and stimulates T lymphocytes (Webb et al., 1998).

PAPLE22 is an endogenous nuclear protein. It acts as immunogen on B lymphocytes in VL patients (Suffia et al., 2000) and also in dogs naturally infected (Fragaki et al., 2001). It is known to contribute to VL pathogenesis and immunosuppression through IL10 production (Suffia et al., 2000).

10.2. Enhancing DNA vaccines

10.2.1. Formulation in liposomes

It is well documented that liposomes are very useful as drug and vaccine delivery vehicles. Today liposomal formulations are trending. Initially, most work focused on its use for gene and drug delivery (Jha et al., 2016; Jiang et al., 2016; Li et al., 2019). Already licensed liposomal formulations, such as Doxil® and AmBisome®, have minimized the non-specific toxicity of treatments. The development of two commercial liposomal vaccines (Inflexal® V and Epaxal®) against influenza and hepatitis A viruses, respectively, increased the interest of these vesicles as vaccine delivery systems. They are not only useful as administration vehicles for directing molecules to antigen-presenting cells, they have also been shown to be immunostimulators (Christensen et al., 2007; Henriksen-Lacey et al., 2011; Kelly et al., 2011).

Liposomes may have different pharmacokinetic characteristics depending on their size, composition, and charge. Very large liposomes (> 500 nm) are only captured by DCs at the injection site, whereas only the smallest liposomes diffuse freely into the lymph nodes, where they can be phagocytosed by DCs and macrophages (Manolova et al., 2008). In contrast, the phagocytosis process would be more efficient in large particles (Thiele et al., 2001). Membrane fluidity can also influence the immunogenicity of these particles. It seems that the induction of the humoral immune response by liposomes would be achieved with an intermediate fluidity of the lipid bilayer (van Houte et al., 1981). Incorporating cholesterol in lipid bilayer composition allows to modulate the fluidity of the membrane and obtain more stable particles (Gregoriadis and Davis, 1979; Kaddah et al., 2018). The polydispersity index (PDI), useful for defining the particle size distribution, can have numerical values ranging from 0 (for a perfectly homogeneous sample) to 1 (for a highly polydisperse sample with particles of multiple sizes). A sample with a PdI above 0.7 is considered to have too wide particle size Distribution (Danaei et al., 2018). Regarding the zeta potential (ζ-potential), which represents the value of the

electrostatic potential at the interface, values between -30 mV and +30 mV are considered to be representative of stabilized particles (Vogel et al., 2017). Cationic liposomes also enhance antigen presentation and result in a stronger cellular immune response, characterized by increased production of IFN-γ and IL17 and proliferation of specific T cells (Henriksen-Lacey et al., 2010a). Cationic liposomes on their own have been shown to be able to stimulate antigen-presenting cells (Vangasseri et al., 2006) and are better phagocytosed by macrophages (Ahsan et al., 2002). Cationic liposomes also effectively protect plasmid DNA molecules from enzymatic degradation in host cells (Crook and K., 1996).

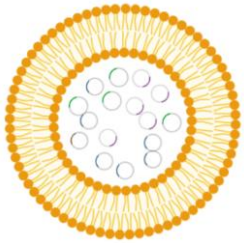


Figure 13. Vaccine designed in our laboratory encapsulated in liposomal nanoparticles.

10.2.2. Immunomodulatory adjuvants

Given the importance of the host immune response in the outcome of *Leishmania* infection, immunomodulatory substances may be very useful in redirecting the immune response toward infection control and enhancing the prophylactic effect of vaccines (Taslimi et al., 2016).

It is common to use interleukins and other immunomodulatory compounds as vaccine adjuvants (Li and Petrovsky, 2017). Numerous studies have been conducted combining vaccine strategies against leishmaniasis with adjuvants, achieving variable successes (Raman et al., 2012; Roatt et al., 2014). The immune response has been induced by cytokine IL12 (Afonso et al., 1994), aluminium salts (Misra et al., 2001), saponins (Borja-Cabrera et al., 2004; Santos et al., 2002), the TLR4 agonist monophosphoryl lipid A (MPL) (Bertholet et al., 2009; Goto et al., 2007), the TLR7/8 agonist imiquimod (Zhang and Matlashewski, 2008)and by rapamycin (Martínez-Flórez et al., 2020), increasing the immunogenic power of the vaccine in question.

The main problems related to the choice of a suitable adjuvant for vaccination are physicochemical incompatibilities with the vaccine content or with other adjuvants, and undesirable reactions associated with its administration (Mutiso et al., 2013; Raman et al., 2012).

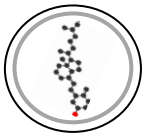
10.2.3. Vitamin D as a combined immunomodulator for CanL prophylaxis

Vitamin D metabolites and their analogues can be purchased in multiple formats. Due to the breadth of its actions it has been recommended and prescribed to prevent and treat diseases such as osteoporosis, kidney disease, hyperparathyroidism, multiple sclerosis or prostate cancer (Jagannath et al., 2018; Trump and Aragon-Ching, 2018; Zand and Kumar, 2017).

Vitamin D strengthens the innate immune system by inducing the expression of peptides that perform defence actions and modulates the adaptive response, changing from a Th1-mediated T cell response to a Th2 response (increased levels of IL4, IL5, IL10 and reduced levels of IL2, IFN-γ, and TNF-α), thus reducing inflammation and pathology (Baeke et al., 2010c; Hansdottir et al., 2010; Hart et al., 2011; Jo, 2010; Di Rosa et al., 2011; Scott and Hancock, 2000). Taken together, current information and knowledge about the regulatory effects of vitamin D on the immune system suggest that it may be useful as adjunctive therapy in vaccination strategies, stimulating the protective immune system and thus enhancing the effect of the vaccine.

Some investigations have examined the influence of vitamin D or its derivatives in response to immunogens vaccines. Several studies have been performed with the influenza vaccine, but they have been partly discrepant and inconclusive (Kriesel and Spruance, 1999; Lee et al., 2018; Sundaram et al., 2013). In a study, humoral immunity was not improved when administering calcitriol together with influenza vaccine in human volunteers (Kriesel and Spruance, 1999). However, lower seroprotection rates have been observed for strain A (A/H3N2) and strain B of influenza virus in patients with vitamin D deficiency compared to patients with normal vitamin D levels (Lee et al., 2018). In addition, Goncalves-Mendes et al. observed a significant decrease in the Th1/Th2 ratio in relation to the reduced levels of TNF-α and IL6 in the group of people who received cholecalciferol prior to vaccination (Goncalves-Mendes et al., 2019). There have also been several researches to combine vitamin D with BCG vaccine against tuberculosis, as it is widely accepted the protective effect of vitamin D against *M. tuberculosis* infection (Wilkinson et al., 2000). One research found that there was a strong inverse association between the IFN-γ response to the PPD (purified protein derivative) skin test against *M. tuberculosis* and vitamin D concentration (Lalor et al., 2011). There are also results indicating that the response to PPD is higher when the vaccine is combined with vitamin A and vitamin D supplements (Zheng et al., 2014).

In this work, the immunomodulatory compound 1α,25-dihydroxyvitamin D_3 was used for the first time to increase the immune response induced by the DNA vaccine against *Leishmania* described above.



HYPOTHESIS AND AIMS

Vitamin D plays a role in CanL progression and its serum concentration could be used as a prognostic marker for this disease. Vitamin D is involved in immune response during CanL, it exerts a parasiticidal effect in *Leishmania*-infected canine macrophages and regulates immune response of these immune cells. If vitamin D is involved in CanL immune response, it could be exploited as a new adjuvant in a vaccination strategy.

AIMS

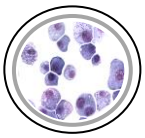
The overall aim of this thesis has been to evaluate if vitamin D serum concentration is associated with CanL progression and weather it could be a prognosis or clinical marker for this disease. It also aims to study the mechanism of action of vitamin D in canine macrophages during *L. infantum* infection and determine if vitamin D could have a therapeutic application. We established 4 different objectives to tackle these topics:

1. **To study whether vitamin D concentration is associated with CanL progression**
   1.1. To determine vitamin D concentration from a cohort of healthy and diseased dogs from a highly endemic area
   1.2. To evaluate the potential correlation between vitamin D serum concentration and the *Leishmania*-specific immune response and the parasitological status of animals
   1.3. To investigate the genetic association of a single nucleotide polymorphism in the *VDR* gene to CanL susceptibility

2. **To determine if there was a vitamin D deficiency prior to develop clinical canine leishmaniasis and if vitamin D levels could be a prognostic or clinical marker for this disease**
   2.1. To evaluate weather vitamin D concentration shows a seasonal variation in dogs
   2.2. To determine vitamin D concentration in a cohort of dogs at the moment of *Leishmania* diagnosis and one-year earlier
   2.3. To determine expression of genes involved in vitamin D pathway at the time of diagnosis of CanL and a year earlier

3. **To study the mechanism of action of vitamin D in canine macrophages during *L. infantum* infection**
   3.1. To evaluate the effect on parasite load of vitamin D pretreatment in *Leishmania*-infected canine macrophages cell line (DH82)
   3.2. To identify genes that vitamin D activates in primary canine macrophages and their role in *L. infantum* control

4. **Use of vitamin D as immunomodulator during a vaccination protocol to boost protection against visceral leishmaniasis in a rodent model**
   4.1. To test the immunogenicity of a DNA *Leishmania* vaccine formulated in liposomal nanoparticle
   4.2. To evaluate if there is an increase of efficacy lead by the vitamin D coadministration



CHAPTERS

CHAPTER 1

Cross-sectional study of vitamin D and canine leishmaniasis

Summary of the experimental approach

This chapter is destined to tackle the first objective of this work:

"To study wether vitamin D serum concentration is associated with canine leishmaniasis progression"

Some studies support that innate immune system play a relevant role by promoting the appropriate inflammatory response against the parasitic invasion. It is currently known that vitamin D strengthens the innate immune system. Besides, recent studies have shown a direct effect of vitamin D on T and B cells, where it reduces the over production of pro-inflammatory Th1 and Th17 derived cytokines B-cell differentiation, and IgG secretion. To investigate if there is an association between vitamin D status and CanL progression, we measured the vitamin D concentration in serum samples from a cohort of 68 healthy and suffering from leishmaniasis dogs from a highly endemic area and we have also studied the relationship of these levels with parasitological and immunological parameters. We also investigated the genetic association of a single nucleotide polymorphism in the vitamin D receptor gene to CanL susceptibility.

MATERIAL AND METHODS

Dogs and samples

All procedures were approved by the Universitat Autònoma de Barcelona's Ethical Committee of Human and Animal Experimentation (Spain) in compliance with national (Royal Decree 1201/2005) and European Union regulations (European Directive 86/609/CE) for projects using animals for research purposes. Sixty-eight dogs attended at the Mon Veterinari Clinical Hospital of Manacor (Mallorca, Spain) were included in the study after their owners' consent. The sample included 31 females and 38 males from different breeds, and ages ranged from 6 months to 15 years (4 ± 2.7 years). Prior to sampling, all dogs were examined for clinical signs compatible with CanL, such as weight loss, lymphadenopathy, cutaneous lesions, and ocular, gastrointestinal, and renal alterations. Blood samples were collected by jugular venipuncture. Serum was obtained after centrifugation at 3 000 rpm for 20 min and stored at −20 °C until further use. Blood-EDTA samples used for *Leishmania* DNA detection were frozen at −20 °C before DNA extraction, and blood-heparin samples were analyzed before 24h.

Clinical signs, biochemistry, and hematological values were scored as previously described with some modifications (Rodríguez-Cortés et al., 2007a). Briefly, clinical signs compatible with leishmaniasis such as cutaneous lesions, ocular signs, and epistaxis were scored 0–3 as indicated in Table 3. Both biochemistry and hematological results scored 1 point for each abnormal value. These scores were added to obtain an overall clinicopathological score for each dog. A score greater than 5 was considered as indicative of patent clinical leishmaniasis.

Table 3. **Guide for scoring clinicopathological signs compatible with canine leishmaniasis.**

Parameter	Alteration	Score
Weight loss	Normal (no weight loss or the animal grows normally)	0
	Weight loss < 10 %	1
	Weight loss between 10 and 20 %	2
	Weight loss > 20 %	3
Cutaneous lesions (Score for each cutaneous clinical sign: erythema, alopecia…)	Normal	0
	Slightly generalized (< 10 % of the body)	1
	Moderately generalized (< 25 % of the body)	2
	Generalized (> 25 % of the body)	3
Lymphadenopathy	Absence	0
	Localized	1
	Generalized	2
Renal disorder	Absence	0
	Polyuria	2
Gastrointestinal disorder	Absence	0
	Diarrhea and/or emesis	2
Splenomegaly	Small increase on spleen size	3
	Large increase on spleen size	5
Ocular	Absence	0
	Slight and Unilateral	1
	Bilateral or severe unilateral	2
	Bilateral and severe	3
Lameness	Absence	0
	Localized	1
	Generalized	2
Epistaxis	Absence	0
	Presence	2
Red blood cells	Normal	0
	Regenerative Anemia	1
	Non-regenerative anemia	2
With blood cells	Normal	0
	One hematological parameter is altered	1
	Two altered parameters	2
	3 altered parameters	3
	Consecutively	
Proteinogram	Normal	0
	One parameter is altered	1
	Two parameters altered	2
	3 parameters altered	3
	Consecutively	

Crude total *L. infantum* antigen (CTLA)-based ELISA

B cell function was analyzed by measuring anti-*Leishmania* antibody levels with an ELISA technique. Antigen used was a whole suspension of sonicated promastigotes (CTLA) provided by Dra Cristina Riera (Universitat de Bacelona, Spain). Microtiter plates were coated with 2 µg of CTLA per well in carbonate buffer 0.1 M, pH =9.6 and sequentially incubated overnight. 100 µL of serum sample at a dilution of 1/400 in phosphate buffered saline (PBS) (Sigma-Aldrich®) containing 0.05 % Tween 20 (PBST) and 1 % dry skimmed milk (PBSTM) was added to each well and incubated at 37 °C for 1 h, followed by three washes with PBST and one final wash with PBS. After, 100 µL of Protein A conjugated to horseradish peroxidase (Pierce) at working dilution of 1/15 000 was added, incubated at 37 °C for 1 h and washed as before. Peroxidase substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich®) was added at 100 µL/well and developed for 5 min at room temperature. The reaction was stopped with 50 µL of H_2SO_4 1 M and absorbance values were read at 492 nm in an automatic microELISA reader (Anthos 2001, Anthos Labtec Instruments). Results were expressed in ELISA units (EU), referred to a known positive serum used as a calibrator, and arbitrarily set to 1 EU. Cut-off value (mean + 3SD) for 76 dogs from a non-endemic area was established at 0.220 OD.

Real-Time PCR amplification of *Leishmania* DNA in blood simples

Parasite load was determined by quantitative real-time PCR (qPCR) in blood samples. DNA was extracted using the High Pure PCR Template Kit (Roche). *Leishmania infantum* DNA was specifically detected and quantified with a TaqMan® qPCR assay (Applied Biosystems™). We employed a previously reported protocol with some modifications (Francino et al., 2006). The qPCR assay targeted a conserved DNA region of the kinetoplast *L. infantum* genome. Primer sequences were LEISH-1 5'-AAC TTT TCT GGT CCT CCG GGT AG-3', LEISH-2 5'-ACC CCC AGT TTC CCG CC-3', and the TaqMan®-MGB probe FAM-5'-AAA AAT GGG TGC AGA AAT-3' - MGB. The thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min. Analyses were performed in a Step One Plus Real Time PCR System device (Applied Biosystems™). Each sample plus a negative control was analyzed in triplicate. The number of parasites per mL of blood was calculated using a standard curve generated with *L. infantum* DNA extracted from 1×10^7 parasites by using serial dilutions from 10^3 to 10^{-3} parasites. This technique was sensitive enough to detect 0.001 parasites per reaction with a dynamic range of 10^7. The median slope of three different standard curves was −3.44, and the qPCR efficiency was 98 %. Quantification was linear between 10^3 and 10^{-2} parasites per reaction tube (correlation = 0.99).

Leishmanin skin test (LST)

In vivo T cell-mediated immunity was determined in each dog by measuring delayed type hypersensitivity (DTH) response against the leishmanin reagent. The leishmanin reagent consisted of a suspension of 3×10^8 inactivated *L. infantum* (MHOM/FR/78/LEM75) promastigotes per mL in a 0.4 % phenol-saline solution. A volume of 0.1 mL of leishmanin solution was intradermally inoculated to dogs. The delayed-type hypersensitivity response was assessed by measuring the size of the indurated and erythematous area (mean of two perpendicular diameters) observed at 72 h post injection. A response against leishmanin reagent > 5 mm was considered as an LST positive result (Pinelli et al., 1994c).

Whole blood assay and cytokine detection

The *in vitro Leishmania*-specific immune response was evaluated by measuring pro-inflammatory (IFN-γ) and regulatory (IL10) cytokine levels expressed by antigen stimulated lymphocytes in whole blood assays (Shifrine et al., 1978). Blood collected in tubes containing heparin anticoagulant was diluted 1:10 in RPMI-1640 medium supplemented with 10 % v/v heat-inactivated foetal calf serum, 10 mM Hepes buffer, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Gibco, Paisley, UK). Cells were incubated in 96-well flat bottom plastic culture plates at 1.8 x 10^6 cells per well with *Leishmania* soluble antigen (LSA) (10 µg/mL), concanavalin A (ConA) (2.5 µg/mL), or media (unstimulated), for a period of 5 days at 37 °C in 5 % CO_2. Supernatants from each of the three replicate wells were pooled and stored at −80 °C until required. Quantikine ELISA kits (R&D systems) were used to detect IFN-γ and IL10 in supernatant cultures following manufacturer's recommendations. Background levels in the non-stimulated control wells were extracted from the values on the antigen-stimulated wells to quantify the antigen-specific cytokine production.

Vitamin D determination

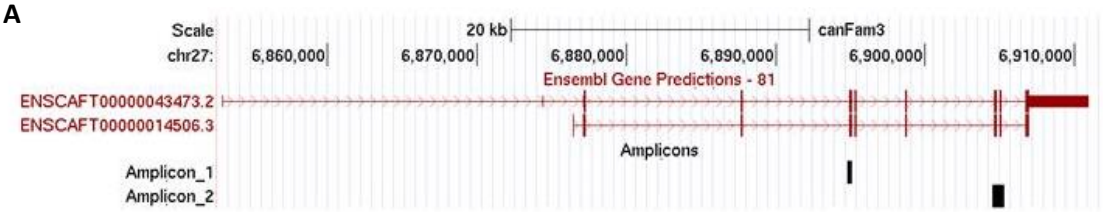
Circulating levels of 25(OH)D are considered to be the most reliable estimate of overall vitamin D status because this is a stable circulating metabolite of vitamin D and its concentration is nearly 1,000-fold higher than that of $1,25(OH)_2D_3$ (Lang et al., 2013). Thus, 25(OH)D levels were assessed in serum samples using a competitive direct enzyme-linked immuno-sorbent assay (IDS 25-Hydroxy Vitamin D Direct EIA kit, Immunodiagnostic Systems Ltd.) according to the manufacturer instructions and employing an automatic micro-ELISA reader (Anthos 2001, Anthos Labtec Instruments). Concentration of 25(OH)D in each sample was calculated using a four-parameter logistic curve fit (GraphPad Prism v3.02), and results were expressed in ng/mL

units. The threshold for 25(OH)D deficiency was set at < 20 ng/mL (< 50 nmol/L) as reported by Ross et al. (Ross et al., 2011).

Genetic variation at the Vitamin D receptor

With the aim to identify DNA variants in the *VDR* gene segregating in our dog population, primers targeting *VDR* exons 5, 8 and 9 and introns 4, 5, 7 and 8 were designed (Figure 14). These regions were PCR amplified and sequenced in ten dogs.

A



B

Amplicon	Position	Size (bp)	Exons covered	Introns covered
1	Chr27:6,894,808-6,895,100	293	5	4, 5
2	Chr27:6,904,465-6,905,288	824	8, 9	7, 8, 9

C

Amplicon	Primer name	Sequence
1	Cfa_VDR_amplicon_1_f	5'-ATCTGTTTGCCTTTAGCACCTG-3'
1	Cfa_VDR_amplicon_1_r	5'-CACCCCGCGGCTCATCGT-3'
2	Cfa_VDR_amplicon_2_f	5'-TGTCCAATCACAGCCTTGAG-3'
2	Cfa_VDR_amplicon_2_r	5'-GAGGGACCCCTCAACATACA-3'

Figure 14. Genomic location of the three amplicons used to identify and genotype genetic variation in the canine VDR gene. The genomic coordinates are based on the dog genome CanFam3.1/canFam3 assembly. (**A**) Figure shows the location of the amplicons in relation to the canine VDR exons. The upper track displays the two known and annotated transcripts of this gene. (**B**) Table showing genomic location, amplicon size and the exons and introns that are fully or partially covered by these amplicons. (**C**) Table showing the sequence of each primer from amplicon 1 and amplicon 2.

PCR reactions were prepared in a final volume of 15 µL containing 1.5 µL 10 × PCR buffer, 0.25 mM of each dideoxynucleotide, 2.5 mM $MgCl_2$, 0.3 µM of each primer, 0.75 U Amplitaq Gold DNA polymerase (ThermoFisher Scientific™), and 6–22 ng of genomic DNA. Thermal cycling conditions included an initial step at 95 °C for 10 min, and 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 62 °C (PCR amplicon 2) or 66 °C (PCR amplicon 1) for 1 min, and extension at 72 °C for 1 min. Subsequently, a final extension step at 72 °C for 7 min was carried out. Amplicons were purified with the ExoSAP-IT PCR cleanup kit (Affymetrix) and sequenced in both directions using the PCR primers and the Big Dye Terminator Cycle Sequencing Kit v1.1

(Applied Biosystems™). Sequencing reactions were run in an ABI 3730 DNA Analyzer (Applied Biosystems™) platform. Sequences were aligned with the SeqScape® v2.1.1.0 software (Applied Biosystems™). Four polymorphic positions were identified and genotyped by direct sequencing of PCR products in a cohort of 51 samples including 15 non-infected, 25 asymptomatic, and 11 symptomatic dogs

Data analysis

In the unadjusted analysis, the comparisons between dog groups were performed using the Mann-Whitney *U* Test and the correlations between different parameters by Spearman's rank correlation coefficient. We then adjusted for potential confounders between clinical leishmaniasis risk and vitamin D deficiency, considering clinical leishmaniasis as the dependent variable (outcome) and vitamin D levels, sex, age, serology, LST and cytokine levels as independent variables (predictors) using both univariate and multivariate regression analyses. To determine the genetic association of each polymorphism with the infection and disease status, the allelic frequencies of the three phenotypic groups were compared using the χ^2 test for independence. All statistical tests were performed using SPSS version 15.0 (SPSS software, SPSS Inc). A *P*-value ≤ 0.05 was considered to be significant.

RESULTS

Characterization of the canine population

Of the 68 dogs included in the study, 18 were negative for *Leishmania* serology, *Leishmania* quantitative real-time PCR (qPCR) in blood, and also for leishmanin skin test (LST), and were thus included in the group of "Non-Infected" animals (Dos-Santos et al., 2008; Maia et al., 2016). The remaining 50 dogs were positive at least one of the above tests, and they were considered as infected. Of these, 35 were clinically asymptomatic and 15 showed clinical signs of leishmaniasis.

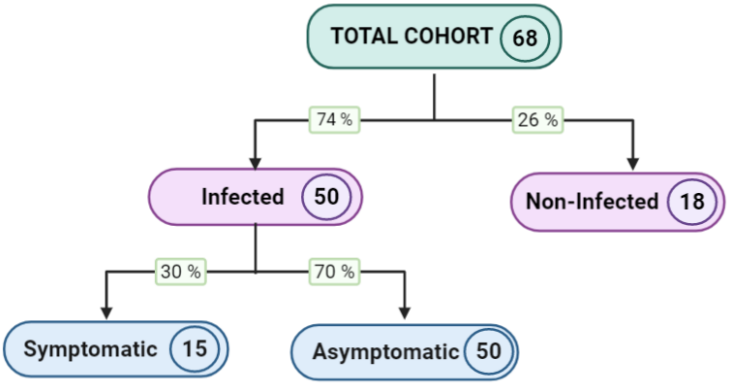


Figure 15. Classification of the animals included in the study. 50 dogs were *Leishmania*-infected and 18 were not. Among the infected dogs, 15 were sick and 24 were clinically healthy.

The baseline characteristics of the three groups are shown in Table 2. The group of dogs suffering from clinical leishmaniasis presented a median [interquartile range] clinicopathological score of 13.0 [10.50–18.50], a value that is significantly higher than the non-infected counterpart ($P < 0.001$). Their main clinical signs were cutaneous lesions and lymphadenopathy accompanied by statistically significant higher levels of β- and γ-globulins ($P = 0.016$, $P < 0.001$, respectively) and lower red blood cell counts ($P < 0.001$). On the other hand, the group of asymptomatic dogs showed a median clinicopathological score of 3.0 [1.00–4.00], a value that was non- statistically different than that from the non-infected cohort (2 [1.00–2.75]) ($P = 0.240$).

Table 4. Baseline characteristics of the dog population used in the current study.

a = statistically different from non-infected dogs ($P < 0.05$); b = statistically different from asymptomatic dogs ($P < 0.05$). [1] ALT: Alanin-amino transferase; ALP: Alkaline phosphatase; LAP: Lymphadenopathy. [2] Number of dogs from each group that showed a value out of the reference range for each one of the parameters under evaluation. Any dog of any group showed urinary signs, gastrointestinal signs, lameness or epistaxis.

PARAMETERS	GROUPS OF ANIMALS						REFERENCE RANGE
	Non-infected (N = 18)		Asymptomatic (N = 35)		Symptomatic (N = 15)		
	Median [IQR]	Positive dogs[2]	Median [IQR]	Positive dogs[2]	Median [IQR]	Positive dogs[2]	
ALT (U/L)[1]	48.5 [36.0–99.8]	5 (28 %)	42.0 [35.2–51.7]	1 (3 %)	38.0 [29.5–82.5]	2 (13 %)	**21–102**
ALP (U/L)[1]	43.3 [29.9–53.4]	0	35.2 [24.7–46.7]	0	54.0 [34.0–85.0]	2 (13 %)	**20–156**
Creatinine (mg/dL)	1.0 [0.9–1.2]	0	1.1 [0.9–1.2]	0	1.0 [0.7–1.2]	1 (7 %)	**0.5–1.5**
Urea (mg/dL)	44.4 [33.4–48.0]	0	47.9 [35.9–56.3]	0	47.2 [31.9–2.7]	2 (13 %)	**21.4–59.9**
Albumin (g/dL)	3.3 [3.0–3.8]	1 (6 %)	3.1 [2.8–3.3][a]	2 (6 %)	2.3 [1.5–2.6] [a]	11 (73 %)	**2.6–3.3**
Beta-globulin (g/dL)	1.4 [1. –1.7]	6 (33 %)	1.7 [1.5–2.0][a]	2 (6 %)	1.8 [1.6–2.1] [a]	11 (73 %)	**0.9–1.6**
Gamma-globulin (g/dL)	0.6 [0.4–0.7]	1 (6 %)	0.7 [0.6–0.8]	3 (9 %)	2.5 [1.2–4.1] [a]	13 (87 %)	**0.3–0.8**
RBC (10^6 cells/µL)	7.1 [6.5–7.6]	1 (6 %)	6.8 [6.3–7.3]	0	5.1 [4.6–6.2] [a]	10 (66 %)	**5.5–8.5**
Platelets (10^3 cells/µL)	296.0 [227.0–353.0]	2 (11 %)	286.5 [230.5–337.5]	2 (57 %)	407.5 [202.5–581.5]	4 (27 %)	**200–500**
Neutrophiles (10^3 cells/µL)	6.7 [5.19–9.9]	4 (22 %)	6.7 [5.4–7.9]	0	6.3 [4.5–7.6]	3 (20 %)	**3–11.5**
Lymphocytes (10^3 cells/µL)	2.6 [1.8–3.4]	1 (46 %)	2.6 [1.8–3.3]	1 (3 %)	1.8 [1.2–2.3] [a]	2 (13 %)	**1–4.8**
LAP[1]	—	2 (11 %)	—	8 (23 %)	—	14 (93 %)	**—**
Cutaneous	—	0	—	5 (14 %)	—	14 (93 %)	**—**
Ocular signs	—	0	—	0	—	6 (40 %)	**—**
Musculuatrophy	—	0	—	1 (3 %)	—	3 (20 %)	**—**
Anti-*Leishmania* Antibodies (EU)		0	15.9 [7.76–38.17][a]	16 (46 %)	300.0 [193.85–300.00][a, b]	15 (100 %)	**< 0.22**
Parasite Load in blood (parasites/mL blood)	0.0 [0.00–0.00]	0	0.0 [0.00–4.32][a]	12 (34 %)	12.4 [5.95–24.69][a, b]	11 (73 %)	**0**
Leishmanin skin test reaction (mm)	0.0 [0.00–0.00]	0	12.0 [0.00–22.75][a]	23 (66 %)	0.0 [0.00–0.00][b]	2 (13 %)	**< 5**

The three groups also showed statistically significant differences in the *Leishmania*-specific immunoglobulin concentration, the parasite load in blood, and the intensity of LST reactions (Table 4). Symptomatic animals developed a higher level of *Leishmania*-specific antibodies than the non-infected dogs ($P < 0.001$). Moreover, 73 % of the sick dogs were positive to qPCR (Ct threshold 39) in blood whilst only 13 % showed a positive LST reaction. In clear contrast, the asymptomatic dogs displayed a significantly weaker specific humoral response when compared to the disease group ($P < 0.001$). Moreover, 66 % of the asymptomatic animals were LST positive, but only 34 % presented detectable parasites in blood. We also measured *Leishmania*-specific cytokine production by peripheral blood cells. These results showed that the IFN-γ levels produced by asymptomatic dogs (615.3 [130.80–1579.00] pg/mL) were significantly higher than those produced by symptomatic (32.7 [1.73–125.42] pg/mL) or non-infected dogs (67.3 [0.00–208.59] pg/mL) ($P = 0.001$, $P = 0.004$, respectively). There were no differences between non-infected and sick animals in IFN-γ levels. When we compared IL10 production among the three groups of dogs, no significant differences were detected.

Vitamin D levels and canine leishmaniasis

As shown in Figure 16, dogs with patent disease presented significantly lower levels of 25(OH)D than the non-infected and asymptomatic groups ($P < 0.001$, respectively). The median [interquartile range] levels of 25(OH)D in non-infected, asymptomatic, and symptomatic dogs were 31.8 [25.95–34.65], 29.6 [24.64–40.03], and 19.6 [10.62–25.14] ng/mL, respectively.

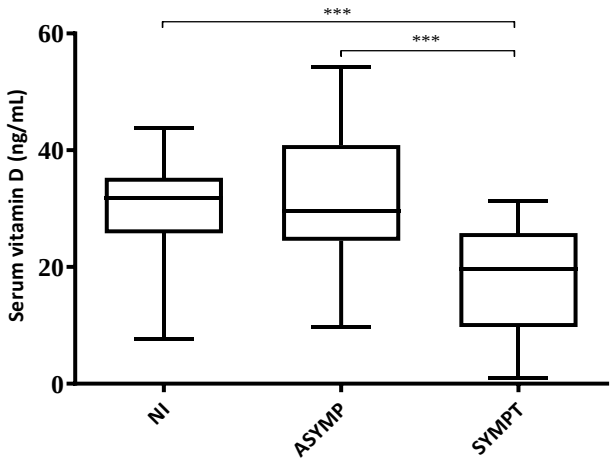


Figure 16. Serum vitamin D levels in dogs living in Mallorca and included in the study. Vitamin D status in dogs was assessed according to the serum levels of 25-hydroxyvitamin D (25(OH)D) estimated with an ELISA test. Non-infected dogs (NI), *Leishmania*-infected asymptomatic animals (ASYMP), and dogs clinically ill (SYMPT) (*** $P < 0.001$).

In addition, vitamin D levels significantly correlated with cliniopathological score ($P < 0.001$), serology ($P = 0.002$), and parasite load ($P = 0.005$) in blood samples (Figure 17). We did not find a significant correlation between 25(OH)D levels and *Leishmania*-specific IFN-γ or IL10 production. Whilst IL10 was not correlated with any of the parameters under evaluation, the IFN-γ levels were positively correlated with LST reaction (ρ Spearman = 0.386, $P = 0.008$) and negatively correlated with clinicopathological score (ρ Spearman = −0.400, $P = 0.006$), serology (ρ Spearman = –0.469, $P = 0.001$) and parasite load in blood (ρ Spearman = –0.369, $P = 0.012$).

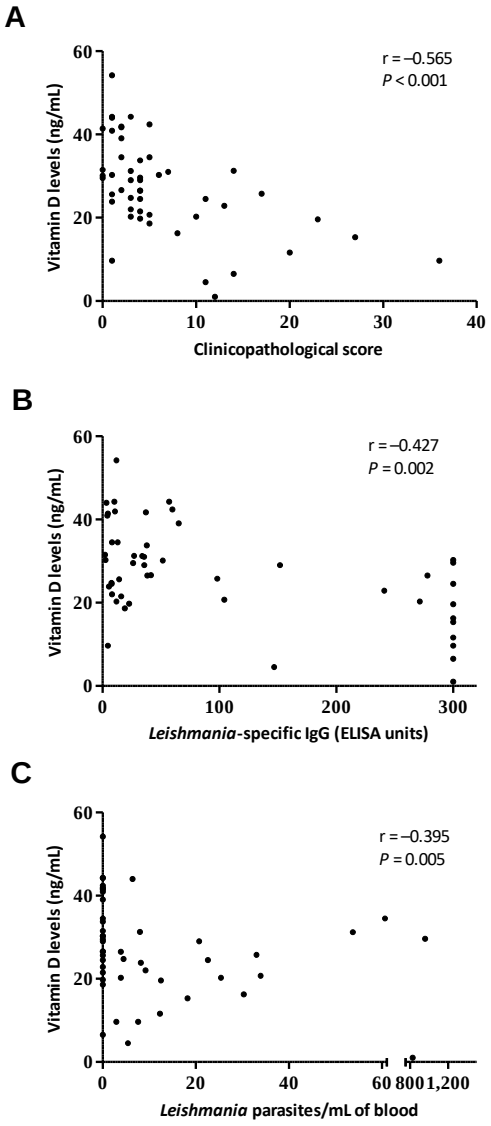


Figure 17. Correlations between vitamin D levels and clinicopathological score (A), *Leishmania*-specific antibody levels (B) and parasite load in blood samples (C) from *Leishmania*-infected dogs. Spearman correlation indexes (r) and $P < 0,05$ are shown in the graphs.

Univariate logistic regression analysis showed that only vitamin D, IFN-γ *Leishmania*-specific antibodies and LST reaction were significant explanatory variables. No association was found

between canine leishmaniasis and age or sex. Table 5 shows the odds ratio (OR) calculated for every significant variable. Thus, for every unit (ng/mL) increase in plasma 25(OH)D, the risk of having leishmaniasis [(1-OR) * 100] declined by 14.09 %. Likewise, for every 1 pg/mL increase in plasma IFN-γ and every 1 mm increase in LST the risk of having leishmaniasis declined by 0.24 % and 14.11 %, respectively. Finally, for every OD increase in ELISA CTLA the risk of having leishmaniasis increased by 1.64 %. In addition, according to the multivariate logistic regression model (Table 5), the increase of both 25(OH)D and LST was independently associated with a decreased risk of leishmaniasis by 21.45 % and 28.73 % per unit, respectively.

Table 5. Risk factors for canine leishmaniasis.

OR: Odds Ratio; CI: Confidence interval

Univariate analysis	OR (CI 95 %)
25(OH)D	0.8591 [0.7810–0.9451]
IFN-γ	0.9976 [0.9954–0.9999]
ELISA CTLA	1.0164 [1.0086–1.0243]
LST	0.8589 [0.7687–0.9596]
Multivariate analysis	**OR (CI 95 %)**
25(OH)D	0.7855 [0.6499–0.9490]
LST	0.7127 [0.5243–0.9689]

Vitamin D receptor polymorphism

Vitamin D exerts its function by activating the VDR transcription factor. We therefore aimed to investigate whether genetic variation within the *VDR* gene locus is associated with the progression of CanL. We sequenced several genomic regions of the canine *VDR* gene (see Materials and Methods) and found four single nucleotide polymorphisms (SNPs), rs851938503 (A > G), rs852643282 (C > G), g.6894812A > G and rs852900542 (T > C). Two of these variants were synonymous (rs851938503 and rs852643282) and mapped to exon 4 of the Ensembl's (www.ensembl.org) annotated transcript ENSCAFT00000014506. The other two SNPs were located in introns 3 (g.6894812A > G) and 7 (rs852900542). Genotyping of the 4 *VDR* SNPs in the cohort of 51 dogs showed intermediate allelic frequencies for SNPs g.6894812A > G and rs851938503, and low minor allele frequencies (MAF) for SNPs rs852643282 and rs852900542 (Tables 6 and 7). Importantly, the allelic frequencies of the 4 SNPs were not statistically different between the three disease classes and between non-infected vs infected (symptomatic and asymptomatic) dogs, as determined with a χ^2 test of independence (Table 8).

Table 6. Genotype distribution for each SNP in the three phenotypic classes.

SNP	Genotype	Non-infected (n = 15)	Ill (n = 11)	Asymptomatic (n = 25)
g.6894812A > G	AA	5	4	11
g.6894812A > G	AG	8	1	10
g.6894812A > G	GG	2	2	4
rs851938503 (A > G)	AA	5	7	12
rs851938503 (A > G)	AG	8	1	9
rs851938503 (A > G)	GG	2	3	4
rs852643282 (C > G)	CC	14	10	21
rs852643282 (C > G)	CG	1	1	4
rs852643282 (C > G)	GG	0	0	0
rs852900542 (T > C)	TT	12	10	20
rs852900542 (T > C)	TC	3	1	5
rs852900542 (T > C)	CC	0	0	0

Table 7. Minor allele frequencies for each of the 4 vitamin D receptor SNPs genotyped in the study. MAF: Minor allele frequency; NI: non-infected dogs; ASYMP: the group of asymptomatic dogs; SYMPT: group of dogs suffering clinical leishmaniasis. In the SNP name column cells, the major and the minor alleles of the SNPs with a rs identifier are indicated between brackets.

SNP name	MAF NI	MAF ASYMP	MAF SYMPT
rs851938503 (A>G)	0.40	0.33	0.32
rs852643282 (C>G)	0.03	0.08	0.05
g.6894812A>G	0.40	0.35	0.36
rs852900542 (T>C)	0.10	0.10	0.05

Table 8. p-values of the χ2 test of independence comparing the allelic frequencies of the 4 SNPs among the phenotypic classes. NI: non-infected dogs; ASYMP: the group of asymptomatic dogs, SYMPT: group of dogs suffering clinical leishmaniasis.

Groups	g.6894812A>G	rs851938503	rs852643282	rs852900542
Infected vs SYMPT	0.68	0.50	0.53	0.46
NI vs ASYMP	0.62	0.49	0.35	0.61
SYMPT vs ASYMP	0.77	0.69	0.45	0.42
NI vs Infected	0.76	0.48	0.45	0.72

CHAPTER 2

Longitudinal retrospective study of vitamin D and canine leishmaniasis. Vitamin D mechanism of action.

Summary of the experimental approach

This chapter is destined to tackle the second and third objective of this work:

"To determine whether there was a vitamin D deficiency prior to developing clinical canine leishmaniasis and if vitamin D levels could be a prognostic or clinical marker for this disease, and to study the mechanism of action of vitamin D in canine macrophages during *Leishmania infantum* infection"

We have shown in the first chapter that dogs with clinical leishmaniasis presented lower vitamin D serum levels than non-infected dogs, and even lower than those with asymptomatic infection. However, if vitamin D deficiency is a risk factor for developing clinical leishmaniasis or it diminishes with the onset of the disease, remains to be answered. For this purpose, we also included 9 dogs with clinical leishmaniasis and 10 non-infected healthy dogs, in which we measured vitamin D levels at the beginning of the study, when all dogs were negative for serology and qPCR, and 1 year later. We also retrospectively analysed serum samples from 36 dogs collected in different periods of the year to study whether there is a seasonal variation in vitamin D concentration. We also aimed to analyze the antiparasitic and immunoregulatory role of this vitamin in the context of canine leishmaniasis. We investigated if adding active vitamin D lead to a reduction in *L. infantum* burden and a regulation in the expression of genes related to vitamin D pathway and immune response in canine macrophages.

MATERIAL AND METHODS

Dog population

Population included in seasonality study: in order to know if there is a seasonal variation in vitamin D levels in dogs, we included serum samples from 36 dogs from different breeds and ages living in Spain, which remained clinically healthy and *Leishmania*-free thorough one year (2016–2017). Serum samples from these dogs obtained in three timing points (February 2016, May/June 2016 and January 2017) were analysed.

Population included in longitudinal study: to study the relationship between vitamin D levels and evolution of *L*. *infantum* infection we included retrospective serum samples from 19 dogs belonging to a longitudinal study of CanL, living in a highly endemic area (Spain). Nine of these dogs became *Leishmania* infected, while 10 remained healthy and *Leishmania*-free along the year of the study. This population included 60 % males and 40 % females from different breeds, and ages ranging from 1 to 11 years (4 ± 3.3 years). Samples at the beginning of the study when all dogs were healthy (T1) and at the end of the study, one year later, when nine of the included dogs suffered from leishmaniasis (T2) were analysed.

Clinicopathological characterization

Clinical signs, clinical chemistry, and haematological values were scored using a clinicopathological score (CPS) as previously described (Rodríguez-Cortés et al., 2007a). Briefly, clinical signs compatible with leishmaniasis such as cutaneous lesions, ocular signs, and epistaxis were scored 0–3. Both clinical chemistry and haematological results scored 1 point for each abnormal value. These scores were added to obtain an overall clinicopathological score for each dog.

Samples

Serum was obtained after centrifugation of peripheral blood at 1600 *g* for 10 min and stored at - 20 °C until serology and biochemical analyses were performed. Lymph node samples collected by fine needle aspiration in sterile 0.9 % NaCl solution were frozen at - 20 °C until DNA extraction. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood in heparin tubes and preserved in liquid nitrogen until processing.

Crude total *L. infantum* antigen (CTLA)-based ELISA

B cell function was analysed through anti-*Leishmania* antibody concentration using an ELISA technique. Microtiter plates were coated with 2 µg of CTLA per well in carbonate buffer 0.1 M, pH = 9.6 and sequentially incubated overnight. 100 µL of serum sample at a dilution of 1/400 in phosphate buffered saline (PBS) (Sigma-Aldrich®) containing 0.05 % Tween 20 (PBST) and 1% dry skimmed milk (PBSTM) was added to each well and incubated at 37 °C for 1 h, followed by three washes with PBST and one final wash with PBS. After, 100 µL of Protein A conjugated to horseradish peroxidase (Pierce) at working dilution of 1/15 000 was added, incubated at 37 °C for 1 h and washed as before. Peroxidase substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich®) was added at 100 µL/well and developed for 5 min at room temperature. The reaction was stopped with 50 µL of H_2SO_4 1 M and absorbance values were read at 450 nm in an automatic microELISA reader (Spark™ 10M, Tecan®). Results were expressed in ELISA units (EU), referred to a known positive serum used as a calibrator and arbitrarily set to 1 EU. Cut-off value (mean + 3 SD) for 76 dogs from a non-endemic area was 0.200 OD.

Real-Time PCR amplification of *Leishmania* DNA

Parasite load was determined by qPCR in lymph nodes. DNA was extracted using the High Pure™ PCR Template Preparation Kit (Roche™). *L. infantum* DNA was specifically detected and quantified with a TaqMan® qPCR Assay (Applied Biosystems™) following a previously reported protocol (Francino et al., 2006) with some modifications. The qPCR assay was designed to target conserved DNA regions of the kinetoplast from *L. infantum* genome. Primer sequences were LEISH-1 5'-AAC TTT TCT GGT CCT CCG GGT AG-3', LEISH-2 5'-ACC CCC AGT TTC CCG CC-3', and the TaqMan®-MGB probe FAM-5'-AAA AAT GGG TGC AGA AAT-3'- MGB. The thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Analyses were performed in a StepOnePlus™ Real Time PCR System device (Applied Biosystems™). Each sample plus a negative control was analysed in triplicate. Parasite quantification was performed by comparison with a standard curve generated with *L. infantum* DNA extracted from 1×10^7 parasites by using serial dilutions from 10^3 to 10^{-3} parasites. This technique was sensitive enough to detect 0.001 parasites per reaction with a dynamic range of 10^7. The median slope of three different standard curves was −3.44, and the qPCR efficiency was 98 %. Quantification was linear between 10^3 and 10^{-2} parasites per reaction tube (correlation = 0.99).

Determination of Vitamin D levels in serum samples

Levels of the stable circulating metabolite of vitamin D, 25(OH)D, were assessed in serum samples using a competitive direct enzyme-linked immuno-sorbent assay (IDS® 25-Hydroxy Vitamin D Direct EIA kit, Immunodiagnostic Systems Ltd.) according to the manufacturer instructions and employing an automatic micro-ELISA reader (Spark™10M, Tecan®). The concentration of 25(OH)D in each sample was calculated using a four-parameter logistic curve fit (Prism® 5, GraphPad Software), and results were expressed in ng/mL units.

In vitro evaluation of Vitamin D effect on *L. infantum* parasite killing

Leishmania infantum promastigotes of the MCAN/ES/92/BCN-83/MON-1 strain were cultured at 26 °C in R15 medium [RPMI 1640 medium (Gibco®) supplemented with 15 % heat-inactivated fetal bovine serum (FBS) (Gibco®), 2 % HEPES 1M (Gibco®) and 1 % 10 000 U/mL penicillin with 10 000 µg/mL streptomycin (Gibco®)]. Weekly passages were performed. Metacyclic promastigotes for *in vitro* infections were obtained from a 6-days-old stationary culture. DH82 dog macrophages were obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in R10 medium [RPMI 1640 medium (Gibco®) supplemented with 10 % FBS (Gibco®) and 1 % Penicillin/Streptomycin (Gibco®)] and kept in a humid atmosphere at 37 °C and in 5 % CO_2.The day of the experiment, cells were cultured in a 24-well plate (250 000 cells per well) and left to adhere for 2h. Different concentrations of 1,25-dihydroxyvitamin D_3 (CAS N 250-963-8, Sigma-Aldrich®) were added to DH82 cells in triplicate (0.01 µM, 0.1 µM and 1 µM) and plates were incubated for 24 h. After 24 hours, pre-treated DH82 cells were infected with metacyclic promastigotes at a parasite:cell ratio of 5:1, incubated for 24 h and then, washed with 1 × PBS to discard non-internalized promastigotes. Cells were treated with trypsin-EDTA 0.05 % (Gibco®) and plated on microscopic slides by cytocentrifugation (Thermo Scientific™ Shandon Cytospin™ 4). Preparations were fixed with methanol and stained with Giemsa 10 %. The number of infected macrophages and the intracellular parasites were recorded by direct microscopic count of 200 cells per sample. Values of infected macrophages and parasite load were expressed as absolute number per 100 macrophages.

Reduction of intracellular amastigotes

$$\left(\frac{\frac{A1}{Mt1} - \frac{A2}{Mt2}}{\frac{A2}{Mt2}}\right) \times 100$$

Reduction of intracellular amastigotes

$$\left(\frac{\frac{Mi1}{Mt1} - \frac{Mi1}{Mt2}}{\frac{Mi2}{Mt2}}\right) \times 100$$

A1: total amastigotes of untreated group

Mt1: total macrophages of untreated group

Mi1: infected macrophages of untreated group

A2: total amastigotes of treated group

Mt2: total macrophages of treated group

Mi2: infected macrophages of treated group

Monocytes isolation

Monocytes were isolated from 2 groups of dogs: a) Nine dogs from the field study, 4 *Leishmania*-infected dogs and 5 non-infected dogs at the end of the study (T2); b)Six healthy dogs whose buffy coats were obtained from the animal blood bank of Spain (Banco de Sangre Animal SL). First, PBMCs were isolated using Ficoll density gradient method. Samples were diluted (1:3) with 1x PBS, gently layered over 15 mL of Ficoll® Paque Plus solution (GE Healthcare®) and centrifuged at 400 *g* for 30 min. The buffy coat cells collected at the interface were washed with 1× PBS and treated with 4 mL of Ammonium-Chloride-Potassium Lysing Buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA) and washed again with 1× PBS. Cells were resuspended in R10 medium. The differential counting was determined by haematological analyser (XN-1500, Sysmex Europe GmbH™). PBMCs from dogs of the field study were frozen in liquid nitrogen until use. PBMCs were cultivated in 24-well plates (2.5×10^6 cells/well) and incubated for 18 h at 37 °C in humidified incubator (5 % CO_2) for adherence. Later, cells were washed to obtain (a) only monocytes and RNA was directly extracted or (b) monocytes were treated with $1,25(OH)_2D_3$ at a concentration of 0.1 µM ofLipopolysaccharides from *Escherichia coli* O111:B4 (LPS) (EC N 297-473-0, Sigma-Aldrich®) at 0.1 µg/mL as positive control for 24 h. Plates were centrifuged at 400 g for 10 min, washed once with 1× PBS and monocytes were collected with 1 mL TRI Reagent® (Ambion™) and stored at −80 °C until RNA extraction.

Gene expression analysis of vitamin D pathway

RNA was extracted from monocytes using the RiboPure™ RNA Purification Kit (Ambion™) following manufacturer's instructions and measured with a NanoDrop™-2000 Spectrophotometer (Isogen Life Science B.V). Retro-transcription was carried out by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™) following a thermal profile of 25 °C for 10 min, 37 °C for 120 min, 95 °C for 5 min. Levels of *CAMP*, *CBD103*, *CYP24A1*, *CYP27B1* and *VDR* expression were determined in addition to *RPL18* as a housekeeping gene. To ensure amplification of cDNA sequences derived from retro-transcription of mRNA of interest, primers were designed including exon boundary (Table 9). Amplification of each sample was carried out in triplicate by using SYBR™ Select Master Mix reagents (Applied Biosystems™) with the aid of Applied Biosystems™ StepOnePlus™ PCR instrument and StepOnePlus™ Software v2.3 (Applied Biosystems™). The thermal cycling profile was 10 min at 50 °C, followed by 40 cycles of 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Melting curves assessed the specificity of our amplification products. Cytokine

mRNA expression levels were calculated by relative analysis using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 9. Sequences of primers used for gene expression determinations.

Gene		Primer sequence
CAMP	F	5'-AGGACACGGGCTACTTTGAC-3'
	R	5'-TTTCGCCAATCTTCTGCCCC-3'
CBD103	F	5'-GCCGCTGCTTACTTGTACCT-3'
	R	5'-CCTCATGACCAACAGGCTTC-3'
CYP24A1	F	5'-ACTCCTTCGGAAGAATGCGG-3'
	R	5'-CGACCGGGGTTACCATCATC-3'
CYP27B1	F	5'- GGCACACCTGACCTACTTCC-3'
	R	5'-AGAGCGTGTTGGATACCGTG-3'
VDR	F	5'-TATCACCAAGGACAACCGCC-3'
	R	5'-CAGGATCATCTCCCGCTTCC -3'
RPL18	F	5'-GTCGACATCCGCCACAACAA-3'
	R	5'-AGGTAGAGTTGGTTCGTCTGG-3'

Acronyms: F and R mean forward primer and reverse primer, respectively.

Data analysis

In the unadjusted analysis, the comparisons between different groups were performed using unpaired *t*-test and comparisons between same groups but different times by paired *t*-test. For analysis in which distribution does not conform to parametric criterion we used Wilcoxon signed-rank test to compare related samples and Mann-Whitney U test for independent samples. One-way ANOVA was used for multiple comparisons in dose-response experiments. All statistical tests were performed using GraphPad Prism 9.0 software. A *P*-value ≤ 0.05 was considered significant.

RESULTS

Vitamin D seasonality in healthy dogs

The mean 25(OH)D concentration of healthy dogs in the three points evaluated were 44.6 ± 12.5 ng/mL, 41.28 ± 12.4 ng/mL and 43.15 ± 14.8 ng/mL for February, May/June and January, respectively. No statistically significant variation was observed in any of the times studied (Wilcoxon matched-pairs signed rank test).

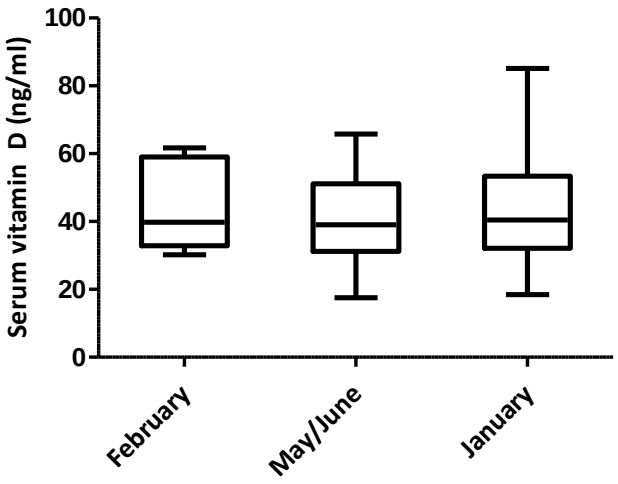


Figure 18. Serum Vitamin D levels of 36 healthy dogs living in Spain at three different time points. Vitamin D status in dogs was assessed according to the serum levels of 25(OH)D estimated with an ELISA test.

Characteristics of dog population included in the vitamin D longitudinal study

We selected 9 *Leishmania*-infected dogs presenting clinicopathological symptoms compatible with this disease (median CPS = 4) and which tested positive for qPCR analysis and/or CTLA-serology at the end of the study (T2) ("Infected" group) and 10 dogs which consistently tested always negative in both test ("Non-Infected" group). The baseline characteristics of both groups at the starting point and at the end are shown in Table 10.

Table 10. **Characteristics of the dog population used in the current study at the two points studied.**

PARAMETERS	GROUPS OF ANIMALS								Ref. RANGE
	Non-Infected T1		Infected T1		Non-Infected T2		Infected T2		
	Median [IQR]	(+)	Median [IQR]	(+)	Median [IQR]	(+)	Median [IQR]	(+)	
CPS	0.0 [0.00–0.00]	0 %	0.0 [0.00–0.00]	0 %	0.0 [0.00–0.00]	0 %	6 [4.00–9.00]	100 %	≥ 4
Anti-*Leish* antibodies (EU)	4.8 [3.55–6.56]	0 %	7.8 [7.04–8.41]	0 %	5.2 [4.68–5.56]	0 %	58.9 [34.91–124.40]	77.8 %	≥ 20
Parasite Load in LN (pp/mL)	0.0 [0.00–0.00]	0 %	0.0 [0.00–0.00]	0 %	0.0 [0.00–0.00]	0 %	1195.1 [4.5–5862.5]	75.0 %	≥ 1

Vitamin D concentration as a risk factor for CanL

No statistically significant differences in vitamin D levels were observed between groups (unpaired *t*-test; $P = 0.9619$) at the beginning of study (T1) (Figure 19). Likewise, there were no statistically significant differences (paired *t*-test; $P = 0.1828$) between initial and final 25(OH)D levels within the healthy groups. Conversely, the infected dogs showed statistically significant lower concentration of 25(OH)D in serum at the end of the study (T2) than at the beginning (T1) (paired *t*-test; $P = 0.0396$). At the end of the study healthy animals showed higher 25(OH)D levels in serum than infected animals (unpaired *t*-test; $P = 0.0032$). Therefore, sick animals show a greater reduction in vitamin D (−35.37 %) than healthy ones (−7.18 %) after a year. The median [interquartile range] levels of 25(OH)D in Non-Infected dogs at the beginning and at the end of the study were 48.3 [37.19–60.00] and 45.2 [39.22–48.80] ng/ml, respectively, and in Infected dogs were 42.9 [39.03–52.57] and 24.5 [20.86–42.27] ng/ml, respectively.

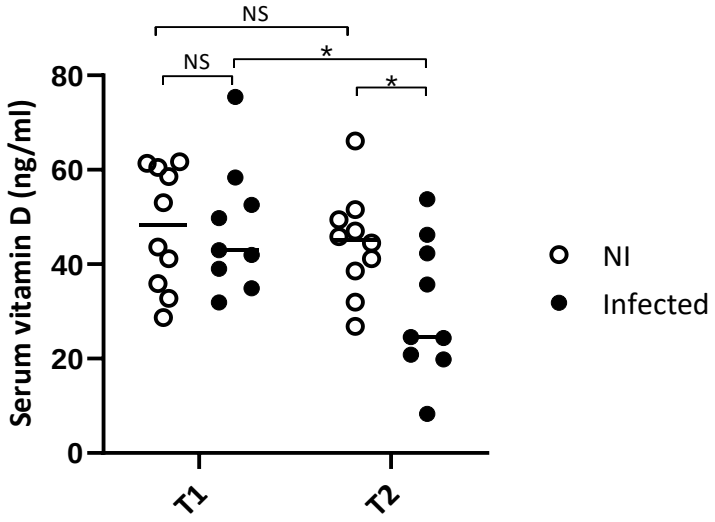


Figure 19. Serum vitamin D levels in a dog population living in Spain. Vitamin D status in dogs was assessed according to the serum levels of 25(OH)D estimated with an ELISA test. Comparison between Non-Infected dogs (NI) and dogs infected during the longitudinal study (Infected) at the inclusion point (T1) and at the end of the study (T2) (* $P < 0.05$).

In vitro effect of vitamin D in *L. infantum* parasite killing

In an *in vitro* model using a canine macrophage cell line (DH82) infected by *L. infantum* we found that addition of active vitamin D lead to a significant reduction in parasite load (Figure 20). Pretreatment of canine macrophages cell line with $1,25(OH)_2D_3$ at a dose of 0.1 µM, achieved a reduction of 26.5 % in the number of infected macrophages (Wilcoxon matched pairs signed rank test; $P = 0.0156$) and 31.4 % in the number of amastigotes per 100 macrophages (Wilcoxon matched pairs signed rank test; $P = 0.0078$) (Figure 20A and 20B). These reductions follow a dose-response effect from 0.01 µM to 1 µM (ANOVA test; $P = 0.0285$ and $P = 0.0107$ for infected macrophages and number of amastigotes reduction, respectively) (Figure 20C and 20D).

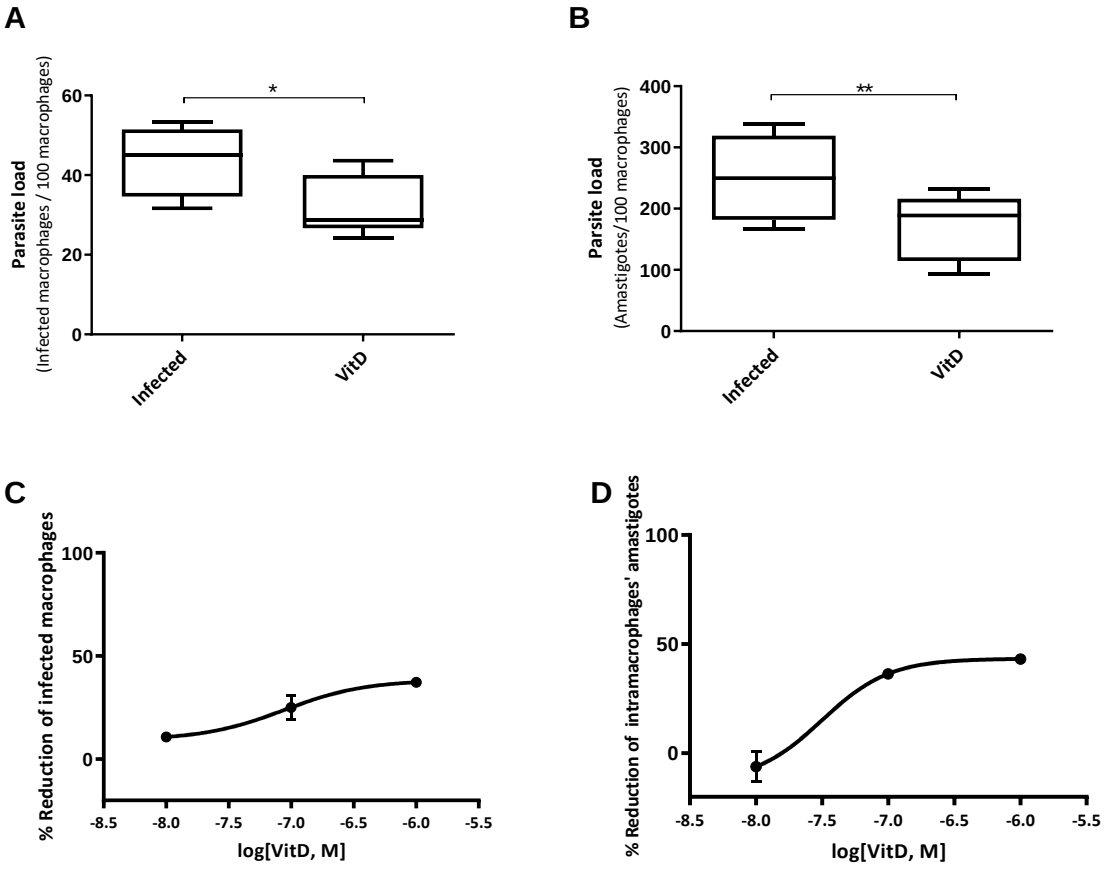


Figure 20. Vitamin D effect on *L. infantum* parasite load in macrophages. Number of infected macrophages per 100 macrophages (**A**) and number of intracellular amastigotes per 100 macrophages (**B**) counted in Giemsa stained-preparations from cells infected with *L. infantum* at ratio 5:1 and from those pre-treated with $1,25(OH)_2D_3$ at 0.1 µM 24 h before infection (* $P < 0.05$, ** $P < 0.01$). Dose response curves showing inhibitory rates of *L. infantum*-infected macrophages (**C**) and intracellular amastigotes growth (**D**) after 24h of treatment with $1,25(OH)_2D_3$ based on the values for the untreated controls.

Vitamin D pathway in primary canine monocytes

In amodel using monocytes obtained from buffy coats of healthy blood donor dogs from an animal blood bank we found that 24 h treatment with $1,25(OH)_2D$ lead to a statistically significant increase of the AMP β-defensin *CBD103* gene compared to the basal expression of non-treated monocytes (Wilcoxon signed rank test; $P = 0.0313$), but not to the cathelicidin AMP *CAMP* gene (Wilcoxon signed rank test; $P = 0.0625$) neither to the *VDR* and *CYP24A1* genes (Wilcoxon signed rank test; $P = 0.0938$ and $P = 0.0625$, respectively) (Figure 21). Addition of LPS at a concentration of 0.1 µg/mL greatly increased *CAMP* and *VDR* expression (Wilcoxon signed rank test; $P = 0.0313$, both), but not that of *CBD103* and *CYP24A1* (Wilcoxon signed rank test; $P = 0.0938$ and $P = 0.8125$, respectively).

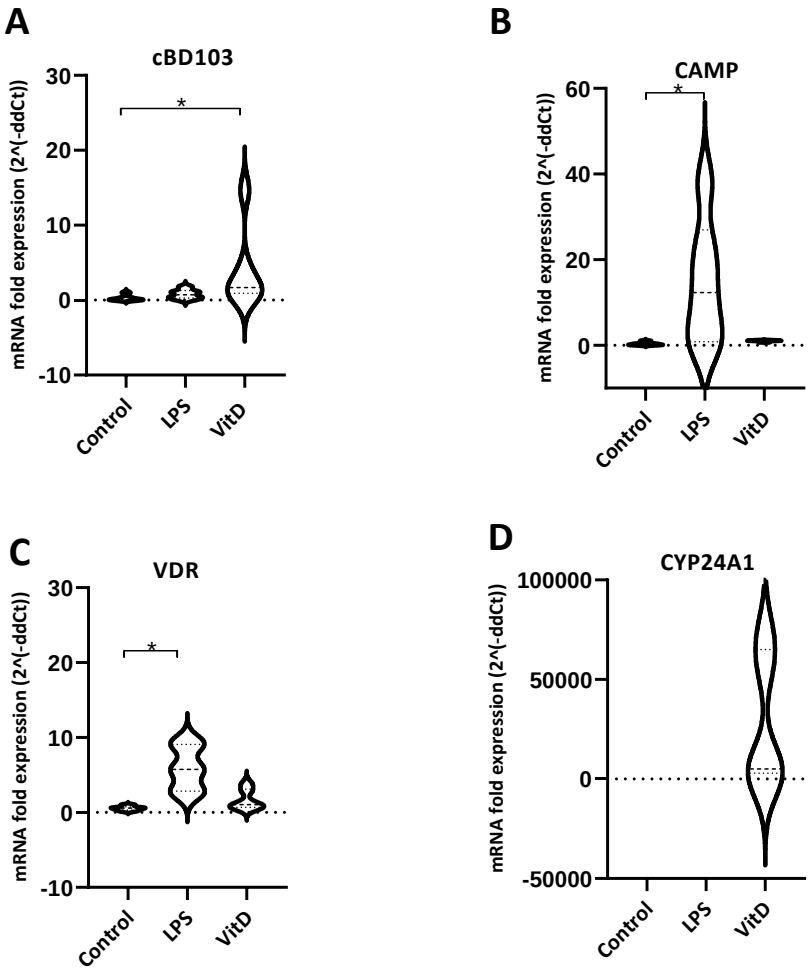


Figure 21. Fold expression of *CBD103* (A), *CAMP* (B), *VDR* (C) and *CYP24A1* (D) genes in canine primary macrophages after adding vitamin D and LPS. mRNA fold-increase from vitamin D and LPS condition was calculated with reference to its negative control (cells of same extraction but untreated). mRNA expression from control condition was normalized from a control with a lowest value (* $P < 0.05$).

Gene expression of monocytes from retrospective field longitudinal study

We have shown that *CBD103* expression increases with the presence of vitamin D, so that it could play a key role in the antiparasitic activity derived from the action of vitamin D. Thus, we analysed mRNA expression of *CAMP*, *CBD103*, *CYP24A1*, *CYP27B1* and *VDR* in monocytes from PBMCs of 9 dogs included in the retrospective longitudinal study from which we were able to collect samples (4 dogs for Infected group and 5 for the Non-Infected group). We found no statistically significant differences in any of the genes studied neither between groups or between T1 and T2. However, results suggest that at the endpoint healthy animals have higher expression of β-defensin *CBD103*, while animals suffering leishmaniasis have higher expression of *CYP27B1*. In the case of *VDR* there are no indications of a differential trend between groups

(Figure 22). *CAMP* expression levels were very close to the quantification limit, and reliable results could not be obtained. *CYP24A1* was undetectable.

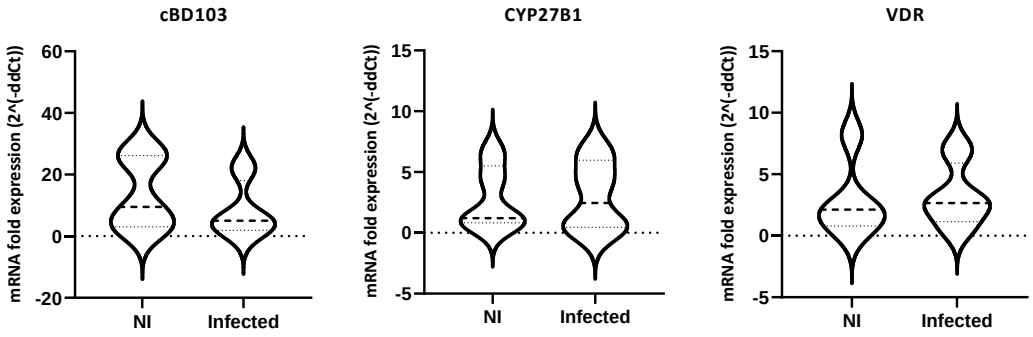


Figure 22. Expression of vitamin D pathway genes in monocytes from dogs included in the retrospective field longitudinal study. Fold expression of *CBD103* (**A**) *CYP27B1* (**B**) and *VDR* (**C**) genes in Non-Infected (NI) and Infected (Infected) groups at the end of the study (T2).

CHAPTER 3

Vitamin D as a vaccine adjuvant against canine visceral leishmaniasis

Summary of the experimental approach

This chapter is destined to tackle the last objective of this work:

"To test if vitamin D coadministration during a DNA vaccination protocol enhances protection against *L. infantum* infection in a rodent model"

In the search for a safe and efficient protective vaccine for VL, we used vitamin D, known for playing an immunomodulatory role by activating innate immune system and modulating the adaptive immune response, as an adjuvant to boost the effect of a DNA vaccine. For this purpose, BALB/c mice were treated with vitamin D concomitantly with a DNA vaccine consisting in four plasmids carrying the *Leishmania* genes *LACK*, *TRYP*, *PAPLE22* and *KMPII* encapsulated in liposomes. Two weeks after the last vaccination, the animals were infected intraperitoneally with *L. infantum* parasites. Parasite load was measured in target tissues and immune response was evaluated before challenge and six weeks post-infection by determining anti-*Leishmania* specific antibodies in combination with cytokine expression in liver and determining percentage of lymphocytes T $CD4^+$ and $CD8^+$.

MATERIAL AND METHODS

Parasites

The *L. infantum* strain MCAN/ES/92/BCN83 (zymodeme MON-1) was kindly provided by Dr. Montserrat Portús, Universitat de Barcelona, Spain. It was obtained from a naturally infected and untreated dog and maintained through hamster passage to retain its full virulence. Mice were intraperitoneally infected with stationary promastigotes and, 6 weeks post-infection, parasites were isolated from spleen and samples incubated in R15 medium (RPMI 1640 medium (Gibco) supplemented with 15 % heat-inactivated fetal calf serum (Gibco), 2 % HEPES 1 M (Gibco) and 1 % of total volume of 10 000 U/mL penicillin, and 10 000 µg/mL streptomycin (Gibco). Promastigotes were maintained at 26 °C in R15 medium, and weekly passages were performed. Stationary promastigotes for infection were obtained from a 6-day-old stationary culture, washed and resuspended in PBS at 1×10^7 parasites/mL.

Animals

A total of 40 female 7-week-old BALB/c female mice (Charles River Laboratories, Barcelona, Spain) were used. The animals were kept in groups of 5 in plastic microfilter cages under biosafety level 2 conditions at the Servei d'Estabulari of Universitat Autònoma de Barcelona (Barcelona, Spain), and food and water were provided *ad libitum*. All procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee following the principles of animal protection according to the Directive 2010/63/UE (Protocol number 3752). Mice were randomly assigned to five experimental groups of 8 animals each: DNA vaccine (pVax-Leish), DNA vaccine in liposomal nanoparticles (NP-pVax-Leish), DNA vaccine in liposomal nanoparticles plus vitamin D treatment (NP-pVax-Leish + VitD), pVax plasmid in liposomal nanoparticles (NP-pVax) and non-vaccinated untreated infected control group (PSS). Three animals per group were euthanized before infection to study immunogenicity. The remaining 5 animals per group were infected to test vaccine protection.

Vitamin D treatment

1α,25-dihydroxyvitamin D_3 (CAS N 32222-06-3) was purchased from Sigma-Aldrich® and kept under condition specified by the manufacturer. 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) stock solution was prepared in miliQ water-15% ethanol. Mice of NP-pVax-Leish + VitD group received on alternated days an intraperitoneal dose of 0.05 mg/kg of $1,25(OH)_2D_3$ in 100 µL of physiological saline solution (PSS) since second vaccine administration until the day before

infection (4 weeks treatment). The remaining animals received 100 uL of PSS following the same pattern of administration.

Vaccine preparation

Sequences of the *L. infantum* genes *TRYP (*AF044679), *PAPLE22 (*AF123892), *LACK* (U49695) and *KMPII* (X95267) were cloned individually into a pVAX vector and then expressed in DH5-alpha *E. coli* cultures as described elsewhere (Rodríguez-Cortés et al., 2007c; Todolí et al., 2012). Each DNA construction was purified using EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. To ensure the presence and integrity of these sequences, 10 µg of each plasmid was digested for 3 h at 37 °C by using the Roche restriction enzymes referred below (Table 11) and DNA fragments were run in a 1 % agarose gel. The immunogenicity of these plasmids was previously tested (Todolí et al., 2012).

Table 11. Fragment lengths and restriction enzymes to ensure the presence and integrity of *LACK*, *PAPLE22*, *TRYP* and *KMPII* in the generated plasmids for naked DNA vaccination.

SEQUENCE	RESTRICTION ENZYME	FRAGMENT LENGTH (pb)
LACK	ECoRI	942
PAPLE22	PstI and NotI	750
TRYP	ECoRI	609
KMPII	ECoRI	279

Liposomal-based vaccines were prepared using the thin-film hydration method. Two vaccine prototypes with pVAX and pVAX-Leish plasmids were developed. Briefly, DMPC, Cholesterol and Cholesterol-PEG were dissolved in chloroform solutions and mixed at 0.5:0.45:0.05 molar ratio, respectively. Total lipid concentration was 40 mM. The organic solvent was removed under vacuum and nitrogen to afford a dry lipid film, which was hydrated under vigorous stirring with a solution of pVax (1 mg/mL) or pVax-Leish (1 mg/mL). Under these conditions, the stacks of liquid crystalline lipid bilayers become fluid and swell, which led to detachment during agitation and self-closure to yield multilamellar large vesicles (MLV). After, the MLV were homogenized using an extruder and a polycarbonate membrane (pore size 400 nm).

Characterization of Liposomal-based vaccine

Encapsulation efficiencies (EE) were calculated according to the following equation, where $C_{plasmid,total}$ is the initial plasmid concentration and $C_{plasmid,out}$ is the concentration of non-encapsulated plasmid.

$$EE(\%) = \frac{(Cplasmid, total - Cplasmid, out)}{Cplasmid, total} \; x \; 100$$

To measure the Cplasmid,out, all liposome suspensions were centrifuged at 3500 *g* for 10 min using Amicon Ultra centrifugal filters MWCO 100 kDa, and plasmid quantification at the supernatants were done using NanoDrop™ 2000 spectrophotometer (260–280 nm).

In order to characterize liposomes, particle size distributions of the liposomes were determined using a dynamic light scattering (DLS) analyser combined with noninvasive backscatter technology. The stability of the liposomes was examined by measuring their electrophoretic mobility using a Malvern Zetasizer (Malvern Instruments, UK). For this, the liposome-based vaccine was diluted 50 times and placed in a cuvette for analysis. Liposome morphology was examined using cryogenic transmission electron microscopy (cryoTEM) in a JEOL-JEM 1400 microscope (Jeol Ltd., Tokyo, Japan).

Table 12. Liposomal-based vaccine characteristics.

	Lipid concentration (mM)	Average size (nM)	Polydispersity index (PdI)	Zeta potential (mV)	Encapsulation yield (%)
NP-pVax	40	358	0.351	−4.15	99.3
NP-pVax-Leish	40	375	0.426	−1.37	98.2

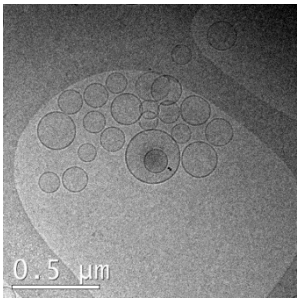


Figure 23. CryoTEM image of liposomal-based vaccine (NP-pVax).

Immunization

Three doses, administered 2 weeks apart, of naked vaccine or liposomal vaccine containing 100 µg of each plasmid in a total volume of 50 µL were administered intramuscularly into the hind limb of the mice of groups pVax-Leish, NP-pVax-Leish and NP-pVax-Leish + VitD. The empty vector control plasmid DNA encapsulated in liposomes (NP-pVax) was also administered in the same way. In order to maintain the same handling conditions as the other groups, the control group was injected with physiological saline solution (PSS) instead.

Infection

Two weeks after the administration of the last vaccine dose, all remaining animals were intraperitoneally inoculated with 1 mL of physiological saline solution containing 1×10^7 *L. infantum* metacyclic promastigotes. Animal weight and typical VL clinical signs in the rodent model —hair and weight loss, and cutaneous lesions— were weekly evaluated to ensure animal welfare.

Necropsy and sample collection

3 animals per group (n = 15) were euthanized the day before infection and 5 animals per group (n = 25) were euthanized 6 weeks post-challenge. Mice were anesthetized with a 100µL intramuscular injection containing ketamine (100 mg/kg) and xylazine (10 mg/kg). An intracardiac puncture was performed to obtain 1 mL of blood sample from each animal, and the animals were then euthanized in a CO_2 chamber. Samples of spleen and liver were aseptically obtained and kept on ice to avoid tissue degradation.

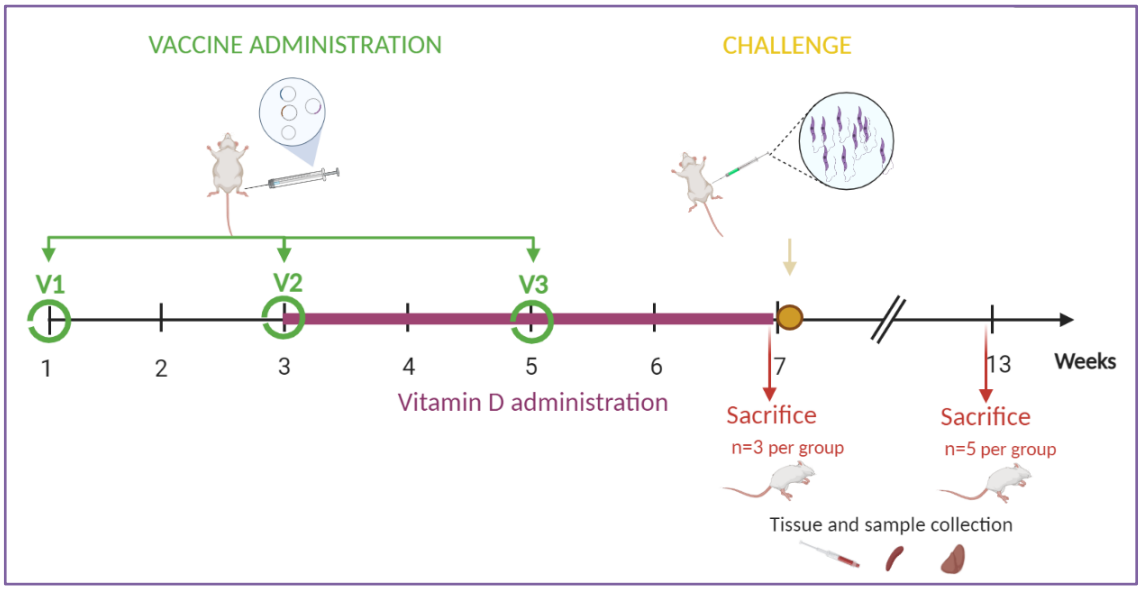


Figure 24. Graphical representation of the experimental design. A total of 40 BALB/c mice were randomly distributed in 5 groups (n = 8 per group): PSS (PSS-inoculated control group), NP-pVax (DNA vaccine plasmid in liposomes-inoculated group), pVax-Leish (DNA vaccinated group), NP-pVax-Leish (encapsulated DNA vaccinated group), NP-pV-Leish + VitD (Vitamin D-treated and encapsulated DNA vaccinated group). Mice from last group received vitamin D in alternate days starting with the second vaccination for 4 consecutive weeks until challenge day. Three vaccine doses were administered, leaving a 2-week period between them. Three mice for all groups were sacrificed two weeks after receiving the last vaccination doses, and the other 5 animals per group were challenged and sacrificed 6 weeks later.

DNA extraction and real-time PCR absolute parasite quantification

Total genomic DNA from all animals was obtained from 50 mg of spleen and liver samples by using the High Pure Template Preparation Kit (Roche) following the mouse tail protocol provided in the manufacturer's guidelines. DNA concentration was measured by using a NanoDrop™ 2000 Spectrophotometer (Isogen Life science). All DNA samples were kept at −20 °C until the qPCR was performed. Primers targeting conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum* and TaqMan®-MGB probes were used as formerly described (Francino et al., 2006). Primer sequences were LEISH-1 5'-AAC TTT TCT GGT CCT CCG GGT AG-3', LEISH-2 5'-ACC CCC AGT TTC CCG CC-3', and the TaqMan®-MGB probe FAM-5'-AAA AAT GGG TGC AGA AAT-3'- MGB. Triplicates of 25 ng of DNA of each sample, a negative control using Nuclease-Free Water (Sigma-Aldrich®) instead of DNA, and a standard curve were run in an Applied Biosystems™ StepOnePlus™ PCR instrument following a thermal cycling profile of 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min. The eukaryotic 18S RNA Pre-Developed TaqMan® Assay Reagents (Applied Biosystems™) were used as internal reference of total genomic DNA. The resulting data were analyzed using StepOnePlus™ Software v2.3 (Applied Biosystems™). The number of parasites per µg of DNA was calculated by interpolation to a standard curve generated by using 10-fold serial dilution from 10^3 to 10^{-3} promastigotes of a *L. infantum* culture (y = −3.44x + 28.43). The median qPCR efficiency (98 %) and slope (−3.44) were calculated from three different replicates. Parasite quantification was linear between 10^3 and 10^{-2} parasites per sample (R^2 = 0.9998).

Evaluation of humoral immune response: total mice IgG anti-*L. infantum*

Blood samples were collected in hematology crystal tubes (BD Vacutainer®) and centrifuged at 2 000 *g* for 15 min to obtain serum samples and were frozen until analyzed. ELISA plates (Costar™ high binding transparent flat bottom) were coated overnight at 4 °C with 2 µg per well of Crude Total *Leishmania* Antigen (CTLA) in 100 µL of carbonate-bicarbonate coating buffer (0.1 M $NaCO_3$-H_2CO_3 pH 9.6). CTLA was provided by Dr. Cristina Riera, Universitat de Barcelona, Spain. Samples were diluted 1:100 in PBS with 0.05 % Tween 20 and 1 % of skimmed milk powder (PBSTM) and serially diluted in the precoated plate. Plates were incubated at 37 °C in a humid atmosphere for 1 h and were then washed 3 times with PBST and once with PBS. Plates were then incubated for 1 h at 37 °C with goat anti-hamster IgG-HRPO (AbDSerotec) diluted 1:2000 in PBSTM. Antibody excess was removed with another cycle of washes as described above. Then, 100 µL of the peroxidase substrate tetramethylbenzidine (TMB) (Sigma-Aldrich®) was added to each well, and the plates were left to develop for 5 min.

The reaction was stopped by the addition of 50 µL of 1 M H_2SO_4, and absorbance was recorded at 450 nm (Anthos 2001 reader). The cut-off value was set as the average optical densities (ODs) serum samples coming from non-vaccinated animals sacrificed in the pre-challenge plus 3 times the standard deviation (Cut-off = 0.150 OD).

Flow cytometry

Splenocytes were isolated using Cell strainers (Falcon™) and 500 000 cellules per well were seeded in 96-well V-shape plates and cultured in R10 medium alone (RPMI 1640 medium (Gibco®) supplemented with 10 % FBS (Gibco®) and 1 % Penicillin/Streptomycin (Gibco®)) or adding LSA at 10 µg/ml and incubated at 37 °C and in 5 % CO_2 for 48 h. 5 h before cytometry we stimulated 3 additional wells with Phorbol 12-myristate 13-acetate at 500 ng/ml and ionomycin at 30 ng/ml (PMA/Ion) as a positive control. Brefeldin A, an inhibitor of protein secretion, was added at 10 µg/ml to all wells for the last 3 h of incubation. Then, splenocytes were incubated in staining buffer (PBS-5% FBS) for 30 min on ice in the presence of anti-mouse CD4-PE clone GK1.5 (BD Pharmingen™) or anti-mouse CD8a-PE-Cy™5 clone 53-6.7 (BD Pharmingen™) antibodies for surface staining (0.25 µl antibody per well). Cells were subsequently washed three times in staining buffer, fixed in 2 % paraformaldehyde-PBS for 20 min on ice, and permeabilized with PermWash solution (PBS-1% FBS-0.1% saponin- 0.1% sodium azide) before applying 0.5 µl per well of anti-mouse IFN-γ-FITC clone XMG1.2 (BD Pharmingen™). Flow cytometric acquisition was performed on a Coulter® Epics XL-MCL™ Flow Cytometer (Beckman Coulter) and data were further analyzed with Flowing software 2.5.1.

IFN-γ measurement

Since we were unable to determine intracellular IFN-γ by flow cytometry, we quantitatively measured this proinflammatory cytokine by the end of the study in mice splenocyte's supernatants isolated using Cell strainers (Falcon™) and cultured in 96-well flat bottom plastic plates at 500 000 cells per well in R10 medium unstimulated or with LSA at 10 µg/mL or ConA at 2.5 µg/mL and incubated at 37 °C in 5 % CO_2 for 48 h. Supernatants from each of the three replicates wells were pooled and stored at −80 °C. IFN-γ levels were assessed using sandwich enzyme-linked immunosorbent assay (Mouse IFN-γ DuoSet ELISA kit, R&D Systems) according to the manufacturer instructions and employing an automatic micro-ELISA reader (Anthos 2001, Anthos Labtec Instruments). Concentration of IFN-γ in each sample was calculated using a four-parameter logistic curve fit (Prism® 5, GraphPad Software), and results were expressed in pg/mL units.

Cytokine expression analysis

Cytokine mRNA expression was studied in liver tissue because of slight parasitological protection found in this organ. Samples maintained in RNA*later*™ (Ambion™) were homogenized with TRI Reagent (Ambion™) and kept frozen until RNA extraction. Total RNA was extracted by using the RiboPure kit (Ambion™) following the manufacturer's instructions, and samples were then treated with TURBO DNA-free™ Kit (Ambion™). Clean RNA samples were stored at −80 °C until analyzed. Retrotranscription was carried out by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™) following a thermal profile of 25 °C for 10 min, 37 °C for 120 min, and 95 °C for 5 min. Levels of *IFNG*, *TNFA*, *IL10* and *IL4* expression were determined in addition to *GAPDH* as a control gene to calculate relative gene expression levels (Deep et al., 2017). To ensure amplification of cDNA sequences derived from the retrotranscription of the mRNAs of interest, primers were designed including exon boundary sequences (Table 13). Amplification of each sample was carried out in triplicate using SYBR™ select Master Mix Reagents (Applied Biosystems™) with the aid of an Applied Biosystems™ StepOnePlus™ PCR instrument and StepOnePlus™ Software v2.3 (Applied Biosystems™). The thermal cycling profile was 10 min at 50 °C, followed by 40 cycles of 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Melting curves assessed the specificity of our amplification products. Cytokine mRNA expression levels were normalized to the expression levels of the housekeeping gene *GAPDH*. Fold induction levels were calculated by using the median values of the C group.

Table 13. Sequences of primers used for cytokine expression determination.

Gene		Primer sequence
GAPDH	F	5'-TTGCCTGGTACGACAACGAA-3'
	R	5'-TGGAAGCCGAAGTCAGGAAC-3'
IFNG	F	5'-GGAACTGGCAAAAGGATGGTG-3'
	R	5'-ATGTTGTTGCTGATGGCCTG-3'
TNFA	F	5'-ACCCTCACACTCAGATCATCTT-3'
	R	5'-GGTTGTCTTTGAGATCCATGC-3'
IL10	F	5'-GGCGCTGTCATCGATTTCTC-3'
	R	5'-ATGGCCTTGTAGACACCTTGG-3'
IL4	F	5'-ACAGGAGAAGGGACGCCAT-3'
	R	5'-GAAGCCCTACAGACGAGCTCA-3'

Acronyms: F and R mean forward primer and reverse primer, respectively.

Statistical analysis

In the unadjusted analysis, the comparisons between groups were performed using the Mann-Whitney U Test. Correlations between different parameters were performed using Spearman's rank correlation coefficient. All statistical tests were performed using GraphPad Prism 9.0 software. A *P*-value ≤ 0.05 was considered significant.

RESULTS

Vitamin D and vaccine tolerance

No mice of any group showed symptoms related to leishmaniasis and no significant differences in weight were found between groups at any point of the study, so we did not have to euthanize any animal during this protocol. Neither adverse reaction was observed after vitamin D, nanoparticles or DNA vaccine administration.

Parasitological protection

Although non-statistically significant, animals from vaccinated groups (pVax-Leish, NP-pVax-Leish and NP-pVax-Leish + VitD) showed a reduction in the number of parasites compared to control groups PSS and NP-pVax in the liver, and though subtle there was also a reduction in the spleen. The mean reduction of NP-pVax-Leish + VitD group compared to control group PSS is 70.9 % and 31.1 % in liver and spleen, respectively. Besides, the group in which we administered the encapsulated vaccine in combination with vitamin D (NP-pVax-Leish + VitD) got a reduction of 54.1 % in liver and 28.4 % in spleen with respect to the group in which we administered only the liposomal vaccine (NP-pVax-Leish). Moreover, only in NP-pVax-Leish + VitD group was achieved total protection against infection in 2 of the 5 animals in both liver and spleen.

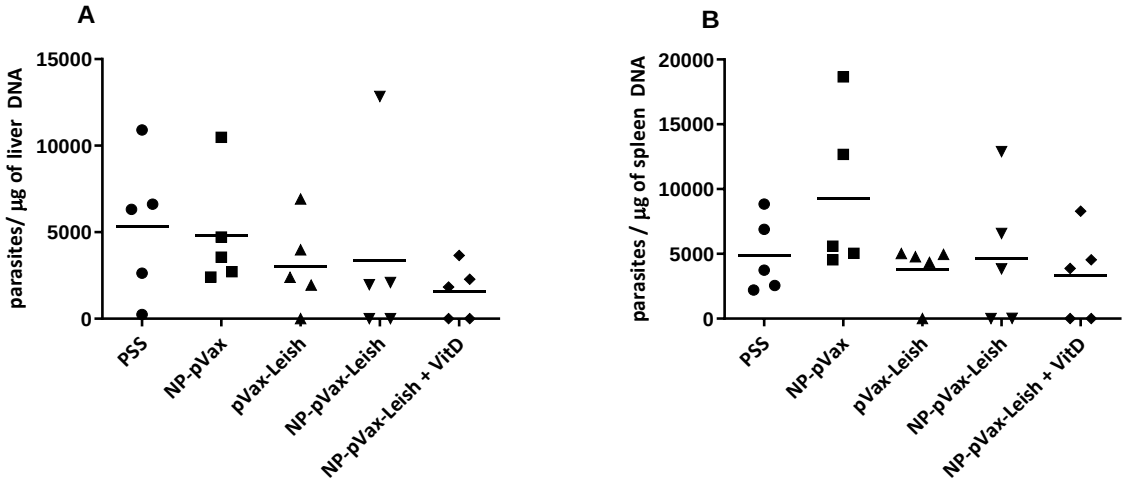


Figure 25. ***L. infantum*** **DNA load.** qPCR analyses showing the number of parasites detected per µg of DNA extracted from the liver (**A**) and spleen (**B**) at the end of the study (6 weeks post-challenge) comparing all groups included (n = 5 per group). Parasite number was calculated by interpolation to a standard curve constructed with a *L. infantum* promastigote culture.

Evaluation of humoral immune response

Anti-*Leishmania* IgG production

Similar levels of *Leishmania*-specific antibodies were detected on average between groups at the two times of sacrifice: pre-infection analysis (n = 3 per group) and 6 weeks post-infection (n = 5 per group) (Figure 26). Most of animals had IgG antibodies against CTLA at the time of sacrifice, but *Leishmania*-specific IgG production was reduced in vaccinated animals which were parasitological protected. Serology positively correlated with parasite load in liver at the end of the study (ρ Spearman = 0.500, *P* = 0.011).

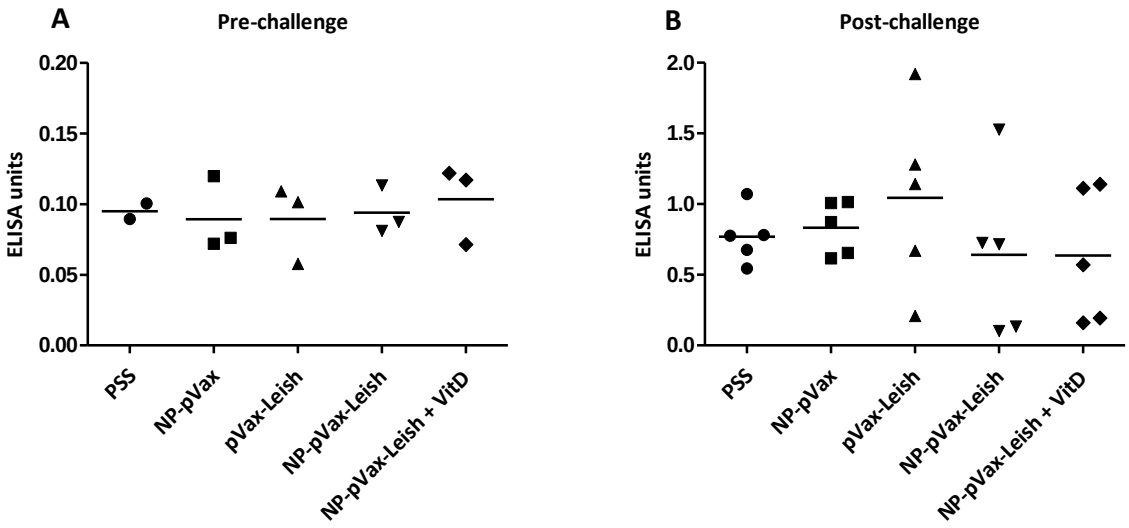


Figure 26. Anti-*Leishmania* IgG levels between groups at pre-challenge (A) and 6 weeks post-challenge (B). Antibody levels were measured by ELISA against crude total *L. infantum* antigen. Cut-off value (mean + 3 SD) was set at 0.150 OD.

Evaluation of cellular immune response

Lymphocytes populations

Statistically significant differences in percentage of lymphocytes T $CD4^+$ and $CD8^+$ were not detected in pre-challenge samples, surely due to the small number of animals included in the analysis (n = 3). However, there seems to be a trend. Vaccinated groups (pVax-Leish, NP-pVax-Leish and NP-pVax-Leish + VitD) had lymphocytes T $CD4^+$ levels a little higher than the PSS control group and also than group receiving encapsulated plasmid (NP-pVax), while all groups (including NP-pVax) had higher levels of lymphocytes T $CD8^+$ than PSS control.

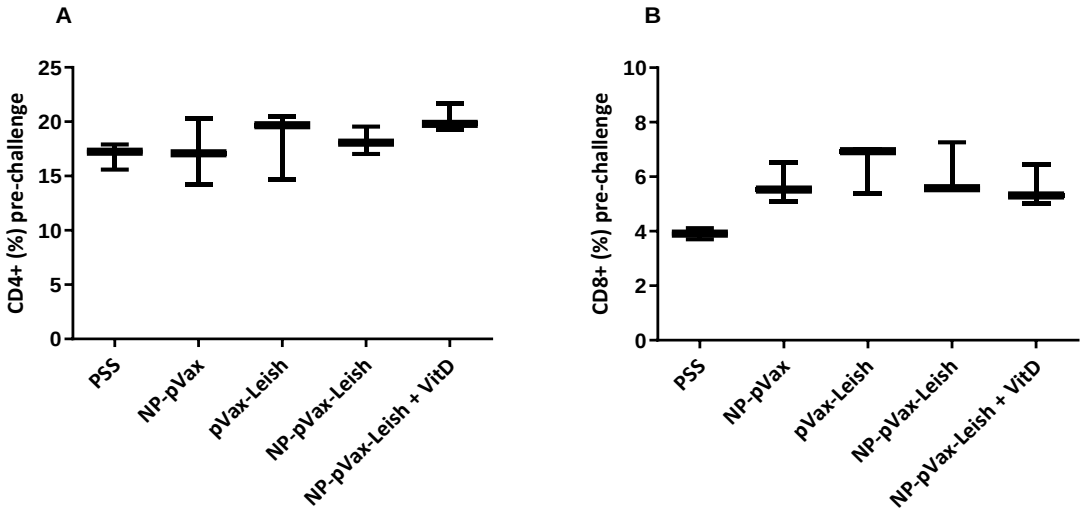


Figure 27. Percentage of CD4⁺ T cells (A) and CD8⁺ T cells (B) for each study group at pre-challenge. CD4⁺ and CD8⁺ T cells were detected from primary cultures of mouse splenocytes.

In the post-challenge analysis, NP-pVax-Leish + VitD showed statistically significant higher percentage of lymphocytes T CD4⁺ with respect to PSS control and with respect to pVax-Leish (P = 0.0317 in both cases). NP-pVax-Leish + VitD group had a higher percentage of CD8⁺ T lymphocytes than the PSS, NP-pVax and pVax-Leish groups (P = 0.0079, P = 0.0317, and P = 0.0079, respectively). The NP-pVax-Leish group also had higher levels of CD8⁺ T lymphocytes compared to the PSS control group (P = 0.0317).

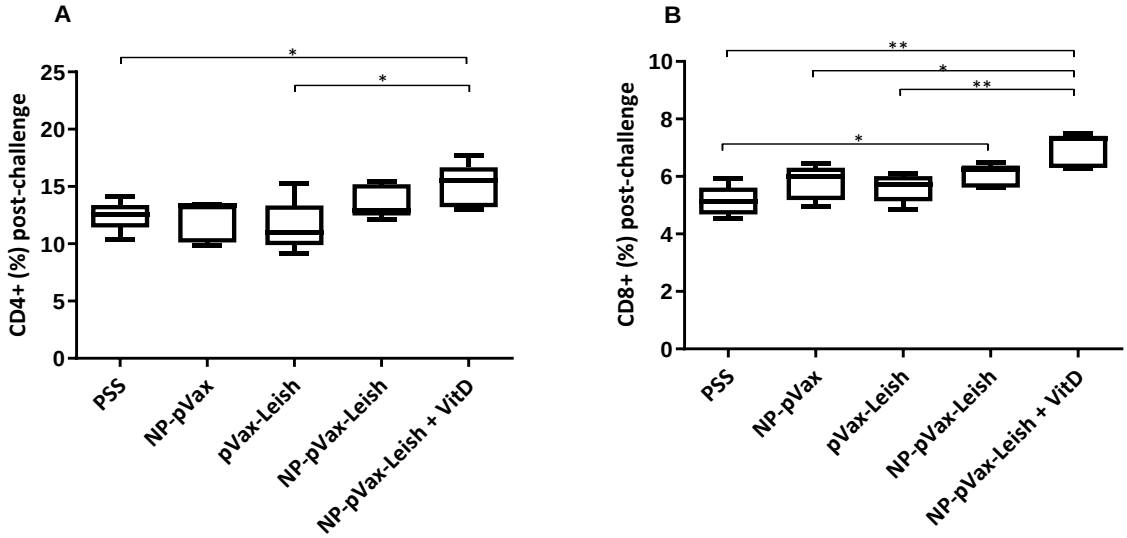


Figure 28. Percentage of CD4⁺ T cells (A) and CD8⁺ T cells (B) for each study group at the end of the study (6 weeks post-challenge). CD4⁺ and CD8+ T cells were detected from primary cultures of mice splenocytes (* P < 0.05; ** P < 0.01).

Percentage of specific lymphocytes T CD4⁺ and CD8⁺ producing IFN-γ were not detected. PMA and ionomycin addition failed to stimulate splenocytes cultures.

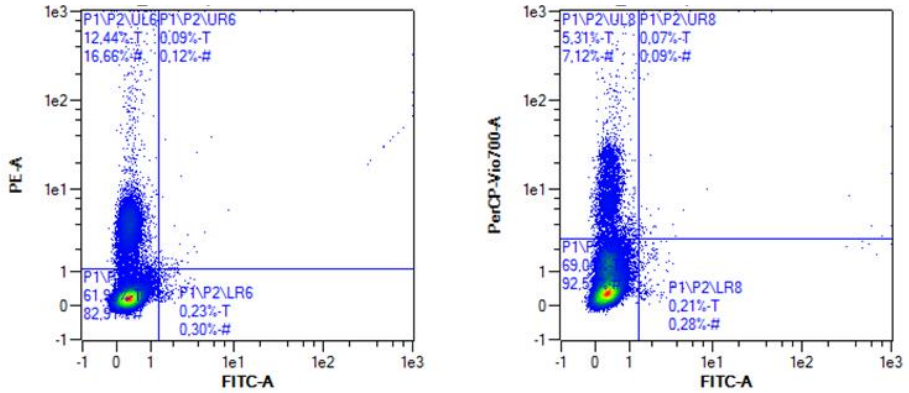


Figure 29. Example of cytometry results for a sample. At top left of are lymphocytes T CD4⁺ (left image) or CD8⁺ (right image).

IFN-γ production

IFN-γ was undetectable in supernatants of unstimulated splenocytes and LSA did not stimulate its production either. We did the analysis considering IFN-γ levels in supernatants of cells stimulated with the T cell stimulus ConA. Despite the variability detected between animals in some groups, it was observed that groups showing higher percentage of CD8⁺ T lymphocytes in flow cytometry analysis did not show higher IFN-γ concentration in supernatants, rather it seems that there were less IFN-γ in NP-pVax-Leish and NP-pVax-Leish + VitD groups when compared to control groups (PSS and NP-pVax). However, there were only statistically significant differences between group NP-pVax and NP-pVax-Leish ($P = 0.0159$).

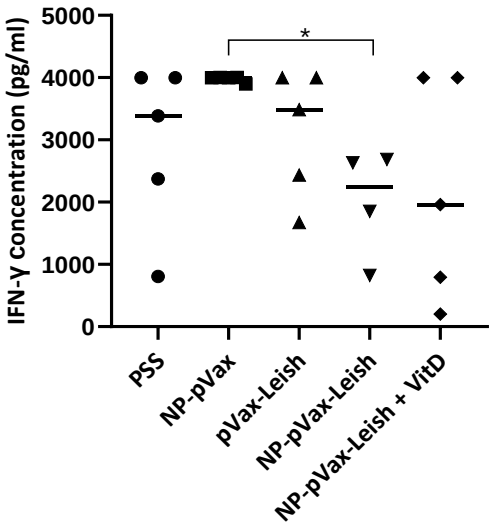


Figure 30. IFN-γ production levels for each study group 6 weeks post-challenge. IFN-γ was measured from supernatants of primary culture of mice splenocytes stimulated with ConA for 48h (* $P < 0.05$).

Cytokine and immune markers expression

There were no significant differences between groups in terms of cytokine expression in liver neither in pre-challenge nor post-challenge analysis (Figures 31 and 32).

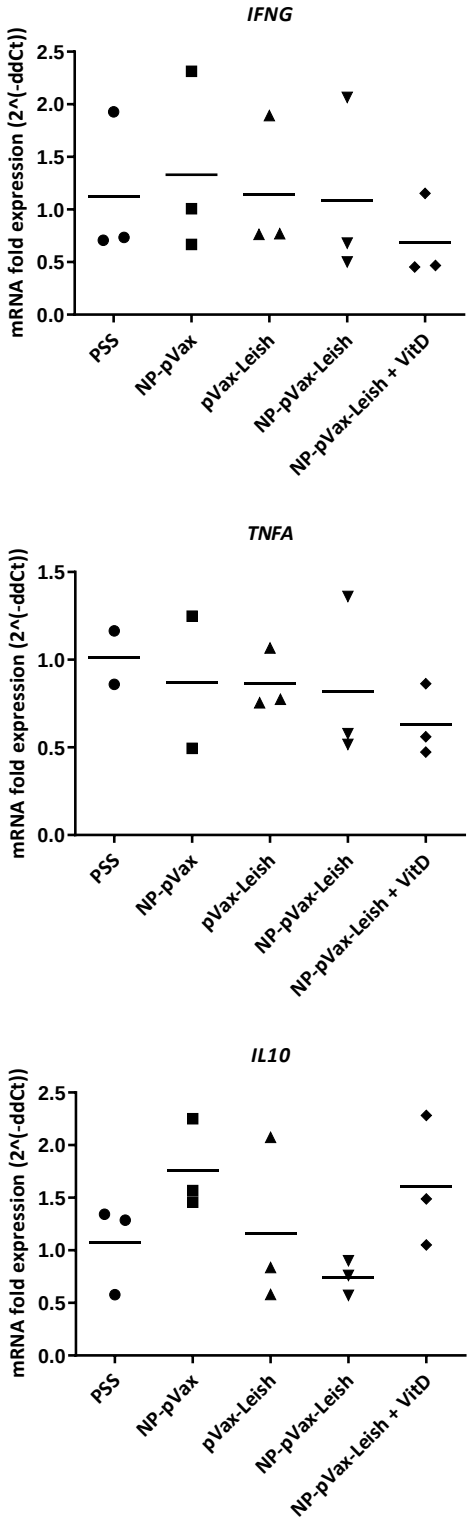


Figure 31. Cytokine expression in liver samples at pre-challenge. *IFNG*, *TNFA*, *IL10* and *IL4* expression was determined by RT-qPCR. *IL4* expression was undetectable. PSS group was taken as a reference.

Although the vaccine itself did not seem to vary cytokine expression in pre-challenge, there was a slight decrease in pro-inflammatory cytokines expression (*IFNG* and *TNFA*) as well as an increase in anti-inflammatory cytokine expression (*IL10*) when vitamin D was administered, but it was not statistically significant (Figure 31).

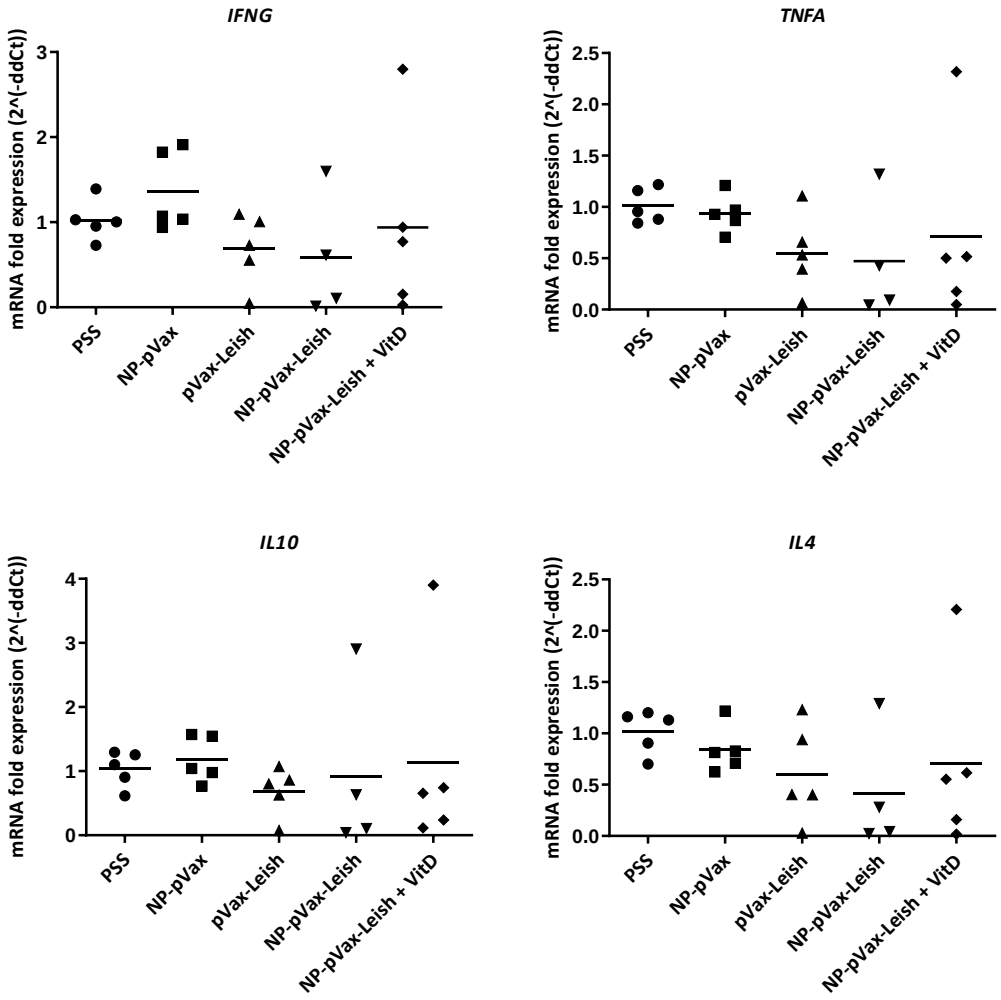


Figure 32. Cytokine expression in liver samples at the end of the study (6 weeks post-challenge). *IFNG*, *TNFA*, *IL4*, and *IL10* expression was determined by RT-qPCR. PSS group was taken as a reference group.

In post challenge analysis, the tendency of the vaccine appeared to be to decrease the expression of all cytokines analyzed (*IFNG*, *TNFA*, *IL10* and *IL4*) (Figure 32).

Parasite load in liver correlated with *IFNG* (ρ Spearman = 0.640, *P* = 0.001), *TNFA* (ρ Spearman = 0.519, *P* = 0.011), *IL4* (ρ Spearman = 0.560, *P* = 0.004) and *IL10* (ρ Spearman = 0.669, *P* = 0.000) expression in 6 weeks post-challenge analysis. Expression of *IFNG* (ρ Spearman = 0.668, *P* = 0.000), *TNFA* (ρ Spearman = 0.539, *P* = 0.008), *IL4* (ρ Spearman = 0.543, *P* = 0.006) and *IL10* (ρ Spearman = 0.640, *P* = 0.001) also correlated with parasite load in spleen at the end of the study.

DISCUSSION

This work was motivated by the urgent need of controlling leishmaniasis, a tropical disease that is responsible for an important number of new infections and deaths every year (World Health Organization, 2017). The dog is the main peridomestic reservoir of zoonotic VL, so that control of the protozoan parasite in this host is the most effective method for controlling this disease (Dye, 1996). This requires a precise diagnosis of infected dogs and the development of a vaccine against CanL. Although there are three licensed and commercialized vaccines for CanL prevention (CaniLeish®, Leish-Tech® and Letifend®) (Bongiorno et al., 2013; Carcelén et al., 2009; Dantas-Torres, 2006; Fernandes et al., 2012), they are not entirely effective (Velez and Gállego, 2020). There is no licensed vaccine against human leishmaniasis yet.

Most *Leishmania*-infected people and dogs have an asymptomatic infection. The protective immunity against *Leishmania* is mediated by both innate and adaptive immune responses. Asymptomatic infections are correlated with the activation of specific cell-mediated immunity and a Th1-proinflammatory immune response (Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A et al., 1986). The effective activation of macrophages, dendritic cells, and antigen-specific $CD4^+$ and $CD8^+$ T cells are needed to kill *Leishmania* (Mougneau et al., 2011; Stanley and Engwerda, 2007). The active disease, however, has been linked to high antibody levels and a progressive Th2-deactivating immune response in the presence of a strong inflammatory reaction (Costa et al., 2012).

It is well-known that vitamin D plays its immunomodulatory role by activating innate immune system and modulating the adaptive immune response against infections (Jeffery et al., 2009). Vitamin D induces expression of AMPs, which are important innate immunity mediators against pathogens (Liu et al., 2007; Yim et al., 2007). Vitamin D also plays a role in regulating adaptive immune response against infection reducing the production of pro-inflammatory Th1 and Th17 derived cytokines (Jeffery et al., 2009; Tang et al., 2009), and IgG secretion and maintaining B-cell homeostasis (Chen et al., 2007).

Vitamin D was hypothesized to play a role in *Leishmania* infection response. Throughout this thesis we have investigated vitamin D implication in canine leishmaniasis and in addition, we tested weather vitamin D immunomodulation could boost DNA vaccine against VL.

DISCUSSION CHAPTER 1

Cross-sectional study of vitamin D and canine leishmaniasis

In this study we show the existence of a clear association between vitamin D levels and the outcome of visceral leishmaniasis. Indeed, 25(OH)D levels were significantly lower in dogs suffering from clinical leishmaniasis than in asymptomatic or non-infected animals living in the same area. In addition, vitamin D deficiency in *Leishmania*-infected dogs was strongly correlated to high parasite load, and the low levels of this vitamin increased the odds of suffering from patent leishmaniasis. To the best of our knowledge, this is the first study that describes a statistically significant relationship between vitamin D deficiency and progression of this parasitic disease. Low vitamin D levels in humans have been associated with a large number of diseases including cancer (Feldman et al., 2014), diabetes (Takiishi et al., 2010), autoimmune diseases (Ponsonby et al., 2005) and infections such as toxoplasmosis (Rajapakse et al., 2007), AIDS (Coussens et al., 2015), influenza (Morris et al., 2016), COVID-19 (Meltzer et al., 2020) or malaria (Cusick et al., 2014). In veterinary medicine, low vitamin D levels in dogs have been recently linked with chronic enteropathy (Titmarsh et al., 2015), neoplasia (Wakshlag et al., 2011), and several infectious diseases (Kuleš et al., 2014; Rosa et al., 2013). The establishment of associations between vitamin D levels and protozoan infections has yielded conflicting results in rodent models (Ehrchen et al., 2007; Rajapakse et al., 2007; Ramos-Martínez et al., 2013; Silva et al., 1993). However, over the last decade some studies have shed light on this issue by demonstrating a role for vitamin D in *Babesia* infections in dogs (Kuleš et al., 2014), as well as in *Plasmodium falciparum* infection in children with vitamin D insufficiency associated with severe cerebral malaria in Uganda (Cusick et al., 2014). Oral administration of vitamin D in rodents before or after *Plasmodium berguei* ANKA infection protected from cerebral malaria by dampening the systemic inflammatory response (He et al., 2014). In addition, a study in Ethiopian children has described vitamin D deficiency (< 20 ng/mL) in more than half of the pediatric VL patients, although the relationship between vitamin D levels and clinical VL could not be established due to the lack of a healthy control group (Diro et al., 2015). In contrast, patients with post-kala-azar dermal leishmaniasis –a clinical form characterized by high levels of anti-inflammatory IL10 cytokine– presented a significant increase in vitamin D levels when compared to healthy controls (Mukhopadhyay et al., 2014).

Due to the cross-sectional nature of this study we were unable to determine whether the low levels of vitamin D found in dogs with VL are the consequence or the cause of this parasitic

disease. One hypothesis states that vitamin D deficiency in sick dogs could be due to an excess of vitamin consumption during the inflammatory process, following a pattern similar to that previously described for vitamin A during chickenpox infection in children (Campos et al., 1987a). Autier et al. performed a meta-analysis investigating the consequences of vitamin D supplementation in humans. They found that the raise of vitamin D levels did not alter the course of the group of health disorders that they studied, and proposed that vitamin D deficiency is the result of inflammatory processes related to age, habits, and/or diseases (Autier et al., 2014). One of the mechanisms that has been put forward to support this hypothesis is the rapid conversion of 25(OH)D to the bioactive $1,25(OH)_2D_3$ by inflammatory cytokines that activate the enzyme catalyzing this reaction, CYP27B1 (Bikle, 2009). Unfortunately, we could not analyze the $1,25(OH)_2D_3$ levels in our canine population, as its half-life is very short and the test for its evaluation is only available at specialized laboratories.

An alternative hypothesis linking vitamin D and leishmaniasis is that dogs with vitamin D deficiency prior to infection might be at a higher risk of developing leishmaniasis. Although vitamin D levels have been determined in other canine infectious diseases (Kuleš et al., 2014; Rosa et al., 2013), no longitudinal studies have been performed so far. The few longitudinal studies conducted in humans reported vitamin D deficiencies prior to clinical manifestations of multiple sclerosis (Décard et al., 2012), pulmonary exacerbations in children with cystic fibrosis (McCauley et al., 2014), and more severe inflammatory bowel disease course (Kabbani et al., 2016). Within malignant neoplasia, colorectal cancer has been consistently associated with low pre-diagnostic vitamin D levels (Fedirko et al., 2012). Low both UVB light exposure and vitamin D-dietary intake have been suggested as the main causes of this prior vitamin D deficiency in humans. Nonetheless, it was unclear whether dogs can synthesize vitamin D from cholesterol precursors and UV skin irradiation as it happens in people (How et al., 1994). In consequence, commercial pet food is routinely supplemented with this ingredient to ensure that the vitamin D nutritional requirements are met. Two different scenarios could have caused the vitamin D deficiency that we observe in the population of dogs that we studied: either the diet differed amongst our groups of dogs, or other factors involved in vitamin D storage/turnover affected serum 25(OH)D concentrations prior to disease.

In any case, regardless of the scenario, as vitamin D has direct effects on the innate and adaptive immune response, a vitamin D deficiency, will —with little doubt—have serious immunological consequences during the course of CanL. The activation of Toll-like receptors (TLR), a family of innate immune system receptors, triggers an antimicrobial response

mediated by vitamin D. Indeed, the expression of *VDR* and *CYP27B1* are up-regulated in response to the activation of TLR2 of human macrophages by *Mycobacterium tuberculosis* antigens (Liu et al., 2006a). Activation of TLR2 in the presence of vitamin D increases the expression of target immunity genes, such as the anti-microbial peptide cathelicidin and β-defensin 4 (Martineau et al., 2007). In the context of *Leishmania* infection, lipophosphoglycans and the cytosolic protein silent information regulator 2 from *L. infantum* activate TLR2 in antigen presenting cells, and the peptide BMAP-28 –a cathelicidin member family – has demonstrated anti-*Leishmania* effects and immuno-modulatory properties in *in vitro* cultures (Lynn et al., 2011; Silvestre et al., 2009). These results would support the hypothesis of a beneficial role of vitamin D in innate immunity against *Leishmania* infection. In addition, vitamin D also exerts its immuno-modulatory role by shaping B cell and T cell responses. Exposing human B cells to $1,25(OH)_2D_3$ inhibits their proliferation, IgG secretion, memory B cell generation, and induces B cell apoptosis (Chen et al., 2007). Although further studies are needed to demonstrate the role of vitamin D on canine B cell immunity, our results seem to tally with this finding, which may explain the strong inverse correlation that we observed between vitamin D and anti-*Leishmania* antibody levels (Figure 17B). On the other hand, there are several studies investigating the relationship between vitamin D and T cell immune response during the course of *Leishmania* infection. However, these studies focused on the cutaneous form in a rodent model and, in addition, they yielded discrepant results (Ehrchen et al., 2007; Ramos-Martínez et al., 2013; Whitcomb et al., 2012). Our analysis did not show any association between *Leishmania*-specific IFN-γ or IL10 production and vitamin D levels. As we have highlighted above, vitamin D function could be more related to the innate immune response rather than to adaptive immunity.

Remarkably, our results show a negative correlation between both LST and IFN-γ levels and the progression of leishmaniasis (clinical signs, serology and blood parasite load). These results are in keeping with the importance that the Th1 immune response has in the control of this disease (Carrillo and Moreno, 2009). Moreover, our study finds a positive correlation between IFN-γ production and the LST reaction suggesting that an LST result could be a marker of IFN-γ status. There are few and poorly standardized assays that evaluate T-cell mediated immunity responses in dogs (Fernández-Bellon et al., 2005). Whilst the techniques based on the detection of IFN-γ levels may represent an expensive and time-consuming technique, the LST test could be a field-adapted tool for the evaluation of *Leishmania*-specific cell-mediated immunity in epidemiological and vaccine studies.

Vitamin D activates the VDR transcription factor, which in turn, modulates the expression of several key genes involved in the immune response (Booth et al., 2016). For this reason, genetic variation in the *VDR* gene changing its binding affinity, activity or protein levels may influence the immunological efficiency of vitamin D. In addition, *in vitro* and knock-out mice experiments have linked VDR to the resistance to *Leishmania major* infection (Ehrchen et al., 2007). Indeed, genetic variants in the *VDR* gene have also been associated with an increased risk of chronic chagasic cardiomyopathy (Rodriguez et al., 2016), malaria severity (Sortica et al., 2014), tuberculosis (Salimi et al., 2015), and some autoimmune disorders (De Azevêdo Silva et al., 2013; Carvalho et al., 2015). Our results showed that the four *VDR* polymorphisms display similar frequencies across the non-infected, asymptomatic, and symptomatic dog groups (Table 7), and no genetic association between these SNPs and disease/infection status were observed (Table 8). The 4 SNPs that we investigated span a 10 kb genomic segment encompassing from intron 3 to intron 7 of the VDR transcript ENSCAFT00000014506. These SNPs are predicted to be either synonymous or intronic and should not alter protein function. They were screened as markers to identify any potential functional and causal polymorphism affecting VDR function. Moreover, two of our variants were present at low allelic frequencies in our population and this could have resulted in a low statistical power to detect a genetic association given the small sample size of our study. However, the canine *VDR* gene has 10 exons and spans a 58 kb long region, so we cannot rule out the hypothesis that other genetic variants elsewhere in the *VDR* gene affects the function or activity of VDR and, ultimately, impacts on the susceptibility to CanL infection. Moreover, a more comprehensive study involving additional genes from the vitamin D pathway such as *CYP2R1*, which converts vitamin D into 25(OH)D, would provide a deeper understanding of the relationship between genetic susceptibility to CanL and vitamin D metabolism. Larger studies involving more individuals and a higher density of genetic variants would increase the power to detect genetic associations in the vitamin D pathway to CanL susceptibility.

In summary, in this study we reported that progression of VL is strongly associated with vitamin D deficiency in dogs. Our findings suggested that vitamin D pathway may be involved in the immune response against *Leishmania* and suggest that vitamin D supplementation could be used both as an adjuvant therapy and to protect against the disease. This study, however, was not enough to determine if low vitamin D concentration found in dogs with clinical CanL were a cause or a consequence of the disease. In order to solve this relevant issue, we planned a longitudinal study which will be discussed in the next section.

DISCUSSION CHAPTER 2

Longitudinal retrospective study of vitamin D and canine leishmaniasis

In the first chapter of this thesis we showed that dogs with clinical leishmaniasis presented lower vitamin D serum concentration than non-infected dogs, and even lower than those with asymptomatic infection. However, it was not possible to prove whether the low vitamin D levels found in dogs with CanL were the consequence or the cause of this parasitic disease. Although vitamin D levels have been determined in other canine infectious diseases such as spirocercosis (Rosa et al., 2013), no longitudinal studies have been performed. To the best of our knowledge, the study showed in chapter 2 is the first longitudinal study describing vitamin D concentration in a canine population living in a highly endemic area of leishmaniasis.

In humans, it has been widely described that vitamin D status is seasonal due to photochemical activation of provitamin D_3 in skin by UVB rays (de Oliveira et al., 2020; Rabufetti et al., 2019), but it remained unclear if the same is true in dogs because of differences in skin hair. Our results show that vitamin D concentration in dogs does not follow a seasonal pattern, with similar concentration in winter and spring. These results are consistent with the few previous studies that investigated vitamin D synthesis in dogs. Whatley and Sherreported low presence of vitamin D precursors in the skin of healthy dogs (Wheatley and Sher, 1961) and How et al. detected a low UV-mediated conversion rate of the precursor 7-dihydrocholesterol to vitamin Din dogs compared to rats (How et al., 1994). However, only one longitudinal study has been performed, enrolling huskies from polar latitudes and showing an inverse relationship between UVB radiation and vitamin D status (Griffiths and Fairney, 1988). This result was accounted for the vitamin D rich diet received by these dogs during the winter. These studies pointed out the importance of vitamin D supplementation in dogs, but they did not rule out some effect of UVB light on this specie. Our results confirm that vitamin D status in dogs is not influenced by the number of hours of exposure to sunlight and would not matter the season of the year when assessing vitamin D levels in clinical practice.

In the retrospective longitudinal study we have shown that non-infected dogs did not present significant changes in 25(OH)D concentration between the beginning and the end of the study one year later, whereas those developing clinical leishmaniasis have a significant 25(OH)D decrease at the end of the study (35 % reduction). When 25(OH)D levels at starting points were compared, no differences between groups were detected. Therefore, vitamin D concentration could not be used as a prognostic marker of clinical leishmaniasis. However, as vitamin D concentration decreased with the onset of clinical symptoms, we suggest that

vitamin D concentration could be useful as clinical marker for the evolution of this disease. It becomes more evident that low vitamin D concentration in sick dogs was due to the infective episode causing an accelerated depletion of vitamin D, as it was demonstrated for vitamin A during a chickenpox infection (Campos et al., 1987b). Decreased vitamin D with the onset of clinical disease would be consistent with studies that have suggested that low vitamin D concentration is a marker of ill health (Autier et al., 2014).

In summary, we have shown that vitamin D concentration is neither seasonal nor a risk factor for developing canine leishmaniasis, but dogs developing clinical leishmaniasis experience a progressive decrease in vitamin D concentration throughout the course of disease suggesting a role in parasitic control. Assessment of vitamin D status in dogs suffering from clinical leishmaniasis may be helpful in improving the management of this disease.

Mechanism of action of vitamin D in canine macrophages during *L. infantum* infection

We also investigated if vitamin D plays a role in the control of *Leishmania* load inside macrophages. Our *in vitro* model using a canine macrophage cell line showed that this hormone has a parasite killing activity, since addition of active vitamin D at 0.1 µM led to a significant reduction in *L. infantum* parasite load at 24 h post-infection. This is in line with the inhibitory effect of $1,25(OH)_2D_3$ described for *Toxoplasma gondii*, *Mycobacterium tuberculosis* and *Leishmania amazonensis* intracellular growth (Liu et al., 2006b; Machado et al., 2020; Rajapakse et al., 2007) and it suggests that vitamin D could have a protective *in vivo* effect against *Leishmania*, as it is the case for *Trypanosoma cruzi* infection (Silva et al., 1993). Studies investigating the relationship between vitamin D and response to *Leishmania* infection yielded discrepant results. Ramos-Martinez et al. reported a significant reduction in the lesion size in *L. mexicana*-infected mice treated with $1,25(OH)_2D_3$ (Ramos-Martínez et al., 2013), while other studies suggest that vitamin D deficiency increases resistance to *L. major* and *L. amazonensis* (Bezerra et al., 2019b; Ehrchen et al., 2007; Whitcomb et al., 2012). However, these results focused on the cutaneous form and/or in a mouse model, which is predisposed to Th1 immune response. A study with VL patients has shown that people suffering from this disease presented significant lower $1,25(OH)_2D_3$ serum concentration than healthy people, in agreement with our results (Das et al., 2017).

Although the molecules and signals involved in vitamin D effects against *Leishmania* infection have not yet been investigated, the mechanism of vitamin D action against tuberculosis

infection is well-known. Following TLR2 activation of human macrophages by *M. tuberculosis* antigens, expression of *CYP27B1* and *VDR* increases (Liu et al., 2006b). This ends up in increased expression of AMPs, mainly cathelicidin (Baeke et al., 2010c). AMPs are important innate immunity mediators against microbial pathogens. They act through direct interaction with and disruption of microbial membranes, and indirectly through modulation of host cell migration and activation (Lehrer et al., 1989). There are evidences that mammalian AMP cathelicidin influences control of cutaneous *Leishmania* infection. A *Leishmania* study using a *CAMP* knock-out mouse model showed that the presence of high inflammatory response in infected animals was *CAMP*-dependent (Kulkarni et al., 2011). In our retrospective longitudinal study, we did not find statistically significant differences in *CAMP* expression between non-infected and *Leishmania*-infected groups at the end of the study. We also investigated *CAMP* expression in primary canine monocytes as an *ex vivo* model. These experiments also showed no-differences in *CAMP* expression after addition of $1,25(OH)_2D_3$, even though *CAMP* expression was increased after LPS stimulation. Although in human macrophages $1,25(OH)_2D_3$ increases the expression of *CAMP* directly via vitamin D response elements in the *CAMP* gene promoter (Das et al., 2017; Gombart et al., 2005), the pathway of action of vitamin D could be different depending on the animal species. In cattle, where $1,25(OH)_2D_3$ modulates the immune response by increasing NO production in peripheral blood mononuclear cells (Waters et al., 2001), *CAMP* was not affected by addition of $1,25(OH)_2D_3$ (Nelson et al., 2010). For this reason, we investigated other candidates that could be induced by vitamin D in canine macrophages.

The other group of AMPs that can fend off bacterial and viral infections are β-defensins (Kim et al., 2018; Midorikawa et al., 2003). Antimicrobial response of vitamin D in human macrophages is also mediated by β-defensins through human TLRs (Liu et al., 2009; Wang et al., 2004). In dogs, canine β-defensin 103 (CBD103) has been found in the epidermis of healthy dogs and its expression was altered in atopic animals (van Damme et al., 2009). In our study, *CBD103* expression was significantly enhanced after $1,25(OH)_2D_3$ addition on primary canine monocytes from blood donors. In addition, we detected that healthy dogs had slightly higher expression of *CBD103* than those suffering from the disease at the end of the study, although this difference was not statistically significant. In agreement with our results, other studies have determined the important role that β-defensins play in host defense against *Leishmania* protozoa (dos Santos et al., 2017; Da Silva et al., 2017). In dogs, some SNP's in *CBD103* gene have been associated with *Leishmania* infection, suggesting that it could be a marker of susceptibility (Da Silva et al., 2017). The expression of β-defensins in *Leishmania*-infected

human macrophage cell line THP-1 was induced by the cytokine IL32γ. The inhibition of IL32 lead to an increase of *Leishmania* infection index in THP-1 cells whereas its overexpression induced parasite control by AMPs (dos Santos et al., 2017). The detection of IL32 in canine macrophages would be very useful to determine if β-defensin expression in dogs is also modulated by this cytokine. Functional studies investigating the direct effect of β-defensin on *Leishmania* growth could confirm this molecule as responsible of the observed antiparasitic activity of vitamin D in canine monocytes.

LPS stimulation did not induce β-defensin expression in canine monocytes. CBD103 has antimicrobial activity against the respiratory pathogen *Bordetella bronchiseptica*, but tracheal epithelial cells stimulated with LPS did not increase β-defensins production (Erles and Brownlie, 2010). Similarly, LPS stimulation was not enough to induce β-defensin expression in cattle monocytes (Merriman et al., 2015). These authors showed that vitamin D was the major driver of the β-defensin response of bovine monocytes. These results suggest that vitamin D pathway in canine macrophages may not be activated via TLR4, but TLR2/1 as in humans (Liu et al., 2006a).

After addition of vitamin D, *VDR* expression remains unchanged in canine macrophages. Treatment of bovine monocytes with the protein translation inhibitor cycloheximide blocked upregulation of β-defensins in response to $1,25(OH)_2D_3$ (Merriman et al., 2015). This suggests that although β-defensins are targets of $1,25(OH)_2D_3$ in cattle, they are not direct targets of the *VDR*. Nurminen *et al*. identified multiple transcriptional regulators that are direct targets of vitamin D in the human THP-1 monocyte cell line. They demonstrated that BCL6 mediated the induction of several of the secondary response genes, and concluded that most of the physiological response of human monocytes to $1,25(OH)_2D_3$ was a secondary response (Nurminen et al., 2015). The same could occur in canine monocytes where we found a significant increase in *CBD103* after adding active vitamin D but not in *VDR*.

In summary, we have described for the first time the parasite killing activity of vitamin D in *Leishmania*-infected canine monocytes. Our results suggest that this relevant effect could be due to a significant induction of expression of genes implicated in host defence, such as the AMP β-defensin 103. Future goals derived from this study would be to investigate if administering calcitriol or active vitamin D may mitigate the symptoms and progression of this disease and if could be a good adjuvant in vaccine strategies.

DISCUSSION CHAPTER 3

Use of vitamin D as immunomodulator during a vaccination protocol to boost protection against visceral leishmaniasis in mice

Obtaining an effective prophylactic vaccine to prevent VL remains a challenge. Different types of vaccine have been assayed up until now, with variable results, but a perfect safety vaccine that achieves a high protective immunization has not yet been discovered. Clinical trials have shown that DNA vaccines are safe and well tolerated but have highlighted the need for finding new boosting strategies to potentiate protection induced by DNA immunization (Donnelly et al., 2003). Although vitamin D have been studied for enhancing protection against some bacterial and viral infections (Goncalves-Mendes et al., 2019; Zheng et al., 2014), nothing is known about their effect in a vaccination protocol against VL. To the best of our knowledge, the present study represents the first that use the immunomodulatory molecule vitamin D in a vaccination strategy against leishmaniasis with the aim of improving the immune response against this parasitic infection. We have tested a naked DNA vaccine and a liposomal DNA vaccine in order to test if encapsulation in nanoparticles could improve this vaccine candidate, as nanoparticles are known to protect from DNA degradation by nucleases and improve delivery to immune cells (Farris et al., 2016). For that purposes, five different experimental groups were defined: PSS (physiological salt solution), NP-pVax (vaccine plasmid encapsulated in liposomes), pVax-Leish (naked DNA vaccine), NP-pVax-Leish (DNA vaccine encapsulated in nanoparticles), NP-pVax-Leish + VitD (DNA vaccine encapsulated in nanoparticles with $1,25(OH)_2D_3$ coadministration). The effect on parasite load and immune mediators were analyzed before *L. infantum*-challenge and six weeks after experimental infection.

The results showed that the naked DNA vaccine containing *KMPII*, *PAPLE22*, *TRYP* and *LACK* sequences designed in our laboratory protected 1 of 5 animals in the main *Leishmania* target organs: liver and spleen. Liposomal DNA vaccination achieved low parasite loads on 2 of 5 animals in these organs and the same liposomal DNA vaccination with concomitant active vitamin D administration achieved full protection in 2 of 5 animals. These results indicate that naked DNA vaccine itself had partial protection that could be improved with liposomal formulation and especially with vitamin D coadministration. However, due to the small size of the sample it is difficult to draw conclusions. This will have to be confirmed with a largest study including more animals. Anyway, the fact that vitamin D led to a *Leishmania* parasite load reduction is supported with our *in vitro* results shown in chapter 2, and with other *in vitro* studies in which vitamin D increased killing of intracellular parasites (Machado et al., 2020;

Rajapakse et al., 2007). Other research conducted in mice showed contradictory results on whether vitamin D allows to reduce the parasitic load of *Leishmania,* finding that there were no difference in the number of *L. mexicana* when received $1,25(OH)_2D_3$ treatment (Ramos-Martínez et al., 2013) and even that vitamin D deficiency increases resistance to *L. amazonensis* (Bezerra et al., 2019a). However, these studies were conducted with strains causing cutaneous form.

Plasmid DNA vaccines are known to promote antibody production (Gurunathan et al., 2000b; Huygen, 2005). However, serology levels in our study revealed no differences of anti-*Leishmania*-specific IgG antibodies neither in pre-challenge nor six weeks post-challenge in any of the groups (Figure 26). In previous experimental trials of this vaccine conducted with hamsters, a reduction in specific IgG antibodies of vaccinated animals was observed (Martínez-Flórez et al., 2020; Todolí et al., 2012). The effect of vitamin D supplementation on humoral immune response was evaluated in influenza vaccination studies, which showed no effect of vitamin D coadministration on antibody production (Goncalves-Mendes et al., 2019; Kriesel and Spruance, 1999; Principi et al., 2013). Detection of specific antibodies against each one of the antigen vaccine candidates would be needed to further characterize the vitamin D response. Nanoparticle vaccine models have been shown to induce high titers of serum antibodies, but it could be highly dependent on the composition of the nanoparticles (Sulczewski et al., 2018). Some liposomal formulation have proved an improved antibody response in different vaccine trials for viral infections (Bale et al., 2017; Lay et al., 2009; Mitchell et al., 2006). This humoral enhancement has not been noticed in our assay.

When T lymphocyte populations were analyzed, we found that plasmid DNA alone could be acting as an adjuvant per se as we detected a noticeable increase in $CD8^+$ T lymphocytes population at pre-challenge in all groups injected with plasmid DNA vaccine (NP-pVax, pVax-Leish, NP-pVax-Leish, and NP-pVax-Leish + VitD). We also observed a moderate increase in $CD4^+$ T lymphocytes in pVax-Leish, NP-pVax-Leish, and NP-pVax-Leish + VitD groups. Although the small size of the sample and variability between animals make it difficult to draw conclusions, these appear to be successful results as ideally, an effective leishmania vaccine should generate a long-lasting protective immune response involving activation of $CD4^+$ and $CD8^+$ T lymphocytes. Several studies demonstrated that CpG motifs naturally present in plasmid DNA help to induce long-term immunity that protects against parasite (Klinman et al., 1997; Lipford et al., 1997). These sequences trigger TLR9 activation in APCs (Hemmi et al., 2000), therefore boosting Th1 responses by promoting non-specific production of IFN-γ and

$CD4^+$ and $CD8^+$ responses, which had been shown to be important in *Leishmania* infection (Belkaid et al., 2002; Gurunathan et al., 2000a; Huygen, 2005; Lipford et al., 1997; Rhee et al., 2002; Stacey and Blackwell, 1999; Walker et al., 1999). However, our previous studies using DNA vaccines against leishmaniasis have showed that this strategy needs to be boosted to improve their efficacy against infection (Martínez-Flórez et al., 2020; Rodríguez-Cortés et al., 2007c; Todolí et al., 2012). For this reason, we have tested nanoparticles and vitamin D coadministration.

In this sense, our results showed that only group receiving vitamin D as an adjuvant during immunization protocol had statistically significant higher percentage of $CD4^+$ and $CD8^+$ T lymphocytes than control group 6 weeks after infection (Figures 28A and 28B). Liposomes may have contributed to $CD8^+$ T cells increase (Christensen et al., 2007; Henriksen-Lacey et al., 2010b; Heuts et al., 2018; Varypataki et al., 2015; Zaks et al., 2006), which would explain the slight increase observed in groups in which nanoparticles have been administered and that was not seen in PSS and pVax-Leish groups (Figure 28B). The increased frequency of $CD8^+$ T cells has been associated with low splenic parasitism during canine visceral leishmaniasis (Guerra et al., 2009). $CD8^+$ T cells are required for primary immunity in mice, as an association between reduction of the parasite load and the accumulation of $CD8^+$ T cells in the skin of *L. major*-infected mice was found (Belkaid et al., 2002). To know if these increased lymphocyte populations were promoting enhanced specific immune response, such as IFN-γ production, it would have been interesting to analyse isolated cell population from animals' spleen directly labelling $CD4^+$ and $CD8^+$ IFN-γ -producing cells. Due to technical problems we were unable to determine IFN-γ by flow cytometry, thereby, we measured this relevant cytokine in supernatants of ConA-stimulated murine splenocytes. We did not find a correlation between the increase of $CD8^+$ T cells and IFN-γ production, suggesting that the $CD8^+$ population stimulated by vitamin D could be regulatory. Recent studies have shown the immunoregulatory role of a population of $CD8^+$ Treg cells (Yu et al., 2018).

We did not neither find statistically significant differences on cytokine expression in liver between groups, as our previous results in a hamster model (Martínez-Flórez et al., 2020). On the contrary, we noticed that protected animals presented very low cytokines expression and there was a positive correlation between *IFNG*, *TNFA*, *IL4* and *IL10* expression in liver and parasite load in liver and spleen. *Leishmania infantum* infection caused a burst of both pro-inflammatory and anti-inflammatory cytokine in mice, in accordance with studies performed in *L. infantum*-infected dogs and humans (Corrêa et al., 2007; Lage et al., 2007; Michelin et al.,

2011; de Oliveira França et al., 2020; Panaro et al., 2009). Although IFN-γ is a cytokine widely accepted to be involved in *Leishmania* control, it is also known to steadily increase during early stages of infection without a clear contribution to parasite control (Melby et al., 2001; Perez et al., 2006). Recent studies have also shown that IFN-γ has a paradoxical effect in promoting parasite growth (Kong et al., 2017). Interestingly, in experimental-infected dogs, *IFNG* was only expressed by tissues with high parasite load, such as spleen and liver, suggesting that the presence of this cytokine is not synonymous of parasite clearance (Maia and Campino, 2012). Further studies are needed to investigate the cell source of IFN-γ and its role during disease in *L. infantum* infection.

Despite parasitological protection observed in liver in two vitamin D-treated mice, we observed a slight decrease of *IFNG* and *TNFA* and little increase of *IL10* expression in NP-pVax-Leish + VitD group with respect to NP-pVax-Leish group in the same tissue at pre-challenge. These tendencies would agree with the fact that vitamin D directs the lymphocyte polarization toward a tolerogenic immune response (Alvarez et al., 2013; Goncalves-Mendes et al., 2019). Vitamin D is known to shift the T-cell response from a Th1 to a Th2-mediated cell response, and thereby reduce inflammation and promote an immunoregulatory state (Cantorna et al., 2008; Hansdottir et al., 2010; Di Rosa et al., 2011). Moreover, it promotes *in vitro* the regulatory T cells (Treg) differentiation (Correale et al., 2011; Rosenblatt et al., 2010). Other studies show the protective role of Treg expansion in *L. panamensis* and *L. amazonensis* infection, favoring an improvement in the disease (Ehrlich et al., 2014; Ji et al., 2005), probably by relieving the hyper-inflammatory state by IFN-γ downregulation. Thus, although we need more experiments to draw conclusions, our results could reaffirm that vitamin D may be an important immune response regulator, notably in vaccine and infection challenges (Avenell et al., 2007; Urashima et al., 2010). Our DNA vaccine may be regulating IFN-γ production by avoiding its over-expression, and possibility its counter-productive effect, which resulted in a little protection due to synergic effects of vitamin D as already happened with sirolimus coadministration (Martínez-Flórez et al., 2020).

To sum it up, co-administration of vitamin D could have potentiated the protection conferred by the liposomal DNA vaccine carrying the *Leishmania* genes *TRYP*, *PAPLE22*, *KMPII* and *LACK* assayed in this study. The protection achieved could be associated with increased lymphocytes T $CD4^+$ and $CD8^+$. Vitamin D could be a promising adjuvant for immune therapies, but tested vaccine was not enough protective. So, our data are not enough to demonstrate the basis of potentiation. More studies to characterize its response in *L. infantum* infection are needed.

FUTURE PERSPECTIVES

Our findings about the role of vitamin D in the immunological system of dogs will allow to exploit calcitriol as a new immunomodulatory or antimicrobial compost and to discover new molecules that can be used for the treatment or immune prophylaxis of CanL. The inducible nature of β-defensins expression suggest that potent inducers could be used to increase their endogenous expression for the prevention or treatment of disease. Or even, the administration of these peptides could be used directly. Given the need of new preventive methods for CanL and because of the limited effectiveness of current vaccines against this disease, a future interesting goal would be to exploit the use of vitamin D or its derivatives as immunomodulators in other vaccine strategies more protective than the DNA vaccine tested in this thesis.

CONCLUSIONS

1st objective: "To study if vitamin D concentration is associated with CanL progression"

1. Dogs with patent leishmaniasis disease present significantly lower levels of 25(OH)D than the non-infected and the asymptomatic groups.
2. 25(OH)D levels are correlated with the clinicopathological score, serology and parasite load in blood samples. 25(OH)D levels are not correlated with *Leishmania*-specific IFN-γ or IL10 production.
3. *VDR* polymorphisms are not associated with CanL.

2nd objective: "To determine if there was a vitamin D deficiency prior to develop clinical CanL and if vitamin D levels could be a prognostic or clinical marker for this disease"

4. The population of dogs studied shows similar vitamin D concentrations throughout the year. So, vitamin D concentration does not follow a seasonal pattern in dogs, ruling out photochemical activation of vitamin D in dog skin by UVB rays.
5. Dogs developing clinical CanL undergo a progressive decrease in vitamin D concentration throughout the course of the disease.
6. At the time of diagnosis, healthy dogs seem to have slightly higher expression of *CBD103* than those suffering from the disease, and dogs suffering leishmaniasis may have slightly higher expression of *CYP27B1*. There are no indications of a differential trend in *VDR* expression between groups. Therefore, vitamin D could act against *Leishmania* infection by activation of AMP *CBD103*. As *VDR* seems unchanged, this regulation could involve other transcriptional regulators.

3rd objective: "To study the mechanism of action of vitamin D in canine macrophages during *L. infantum* infection"

7. Active vitamin D shows parasite-killing properties on *Leishmania infantum*. It reduces the parasite load of *Leishmania*-infected canine macrophages in a dose-dependent manner.
8. *β-defensin 103* expression is upregulated after vitamin D addition in canine macrophages. Vitamin D regulates *Leishmania infantum* infections in dogs through β-defensin 103.

4th objective: "Use of vitamin D as immunomodulator during a vaccination protocol to boost protection against visceral leishmaniasis in a rodent model"

9. The DNA vaccine carrying the *Leishmania* genes TRYP, PAPLE22, KMPII and LACK in the pVAX vector assayed in this study does not significantly reduced parasite load in liver nor spleen of challenged mice.
10. DNA vaccine and vitamin D do not promote specific antibody production.
11. Vitamin D and liposomal nanoparticles could act as immunogens for T cells in mice.
12. The co-administration of vitamin D during DNA vaccination in mice expanded the achieved protection by the vaccine itself. Vitamin D could be a good candidate for VL vaccine boosting, but more studies are needed to confirm it.

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ANNEX

PUBLICATIONS

List of publications derived from this thesis

- Rodriguez-Cortes, A., Martori, C., Martinez-Florez, A., Clop, A., Amills, M., Kubejko, J., Lull, J., Nadal, J. M., & Alberola, J. (2017). Canine leishmaniasis progression is associated with vitamin D deficiency. *Scientific reports*, *7*(1), 1-10. https://doi.org/10.1038/s41598-017-03662-4
  *Footnote: A. Rodriguez-Cortes and C. Martori contributed equally to this work

- Martori, C., Vélez, R., Gállego, M., Mesa, I., Ferreira, R., Alberola, J., & Rodriguez-Cortes, A. (2021). Vitamin D and leishmaniasis: neither seasonal nor risk factor in canine host but potential adjuvant treatment through *CBD103* expression. Under review in *Plos neglected tropical diseases*.

List of other publications during this thesis

- Martínez-Flórez, A., Martori, C., Monteagudo, P. L., Rodriguez, F., Alberola, J., & Rodríguez-Cortés, A. (2020). Sirolimus enhances the protection achieved by a DNA vaccine against *Leishmania* infantum. *Parasites & Vectors*, *13*(1), 1-12. https://doi.org/10.1186/s13071-020-04165-4

- Martinez-Peinado, N., Martori, C., Cortes-Serra, N., Sherman, J., Rodriguez, A., Gascon, J., Alberola, J., Pinazo, MJ., Rodriguez-Cortes, A., & Alonso-Padilla, J. (2021). Anti-Trypanosoma cruzi Activity of Metabolism Modifier Compounds. *International Journal of Molecular Sciences*, *22*(2), 688. https://doi.org/10.3390/ijms22020688

- Sanchez-Moral, L., Ràfols, N., Martori, C., Paul, T., Téllez, E. & Sarrias, MR. (2021). Multifaceted roles of CD5L in infectious and sterile inflammation. *International Journal of Molecular Sciences,* 22(8), 4076. https://doi.org/10.3390/ijms22084076