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A single-centre, open-label, controlled, randomized clinical trial to assess the preventive efficacy of a domperidone-based treatment programme against clinical canine leishmaniasis in a high prevalence area

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ABSTRACT

The innate immune response acting immediately after initial infection with *Leishmania* parasites is known to play a relevant role in prevention against clinical progression of the disease. Domperidone is a dopamine D2 receptor antagonist that has shown to enhance the innate cell-mediated immune response.

The aim of this study was to assess the preventive efficacy of a domperidone-based treatment programme against clinical canine leishmaniasis (CanL) in a high prevalence area. The study was performed with 90 healthy, seronegative dogs of different sex, age, weight and breed from a single veterinary clinic located in Valencia (Spain). Dogs were randomly allocated into two groups. Dogs in one group (domperidone-treated group; $n = 44$) were administered an oral suspension of domperidone at 0.5 mg/kg bw/day during 30 consecutive days, every 4 months. Dogs in the other group (negative control group; $n = 46$) were left untreated. A 21-month follow-up period was implemented covering two seasonal phases of the sand fly vector. During this period all animals underwent periodic clinical examinations and blood samplings for anti-*Leishmania* serological testing. Dogs seropositive for *Leishmania* (IFAT antibody titre $\geq$ 1:80) plus at least one clinical sign consistent with CanL (indicative of active infection and incipient disease progression) were categorized as a 'prevention failure'. These dogs were withdrawn from the study after confirming the infection by direct observation of the parasite in smears of lymph nodes and/or bone marrow aspirates.

The cumulative percentage of 'prevention failure' after 12 months was significantly lower in the domperidone-treated group than in the negative control group (7% *versus* 35%, $p = 0.003$). Differences between groups persisted after 21 months (11% *versus* 48%, $p < 0.001$). The prevention rate provided by domperidone was 80% during the first 12 months and 77% throughout the complete 21-month follow-up period, with odds ratios of 7.3 ($p = 0.001$) and 7.15 ($p < 0.001$), respectively, this indicating that the risk for domperidone-treated dogs to develop the clinical disease is quite 7 times lower than for dogs left untreated.

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The results of this study demonstrate that the implementation of a strategic domperidone-based treatment programme consisting in quarterly repeated 30-day treatments with domperidone effectively reduces the risk to develop clinical CanL in areas with high prevalence of the disease.

1. Introduction

Canine leishmaniasis (CanL) is a zoonotic disease caused by a protozoan parasite (*Leishmania infantum* or its New World synonym *Leishmania chagasi*) and transmitted by the bite of a phlebotomine sand fly vector. Infected domestic dogs constitute the major reservoir of the parasite and play a key role in transmission to humans, in which the infection causes visceral leishmaniasis (Alvar et al., 2004; Gramiccia and Gradoni, 2005; Maroli et al., 2012). CanL is present in some areas of southern Europe, Africa, Asia, South and Central America (Baneth et al., 2008) and it has been recently reported in previously non-endemic areas of northern Europe, southern Canada and from northern Argentina to northern United States (Maroli et al., 2008; Otranto et al., 2009; Chamaille et al., 2010; Petersen and Barr, 2009; Mencke, 2011; Dantas-Torres et al., 2012). Expansion of human and canine leishmaniasis from endemic to non-endemic areas has been attributed to the geographical spread of sand fly vectors due to global warming, among other causes (Ferroglio et al., 2005; Shaw et al., 2009; Dantas-Torres et al., 2012). A unified medical-veterinary approach has recently been proposed to avoid the expansion of visceral leishmaniasis to new areas of the planet as a consequence of global warning. Prevention of the disease in dogs is one of the main action lines of this approach (Reis et al., 2010; Palatnik-de-Sousa and Day, 2011).

During several decades, prevention strategies against CanL have been mainly focused on the use of veterinary registered products containing synthetic pyrethroids, permethrin, or deltamethrin with a repellent effect against phlebotomine sand flies (Maroli et al., 2010; Solano-Gallego et al., 2011). A 'fructose mannose ligand' (FML)-based vaccine is commercialized in Brazil since the early 2000s and, more recently, one vaccine based on the recombinant A2 protein and another vaccine based on cultured *L. infantum* purified excreted/secreted antigens have been approved for use in dogs in Brazil and Europe, respectively (Solano-Gallego et al., 2011). A domperidone-based product has also been approved in several European countries for reduction of the risk of developing an active infection and clinical disease in case of contact with *L. infantum*, as well as for the control of clinical progression of CanL at early stages of the disease (HMA, 2013).

Domperidone is a dopamine D2 receptor antagonist developed during the 1970s as a prokinetic and antiemetic agent (Prakash and Wagstaff, 1998; Barone, 1999). In addition to these effects, domperidone has an immunomodulatory effect, mainly thorough a reversible increase of prolactin blood levels (Rovensky et al., 1995, 1996, 1999). Prolactin is a neuroendocrine hormone mainly produced in the pituitary gland that is largely known to play a stimulatory role of the immune system as a pro-inflammatory lymphocyte-derived cytokine (Reber, 1993; Hinterberger-Fischer, 2000; Vera-Lastra et al., 2002; Chavez-Rueda et al., 2005; Kelley et al., 2007). Specifically, an elevation of prolactin blood levels has been shown to induce an increase in the CD4 + Th1 subsets and the release of interleukin (IL)-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, leading to a natural killer (NK) cell and macrophage activation, followed by a decrease in CD4 + Th2 subsets and TNF-β (Di Carlo et al., 1993; Richards et al., 1998; Majumder et al., 2002).

In CanL, it is well known that resistance to disease progression is associated with a protective CD4 + Th1 cell-mediated immune response, while susceptibility to clinical disease is associated with a high humoral CD4 + Th2 mediated immune response and a reduced or depressed CD4 + Th1 cell-mediated immunity (Paltrinieri et al., 2010; Solano-Gallego et al., 2011). Through its stimulatory effect on the Th1 cell-mediated immune response, domperidone helps dogs with CanL to control and reduce clinical signs and anti-*Leishmania* antibody titres, particularly at early stages of the disease (Gómez-Ochoa et al., 2009).

In clinically healthy, seronegative dogs, repeated administration of domperidone has been shown to induce a significant increase in the percentage of activated phagocytic polymorphonuclear cells involved in the innate immune response such as neutrophils and macrophages (Gómez-Ochoa et al., 2012). These cells constitute the first line of defence encountered by *Leishmania* parasites when entering the susceptible host. Indeed, after initial infection, neutrophils and macrophages, among others, are recruited to the infection site and the interaction between them as well as with the parasites significantly influence the outcome of infection (Novais et al., 2009; Ribeiro-Gomes and Sacks, 2012). An appropriate activation of these cell populations is crucial for rapid elimination of the phagocytosed parasites and further antigen presentation by dendritic cells (Bonilla-Escobar, 2005; Liu and Uzonna, 2012).

According to the above mentioned, by activating the phagocytic polymorphonuclear cells involved in the innate immune response domperidone might hypothetically control the early infection by *Leishmania* parasites as well as prevent the progression of the disease. The present clinical trial was performed in order to contrast this hypothesis under real field conditions in a high prevalence area. The study was carried out under the approval of the Spanish Medicines Agency (AEMPS, *Agencia Española de Medicamentos y Productos Sanitarios*).

The results of this study were partially presented at the Southern European Veterinary Conference (SEVC) – 46th National Congress of AVEPA, Barcelona, 2011.

2. Methods

2.1. Trial design

This was a single-centre, open-label, controlled, randomized clinical trial with a one-to-one allocation ratio performed in a veterinary clinic located in Silla, Valencia (Spain), an area with a reported high average seroprevalence of CanL (around 20%) (Miró et al., 2013) and a long vector season (estimated from March to November).

2.2. Dog enrolment

Ninety privately owned dogs were recruited for enrolment in the study. All dogs were living in the surrounding area of the veterinary clinic, within a radius of 20 km. Screening visits were performed from June to early August 2007.

Eligibility criteria were as follows: (a) dogs of any sex, age, weight and breed with a normal clinical status confirmed by clinical examination at the screening visit, (b) serologically negative for *L. infantum* (indirect immunofluorescence antibody test, IFAT < 1:40) based on a blood sample obtained at the screening visit, which was analysed in a veterinary reference laboratory (IDEXX Laboratories, Inc.), and (c) whose owners were voluntarily not using products containing synthetic pyrethroids, permethrin or deltamethrin with specific repellent effect against sand flies as a preventive approach against CanL. Instead, at their inclusion all dogs were following an early detection plan for the disease based on regular clinical examinations and serological testing, which was continued during the study.

A written informed consent was signed by the owner of each dog prior to the enrolment of the animal in the study. Owners undertook also to comply with product administration and follow-up schedules as indicated by the participating veterinarian.

2.3. Group assignment

Dogs meeting the eligibility criteria were sequentially distributed by the veterinarian into two groups (domperidone-treated group and negative control group) according to a simple randomization list previously generated using the nQueryAdvisor.

2.4. Treatments

Dogs in the domperidone-treated group were treated with an oral suspension of domperidone (Leisguard®, Lab. Dr. ESTEVE, S.A.), at 0.5 mg/kg bw/day during 30 consecutive days, every 4 months, starting the first treatment immediately after enrolment. Product intake was checked at all administrations and recorded by the owners in a daily treatment history record. In the absence of a comparable reference product registered in Spain at the study time, dogs in the negative control group were left untreated. Use of products with specific repellent effect against sand flies was not allowed. Any other products administered to the dogs of both groups during the study were recorded to evaluate possible interferences with the results.

2.5. Follow-up

A 21-month follow-up period was fixed for each dog with day 0 being the day of enrolment, covering two complete seasonal phases of the phlebotomine sand fly vector. During this period, regular visits for clinical examination and serological testing were performed at the veterinary clinic (mean number of follow-up visits was 4 per animal). At each visit, dogs underwent a complete clinical examination and a blood sampling for serological testing by IFAT (Leishmania antibody IFAT, IDEXX Laboratories, Inc.). This test provides a specificity and sensitivity of respectively 98.6% and 99.1%. Dogs seropositive for *Leishmania* (antibody titre $\geq$ 1:80) that as well showed at least one clinical sign consistent with CanL (indicative of active infection and incipient disease progression) were categorized as 'prevention failure' cases. 'Prevention failure' resulted in the dog being withdrawn from the study and starting a therapeutic treatment as indicated by the practitioner. In these dogs, Diff-Quick-stained smears of lymph node and/or bone marrow aspirates were used for direct observation of *Leishmania* amastigotes. Special attention was paid throughout the follow-up period to dogs in the domperidone-treated group to detect potential drug-related adverse events such as episodes of gastrointestinal disturbances or galactorrhoea, by maintaining close phone contact with the owners.

2.6. Study endpoints

Primary endpoint was the number of dogs withdrawn from the study because of a 'prevention failure' at the end of the 21-month follow-up period. Cumulative incidence of 'prevention failure' (percent rates based on the initial number of dogs in the group) was assessed. Results at the end of the 12-month follow-up period were also evaluated as a secondary endpoint. The prevention rate was calculated as follows: 100 – (['prevention failure' percent rate in treatment group $\times$ 100]/'prevention failure' percent rate in control group).

2.7. Statistical procedures

The experimental unit in the study was the dog. Sample size calculation was based on an assumed 70% cumulative incidence of 'prevention failure' in the untreated control group during the 21-month follow-up treatment period. A reduction by 30% in this rate was considered to be clinically relevant. A sample of 42 dogs per group would be needed to have a 80% statistical power to detect such a difference in the primary endpoint using a Pearson's chi-squared with a two-sided $\alpha = 0.05$. Sample size calculation was performed by using the nQueryAdvisor software.

Homogeneity analysis of baseline characteristics among groups was done by using the Student's *t*-test or the Pearson's Chi-squared test, according to the type of variable. Both primary and secondary endpoints were analysed by

means of Pearson's chi-squared test, after a 21-month and 12-month follow-up, respectively (*i.e.*, after two and one complete sand fly seasons, respectively). The Mantel–Haenszel method was used to adjust the analyses of the primary and the secondary endpoints for the unbalanced baseline co-variables.

For both the primary and the secondary endpoints, survival curves of Kaplan–Meier estimates were calculated for each group and differences between curves were assessed with a log-rank test. A Cox regression model was used to adjust the results for unbalanced baseline co-variables. The Mann–Whitney Rank Sum test was also used for comparison of anti-*Leishmania* antibody titres of those dogs categorized as 'prevention failure' cases in each group. All the statistical analyses were performed by using the SAS 9.2 software package using a two-sided significance level $\alpha = 0.05$.

3. Results

3.1. Participant flow

Among the 90 dogs included in the study, 44 were assigned to the domperidone-treated group and 46 to the negative control group (Fig. 1). During the study, 10 dogs were withdrawn for reasons other than 'prevention failure' (8 dogs because their owners moved out and could no longer continue with the follow-up procedure, and 2 dogs because of death from fibrosarcoma and lymphosarcoma, respectively). At the withdrawal time, all these dogs were seronegative to *Leishmania*. On the other hand, at the end of the study a total of 27 dogs had been withdrawn because of 'prevention failure' while the 53 remaining dogs were seronegative to *Leishmania* and did not have any clinical sign related to CanL. The last-observation-carried-forward imputation method was used during data analysis for dogs withdrawn during the study, including those dogs withdrawn for reasons other than 'prevention failure'. Consequently, data from all the dogs included in the study was used for the statistical analysis of the primary and secondary endpoints.

3.2. Baseline homogeneity

As shown in Table 1, characteristics of dogs recorded at enrolment were well balanced in both groups except for housing conditions, the proportion of dogs living mostly outdoors and sleeping in their garden hut being significantly higher in the domperidone-treated group. Consequently, an adjustment for this co-variable was included in the statistical analyses of the results to check the possible impact of this unbalance.

3.3. Serological and clinical findings

During the 21-month follow-up period, 27 dogs (5 out of 44 in the domperidone-treated group and 22 out of 46 in the negative control group) showed an antibody titre $\geq$ 1:80. All of them also showed clinical signs consistent with the disease so they were categorized as 'prevention failure' cases. Most common clinical signs observed in these dogs were mild to moderate peripheral lymphadenomegaly, exfoliative dermatitis, and weight loss. In all cases the infection was confirmed by direct observation of the parasite in smears of lymph nodes and/or bone marrow aspirates. On the other hand, 7 dogs (4 in the domperidone-treated group and 2 in the negative control group) showed an antibody titre = 1:40 with no clinical signs while the remaining dogs showed titres < 1:40 with no clinical signs.

Among dogs categorized as 'prevention failure' cases, those in the domperidone-treated group showed lower antibody titres than those in the negative control group (Mann–Whitney Rank Sum test, $p = 0.056$). Specifically, in the domperidone-treated group four dogs showed a titre of 1:80 and another one a titre of 1:640, while in the negative

Table 1
Baseline characteristics of dogs assigned to the two study groups and homogeneity analysis in a clinical trial to assess the preventive efficacy of a domperidone-based treatment programme against canine leishmaniasis.

	Domperidone-treated group ($n = 44$)	Negative control group ($n = 46$)	p-Value
Sex, n (%)			
Male	25 (56.8%)	25 (54.3%)	0.981[a]
Female	19 (43.2%)	21 (45.7%)	
Age, years			
Mean (SD)	5 (2.2)	5 (2.3)	0.595[b]
Range	1–10	1–10	
Bodyweight, kg			
Mean (SD)	20.3 (10.83)	20.4 (8.46)	0.683[b]
Range	6.5–54	7–43	
Breed, n (%)			
Mixed-breed	13 (29.5%)	23 (50.0%)	
German Shepherd Dog	4 (9.1%)	3 (6.5%)	
Belgian Shepherd Dog	1 (2.3%)	2 (4.3%)	
Golden Retrevier	2 (4.5%)	1 (2.2%)	
English Staffordshire	4 (9.1%)	2 (4.3%)	
Labrador Retrevier	1 (2.3%)	2 (4.3%)	
English Bulldog	1 (2.3%)	2 (4.3%)	
French Bulldog	2 (4.5%)	2 (4.3%)	
Boxer	2 (4.5%)	1 (2.2%)	
St. Bernard	1 (2.3%)	0	
Dalmatian	1 (2.3%)	0	
Miniature Schnauzer	1 (2.3%)	0	
Giant Schnauzer	1 (2.3%)	0	0.078[a,*]
Deutsche Bracke	3 (6.8%)	1 (2.2%)	
Westinghouse Terrier	2 (4.5%)	0	
Pointer	1 (2.3%)	2 (4.3%)	
Carlin Pinscher	1 (2.3%)	0	
Cocker Spaniel	1 (2.3%)	0	
King Charles Spaniel	1 (2.3%)	0	
Shar Pei	1 (2.3%)	0	
Samoyed	0	1 (2.2%)	
Spanish Water Dog	0	1 (2.2%)	
Valencian rat hunting Dog	0	1 (2.2%)	
Fox Terrier	0	1 (2.2%)	
Airedale Terrier	0	1 (2.2%)	
Housing conditions, n (%)			
Outdoor living	32 (72.7%)	8 (17.4%)	<0.001[a]
Indoor living	12 (27.3%)	38 (82.6%)	

[a] Pearson's chi-squared test.
[b] Student's t-test.
[*] Comparison performed with data grouped in two classes (Mixed-breed *versus* Pure breed) in order to meet the requirements of the Pearson's chi-squared test.

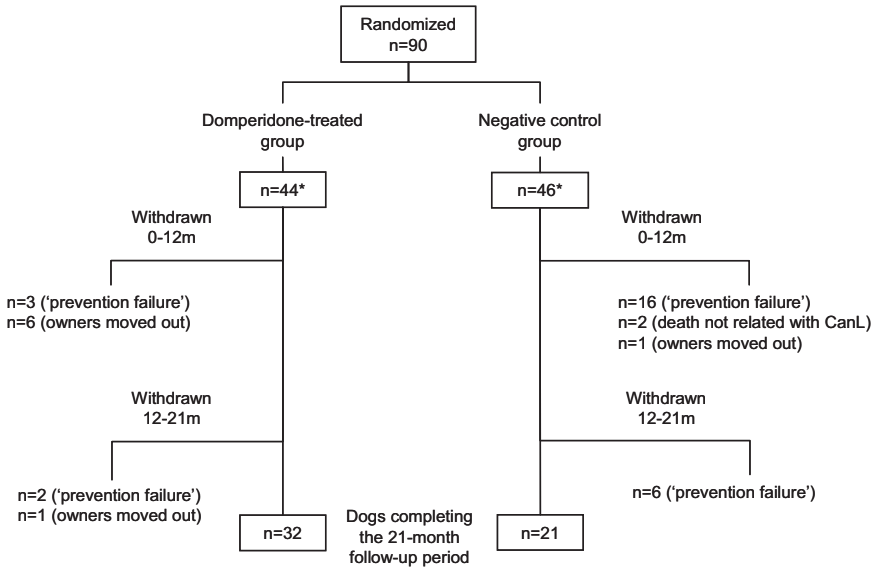

Fig. 1. Flow chart depicting the passage of dogs through a clinical trial to assess the preventive efficacy of a domperidone-based treatment programme consisting on quarterly repeated 30-day treatments with domperidone at 0.5 mg/kg bw/day against canine leishmaniasis. *Dogs which data was used for the statistical analysis of both the primary (number of dogs withdrawn because of a 'prevention failure' at the end of the 21-month follow-up period) and the secondary (number of dogs withdrawn because of a 'prevention failure' at the end of the 12-month follow-up period) study endpoints. The last observation-carried-forward imputation method was used during data analysis for all dogs withdrawn during the study.

control group four dogs showed a titre of 1:80, five a titre of 1:160, four a titre of 1:320, eight a titre of 1:640, and one a titre of 1:1280.

3.4. Efficacy assessment

As shown in Fig. 2, the cumulative percentage of cases with 'prevention failure' after 21 months was significantly lower in the domperidone-treated group than in the negative control group: 5 out of 44 dogs (11%) *versus* 22 out of 46 dogs (48%), (Pearson's chi-squared test; $p < 0.001$). The

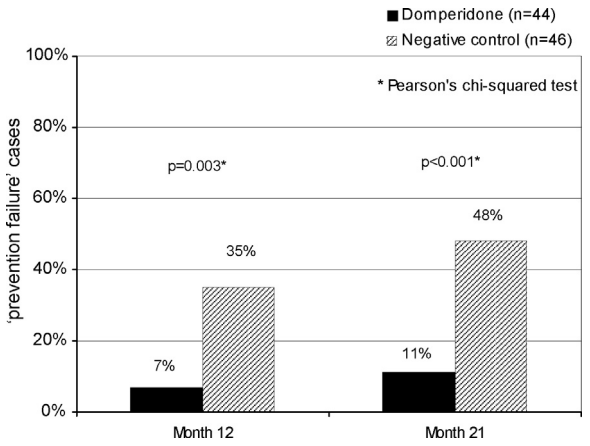

Fig. 2. Comparison of cumulative percentages of dogs with an anti-*Leishmania* antibody titre (IFAT) ≥ 1:80 and at least one clinical sign of canine leishmaniasis ('prevention failure' cases) in the two study groups, 12 and 21 months after enrolment in a clinical trial to assess the preventive efficacy of a domperidone-based treatment programme consisting on quarterly repeated 30-day treatments with domperidone at 0.5 mg/kg bw/day against canine leishmaniasis.

difference remained statistically significant after adjusting the analysis for the only unbalanced co-variable (housing conditions) (Mantel-Haenszel test, $p = 0.0009$). The prevention rate provided by domperidone throughout the complete 21-month follow-up period was 77%, with an odds ratio (OR) [95% CI] = 7.15 [2.4–21.4], $p < 0.001$. Time to withdrawal due to 'prevention failure' was significantly longer in the domperidone-treated group than in the negative control group (log-rank test, $p = 0.0003$); survival Kaplan–Meier curves shown in Fig. 3. The difference remained statistically significant after adjusting the analysis for housing conditions by means of a Cox regression model ($p = 0.0023$).

The cumulative percentage of 'prevention failure' after 12 months was also significantly lower in the domperidone-treated group than in the negative control group: 3 out of 44 dogs (7%) *versus* 16 out of 46 dogs (35%) (Pearson's chi-squared test, $p = 0.003$), and persisted in the analysis adjusted for housing conditions (Mantel–Haenszel test, $p = 0.0008$). The prevention rate provided by domperidone throughout the 12-month follow-up period was 80%, with an OR [95% CI] = 7.3 [1.9–27.3], $p = 0.001$.

3.5. Side effects and product acceptance

Among the domperidone-treated dogs, 4 out of 44 dogs (9%) presented side effects attributed to the product. Specifically, two dogs showed mild galactorrhoea 7 and 9 days after the start of treatment, which subsided at the end of the dosage period; and two other dogs showed mild gastrointestinal disturbances (soft stools and diarrhoea, respectively), which subsided after the dose was reduced by half for a few days. None of these animals had to be withdrawn from the study. Finally, according to the daily

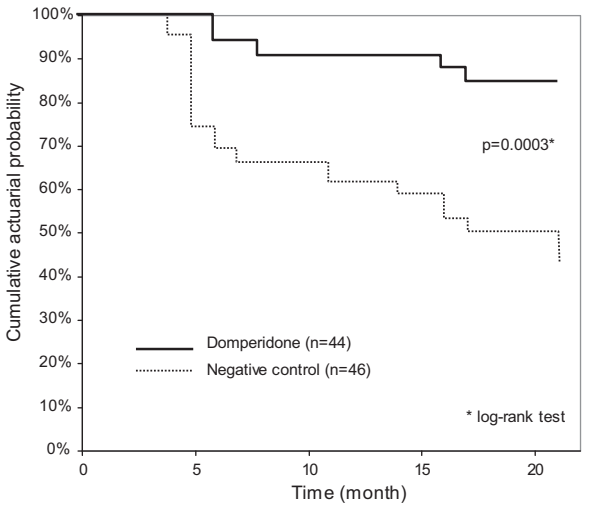

Fig. 3. Evolution (Kaplan Meyer estimates) of cumulative probability of remaining healthy (seronegative without clinical signs of canine leishmaniasis) in the two study groups during the 21-month follow-up period in a clinical trial to assess the preventive efficacy of a domperidone-based treatment programme consisting on quarterly repeated 30-day treatments with domperidone at 0.5 mg/kg bw/day against canine leishmaniasis.

treatment history records, adherence to treatment among the domperidone-treated dogs was good, with only a few dogs partially rejecting the product during the first days of treatment. The full treatment programme was discontinued only in the 12 dogs of the domperidone-treated group that were withdrawn from the study.

4. Discussion

Vaccination of dogs against CanL has been traditionally seen as the definitive solution to prevent the establishment of an active infection and the clinical disease after contact with the causing parasite (Reis et al., 2010; Miro et al., 2008; Solano-Gallego et al., 2011). However, immunomodulatory agents that stimulate the host defence mechanisms by enhancing endogenous production of cytokines have also been claimed to be a potentially useful tool for prophylaxis and treatment of parasitic diseases (Masihi, 2000). In the present study, we evaluated the efficacy of a unique alternative approach to the use of vaccines for the reduction of the risk to develop clinical CanL, consisting on the administration of domperidone, a drug with immunomodulatory activity that has been previously demonstrated to be efficacious in the treatment of naturally infected, seropositive dogs, especially at early stages of the disease (Gómez-Ochoa et al., 2009).

Although it is well known that the final control of CanL mainly depends on the adaptive immune response set after the first week of infection, the innate immune response acting immediately after initial infection has also been described to have a relevant role in protection against the disease (Bonilla-Escobar, 2005; Reis et al., 2010; Novais et al., 2009; Ribeiro-Gomes and Sacks, 2012). In previous studies we have demonstrated that oral administration of domperidone to healthy, seronegative dogs results in a transient stimulatory effect on the innate immune response leading to a statistically significant increase in the percentage of activated neutrophils and macrophages (Gómez-Ochoa et al., 2012). In absence of the infection, the stimulatory effect of a 30-day course treatment with domperidone on the phagocytic polymorphonuclear cells is only temporary. Consequently, in the present study it was considered necessary to repeat this treatment periodically during the vector season in order to keep the animal protected against an eventual contact with the parasite. Because of the high prevalence of CanL and the long vector season in the geographical area where the study was performed, 30-day treatments with domperidone were strategically programmed to be repeated quarterly during the 21-month follow-up. The cumulative percentage of dogs showing an antibody titre $\geq$ 1:80 plus clinical sings of CanL in the negative control group at the end of the study confirms the high infection pressure in the area where it was performed and highlights the validity of this group for controlling the efficacy of domperidone.

Molecular assays were not employed at enrolment of dogs in the study. Because it is well known that PCR-positive findings may anticipate the infection progression in dogs as detected by serology and microscopy, it could reasonably be assumed that some dogs from both groups could have already been infected at time of enrolment. Notwithstanding this, inclusion of potentially infected seronegative dogs can be assumed to have been balanced between groups and consequently, to not have affected the general conclusions of the study.

Unfortunately, in spite of random assignment of dogs to the two groups, housing conditions were not homogenously distributed between them. Specifically, the proportion of dogs living mostly outdoors was significantly higher in the domperidone-treated group, so the risk of sand fly biting was theoretically higher in this group. Although an adjustment for this co-variable was included in the statistical analyses of data, its worth mentioning that the obtained results did not vary substantially in respect to those obtained without adjusting. Consequently, the impact of this unbalance was confirmed to be irrelevant.

A very high prevention rate was showed by the domperidone-based treatment programme implemented in the present study, being similar to or even better than those described by the currently available vaccines against CanL. Indeed, the risk for domperidone-treated dogs to develop a symptomatic disease was quite 7 times lower than for dogs left untreated, while the reported risk for dogs vaccinated with one of the recently approved vaccines is around 4 times lower than for controls, under similar field conditions (EMA, 2012).

On the other hand, among all dogs withdrawn because of 'prevention failure' in the study, those treated with domperidone showed lower anti-*Leishmania* antibody titres than dogs in the negative control group. This is consistent with the results of previous studies evidencing that domperidone is efficacious in reducing antibody titres of diseased animals at early stages of the disease (Gómez-Ochoa et al., 2009). Consequently, the obtained results may also indicate that, although not specifically proven, domperidone treatment helps the already infected

dogs to control the infection progression from sub-patent conditions.

Finally, domperidone was well accepted by dogs and drug-related side effects were very mild and uncommon, the latter confirming the good safety of the implemented treatment programme.

As described in Section 3.1, the last observation carried-forward imputation method was implemented during data analysis, even for dogs withdrawn for reasons other than 'prevention failure'. In order to check whether this method could have affected the results, the 'worst scenario' (considering all withdrawn animals as 'prevention failure' cases) was evaluated, by analysing again the primary and secondary endpoints with the new imputation method. The results of these analyses were similar to those obtained during the main analysis of data, with statistically significant differences evidenced in favour of the domperidone-treated group for both the primary ($p = 0.009$) and the secondary ($p = 0.0327$) endpoints.

According to all the above mentioned, periodic immunomodulation with domperidone can be considered a safety approach for reducing the risk to develop clinical CanL, with comparable effectiveness to the currently available vaccines. Therefore, when implemented together with the well established repellents or anti-feeding compounds, it would be also effective as part of the integrated approach that has been proposed in the current guidelines for the future control of CanL (Paltrinieri et al., 2010; Solano-Gallego et al., 2011; Otranto and Dantas-Torres, 2013).

5. Conclusion

The results of the present study demonstrate that the implementation of a strategic domperidone-based treatment programme consisting on quarterly repeated 30-day treatments with domperidone at 0.5 mg/kg bw/day reduces the risk to develop clinical CanL in areas with high prevalence of the disease.

Conflict of interest statement

DS, JH, and MS are employees of ESTEVE, which has funded the study.

References

Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Adv. Parasitol. 57, 1–88.

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 24, 324–330.

Barone, J.A., 1999. Domperidone: a peripherally acting dopamine2-receptor antagonist. Ann. Pharmacother. 33, 429–440.

Bonilla-Escobar, D., 2005. Respuesta immune a la leishmaniasis: algo más que linfocitos T. Piel 20 (8), 383–385.

Chamaille, L., Tran, A., Meunier, A., Bourdoiseau, G., Ready, P., Dedet, J.P., 2010. Environmental risk mapping of canine leishmaniasis in France. Parasit. Vectors 3, 31.

Chavez-Rueda, K., Hernandez, J., Zenteno, E., Leanos-Miranda, A., Legorreta-Haquet, M.V., Blanco-Favela, F., 2005. Identification of prolactin as a novel immunomodulator on the expression of co-stimulatory molecules and cytokine secretions on T and B human lymphocytes. Clin. Immunol. 116, 182–191.

Dantas-Torres, F., Solano-Gallego, L., Baneth, G., Ribeiro, V.M., de Paiva-Cavalcanti, M., 2012. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends Parasitol. 28, 531–538.

Di Carlo, R., Meli, R., Galdiero, M., Nuzzo, I., Bentivoglio, C., Carratelli, C.R., 1993. Prolactin protection against lethal effects of *Salmonella typhimurium*. Life Sci. 53, 981–989.

European Medicinal Agencies (EMA), 2011. CaniLeish: EPAR – Public Assessment Report, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/veterinary/002232/WC500104953.pdf

Ferroglio, E., Maroli, M., Gastaldo, S., Mignone, W., Rossi, L., 2005. Canine leishmaniasis, Italy. Emerg. Infect. Dis. 11, 1618–1620.

Gómez-Ochoa, P., Castillo, J.A., Gascon, M., Zarate, J.J., Alvarez, F., Couto, C.G., 2009. Use of domperidone in the treatment of canine visceral leishmaniasis: a clinical trial. Vet. J. 179, 259–263.

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

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. Int. J. Parasitol. 35, 1169–1180.

Hinterberger-Fischer, M., 2000. Prolactin as pro-inflammatory cytokine – considerations on consolidated immunotherapy after high dosage therapy. Acta Med. Austriaca Suppl. 52, 16–20.

Heads of Medicines Agencies (HMA), 2013. Veterinary MRIndex: Leisguard 5 mg/ml Oral Suspension for Dogs, http://mri.medagencies.org/download/ES_V_0170_001_FinalPI.pdf

Kelley, K.W., Weigent, D.A., Kooijman, R., 2007. Protein hormones and immunity. Brain Behav. Immun. 21, 384–392.

Liu, D., Uzonna, J.E., 2012. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Front. Cell. Infect. Microbiol. 2, 1–8.

Majumder, B., Biswas, R., Chattopadhyay, U., 2002. Prolactin regulates antitumor immune response through induction of tumoricidal macrophages and release of IL-12. Int. J. Cancer 97, 493–500.

Maroli, M., Rossi, L., Baldelli, R., Capelli, G., Ferroglio, E., Genchi, C., Gramiccia, M., Mortarino, M., Pietrobelli, M., Gradoni, L., 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop. Med. Int. Health 13, 256–264.

Maroli, M., Gradoni, L., Oliva, G., Castagnaro, M., Crotti, A., Lubas, G., Paltrinieri, S., Roura, X., Zini, E., Zatelli, A., 2010. Guidelines for prevention of leishmaniasis in dogs. J. Am. Vet. Med. Assoc. 236, 1200–1206.

Maroli, M., Feliciangeli, M.D., Bichaud, L., Charrel, R.N., Gradoni, L., 2012. Phlebotomine sand flies and the spreading of leishmaniases and other diseases of public health concern. Med. Vet. Entomol. 27, 123–147.

Masihi, K.N., 2000. Immunomodulatory agents for prophylaxis and therapy of infections. Int. J. Antimicrob. Agents 14, 181–191.

Mencke, N., 2011. The importance of canine leishmaniosis in non-endemic areas, with special emphasis on the situation in Germany. Berl. Munch. Tierarztl. Wochenschr. 124, 434–442.

Miro, G., Cardoso, L., Pennisi, M.G., Oliva, G., Baneth, G., 2008. Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part two. Trends Parasitol. 24, 371–377.

Miró, G., Montoya, A., Roura, X., Gálvez, R., Sainz, A., 2013. Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicentre study. Parasit. Vectors 6, 117.

Novais, F.O., Santiago, R.C., Bafica, A., Khouri, R., Afonso, L., Borges, V.M., Brodskyn, C., Barral-Netto, M., Barral, A., de Oliveira, C.I., 2009. Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. J. Immunol. 183, 8088–8098.

Otranto, D., Capelli, G., Genchi, C., 2009. Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. Parasit. Vectors 2, S2.

Otranto, D., Dantas-Torres, F., 2013. The prevention of canine Leishmaniasis and its impact on public health. Trends Parasitol. 29 (7), 339–345.

Palatnik-de-Sousa, C.B., Day, M.J., 2011. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit. Vectors 4, 197.

Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Crotti, A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., Zini, E., Canine Leishmaniasis Working Group, Italian Society of Veterinarians of Companion Animals, 2010. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. J. Am. Vet. Med. Assoc. 236, 1184–1191.

Petersen, C.A., Barr, S.C., 2009. Canine leishmaniasis in North America: emerging or newly recognized? Vet. Clin. North Am. Small Anim. Pract. 39, 1065–1074.

Prakash, A., Wagstaff, A.J., 1998. Domperidone. A review of its use in diabetic gastropathy. Drugs 56, 429–445.

Reis, A.B., Giunchetti, R.C., Carrillo, E., Martins-Filho, O.A., Moreno, J., 2010. Immunity to leishmania and the rational search for vaccines against canine leishmaniasis. Trends Parasitol. 26, 341–349.

Reber, P.M., 1993. Prolactin and immunomodulation. Am. J. Med. 95, 637–644.

Ribeiro-Gomes, F.L., Sacks, D., 2012. The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front. Cell. Infect. Microbiol. 2, 1–8.

Richards, S.M., Garman, R.D., Keyes, L., Kavanagh, B., McPherson, J.M., 1998. Prolactin is an antagonist of TGF-beta activity and promotes proliferation of murine B cell hybridomas. Cell. Immunol. 184, 85–91.

Rovensky, J., Buc, M., Lojda, Z., Ruzickova, M., Blazickova, S., Rrauova, L., Mistina, T., Vigas, M., Lackovic, V., 1995. Effect of domperidone-induced hyperprolactinemia on selected immune parameters in healthy women. Arch. Immunol. Ther. Exp. 43, 221–227.

Rovensky, J., Ferencik, M., Vigas, M., 1996. Effect of domperidone-induced hyperprolactinemia on the activity of some lysosomal enzymes in peripheral polymorphonuclear leukocytes of healthy women. Int. J. Immunotherapy XII, 25–31.

Rovensky, J., Lackovic, V., Veselkova, Z., Horvathova, M., Koska, J., Blazickova, S., Vigas, M., 1999. Plasma cytokine concentration and the cytokine producing ability of whole blood cell cultures from healthy females with pharmacologically induced hyperprolactinemia. Int. J. Tissue React. XXI, 43–49.

Shaw, S.E., Langton, D.A., Hillman, T.J., 2009. Canine leishmaniosis in the United Kingdom: a zoonotic disease waiting for a vector? Vet. Parasitol. 163, 281–285.

Solano-Gallego, L., Miro, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., The LeishVet, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit. Vectors 4, 86.

Vera-Lastra, O., Jara, L.J., Espinoza, L.R., 2002. Prolactin and autoimmunity. Autoimmun. Rev. 1, 360–364.