Retrospective study on the outcome of treatment with miltefosine for canine Leishmaniasis in veterinary first-line practice.

Zoë T.I. Feenstra
5809339
Research Track, Veterinary Medicine of Companion Animals, November 2022
Faculty of Veterinary Medicine, Utrecht University
Supervisor: Dr. C.J. Piek

Abstract

Canine Leishmaniasis is a vector-borne disease caused by the intracellular protozoa Leishmania infantum, which continues to be a difficult and challenging disease to effectively treat and cure. Among variable antileishmanial drugs, the use of miltefosine appeared to be safe and effective for treatment of canine Leishmaniasis and is nowadays widely used in veterinary medicine. This study was aimed to evaluate the use of miltefosine in Dutch veterinary first-line practice, a non-endemic country, by assessing survival, efficacy of a first miltefosine therapy cycle as measured by recovery of clinical signs and clinicopathological parameters, and side effects as outcome parameters. Patient files of dogs with a confirmed diagnosis and treated with miltefosine in 2014, 2015, 2016 and 2017, were retrospectively reviewed. Survival analysis and assessment of side effect was performed on the complete dataset (n=53), while the efficacy of therapy was assessed only on a subset (n=25) of included dogs ("Inclusion T0-T1"). A survival probability of 78% and 71%, three-years from diagnosis and commencement of the first miltefosine therapy cycle, respectively, was shown. Gastrointestinal side effects were reported in 23% of treatments. For the efficacy of the first miltefosine therapy cycle, the clinical signs lymphadenomegaly and scaling skin, and the laboratory parameters hematocrit, thrombocytes, total protein, globulins, albumin, and A/G-ratio improved significantly after treatment. Administration of allopurinol was very common and may have had a positive effect on the outcome. This study provides an accurate overview of the use of miltefosine in Dutch veterinary first-line practice and therewith provided useful and promising results.

Keywords canine Leishmaniasis, miltefosine, therapy efficacy, clinical signs, clinicopathological parameters, survival time, side effects, use of treatment

Introduction

Canine Leishmaniasis due to Leishmania infantum is a vector-borne disease and zoonosis. A sand fly vector of the genus Phlebotomus, transmits the flagellated infective promastigote form through bites when consuming blood meals from dogs. In this second and main reservoir host, an intracellular amastigote form develops in macrophages and replicates. The vector is present in Europe, especially Mediterranean countries and in America, Asia, and Africa [1 2 3 4]. However, canine Leishmaniasis is also of great importance in non-endemic countries, due to import of infected companion animals or dogs travelling to endemic areas [2 3]. Other described non-sand fly transmission include infection through blood, venereal and vertical transmission [2 5].

The incubation period for Leishmania infantum is very variable [4]. Some infected dogs remain asymptomatic for many months to years, while others develop canine Leishmaniasis with different clinical signs and clinicopathological abnormalities, shortly after infection. This strongly depends on the immune response, as dogs with an enhanced humoral but a reduced cellular response are more likely to be affected by a severe clinical manifestation [3 4 5]. Various (nonspecific) clinical signs and altered laboratory values are described in symptomatic dogs due to inflammatory reactions and deposition of immune complexes in multiple organs such as skin, kidney, intestines, eyes, and

mucous membranes. Therefore, common described symptoms are skin lesions, generalized lymphadenomegaly, weight loss, onychogryphosis, ulcerous lesions, pale mucous membranes, anorexia, and ocular lesions [1 2 3 5 6 7 8]. Hyperproteinemia, hypergammaglobulinemia, hypoalbuminemia, anemia, and proteinuria are reported most often as altered laboratory values [8 9 10 11]. Thus, the degree of severity is variable as well and can be fatal, most likely due to chronic renal failure [2 3 6 12 13].

Multiple direct or indirect methods of diagnostics are available. Quantitative serology, such as ELISA, IFAT, and DAT, as an indirect test, detects anti-Leishmania antibodies in blood of infected animals, and is a frequently used diagnostic method. A high positive anti-Leishmania titer, in combination with compatible clinical signs and hematological, biochemical and urinalysis test abnormalities, confirms canine Leishmaniasis. If a quantitative serology test result is negative or low positive, direct tests are recommended. Direct tests, which detect the presence of Leishmania infantum parasites or DNA from sampled cutaneous lesions, enlarged lymph nodes and/or bone marrow, include cytology, histology, and PCR. PCR is used when cytology and histology are not diagnostic [1 2].

Many different drugs can be used in the treatment of canine Leishmaniasis depending on the animal's clinical condition and severity of the disease. Therefore, guidelines were published by the LeishVet Group and the Canine Leishmaniasis Working Group, based on a staging system and recommended drugs include allopurinol alone, or in combination with meglumine antimoniate (Glucantime®) or miltefosine (Milteforan®). However, therapy is not always effective and parasitological cure is rarely achieved which often results in relapses. Therefore, lifelong veterinary checkups should be applied [1 2 3 14 15].

Miltefosine (hexadecylphosphocholine), an alkyphosphocholine ether-lipid analogue, was originally developed as an anticancer drug while its leishmaniacidal potential was discovered in the 1980s. It is presumed that miltefosine perturbates the signaling pathway, phospholipid metabolism and membrane biosynthesis of the parasite, which causes apoptosis-like cell death [16 17 18 19 20]. Because miltefosine has no effect on kidneys, it is the recommended choice of treatment for dogs with renal insufficiency as meglumine antimoniate has nephrotoxicity and long-term use of allopurinol can cause renal mineralization and xanthine urolithiases as a side effect [1 2 5 21 22 23]. Miltefosine appeared to be safe and effective against canine Leishmaniasis, at an administered dosage of 2 mg/kg bodyweight once a day orally, for twenty-eight consecutive days. Gastro-intestinal symptoms such as anorexia, lethargy, vomiting and diarrhea, are reported as side effects of miltefosine [1 16 24 25 26].

The aim of this study was to evaluate the use of miltefosine in Dutch veterinary first-line practice, a non-endemic country, by assessing survival, efficacy of a first miltefosine therapy cycle as measured by recovery of clinical signs and clinicopathological parameters, and side effects as outcome parameters.

Materials and methods

Animals

The distributor of miltefosine in the Netherlands, Virbac Netherlands, provided all written prescriptions for miltefosine for the period from January 1, 2014, to December 31, 2017. Veterinary practices were contacted by phone and email by four students to obtain informed consent and patient files.

Dogs of all breeds, sex, and age, with a confirmed diagnosis of canine Leishmaniasis and treated with miltefosine in 2014, 2015, 2016 and 2017 by first-line veterinarians in the Netherlands were included in the study. The diagnosis of canine Leishmaniasis was based on the combination of

clinical signs, clinicopathological parameters and a positive antibody titer (serology) and/or the proven presence of Leishmania amastigotes on cytology, histology, or PCR.

Dogs with a concurrent CVBD such as Ehrlichia canis, Babesia canis, Borrelia burgdorferi, Anaplasma platys, and Dirofilaria immitis were excluded from the study based on a positive antibody titer (serology), blood smear and/or PCR. Dogs that received other treatments such as systemic corticosteroids, ivermectin and oclacitinib prior to diagnosis, between diagnosis and miltefosine treatment or during miltefosine treatment were excluded from the start of the study. However, if dogs received these drugs after miltefosine treatment, they were excluded during the study. Other exclusion criteria were pretreatment with meglumine antimoniate, original diagnosis was made in the country of origin or too much missing information in the patient file.

For assessment of a first miltefosine therapy cycle efficacy, additional inclusion criteria were the presence of clinical signs and clinicopathological parameters, within a set timeframe of one to four months before and after commencement of miltefosine treatment, respectively. Also, the administered dosage of 2 mg/kg bodyweight/day and duration of at least 20 consecutive days of miltefosine treatment were additional inclusion criteria. Dogs that met these criteria were put in a subset of the included dogs, the group named “Inclusion T0-T1”.

Patient files were obtained with informed consent from both referring veterinarian and owner. The following data were collected from patient files: 1) signalment and travel history, 2) diagnostics, 3) information on clinical presentation and laboratory findings at diagnosis and checkups, 4) concurrent CVBD, 5) information on prescribed treatment for canine Leishmaniasis and other drugs and 6) censoring.

Data analysis

The assessment of frequency and type of possible side effects and survival analysis was performed on the complete dataset. Efficacy of therapy was assessed only for those dogs for which data on dosage, duration, and presence of clinical signs and clinicopathological parameters prior and after treatment was available and met the additional criteria (“Inclusion T0-T1”). To eliminate the introduction of inclusion bias by the data “Inclusion T0-T1”, a comparison of data was made with the dogs in the part of the dataset “Exclusion T0-T1” for whom these data on efficacy of therapy were not suitable.

Statistical analysis

For the statistical comparison of a continuous variable between two dependent group of dogs, both groups were evaluated for normal distribution and equal variance. Evaluation of normality was performed using the generalized linear model function, normal Q-Q residuals, and Shapiro-Wilk normality test of the residuals. The var.test was used for evaluation of equal variance (homoscedasticity). If the data was normally distributed and the variance was equal, a two-sample paired t-test was performed. Otherwise, a Wilcoxon signed rank test was used (paired is true). The McNemar’s test was performed for a comparison of a binary variable between two dependent groups. A Kaplan-Meier curve was used to show the probability of survival until the event. Survival time was defined as time in days from diagnosis or commencement of first miltefosine treatment until time of death due to canine Leishmaniasis (the event). Dogs were censored if they received treatment with corticosteroids or meglumine antimoniate, if lost to follow-up or if still alive. It was assumed that being censored or not, was not related to the probability of the event occurring (non-informative). The significance level used for the statistical tests was 5%. RStudio Team (2022) [27] was used for the calculations.

Results

Figure 1 presents the sum of receipts, informed consent and patient files, and in- and excluded dogs from four consecutive years (2014-2017). The number of miltefosine receipts received from Virbac Netherlands are not corrected for overlapping patients (*). Only three out of 53 dogs (5.7%) from Dutch veterinary first-line practices were referred to UKG, however many veterinarians consulted a Leishmaniasis specialist by phone.

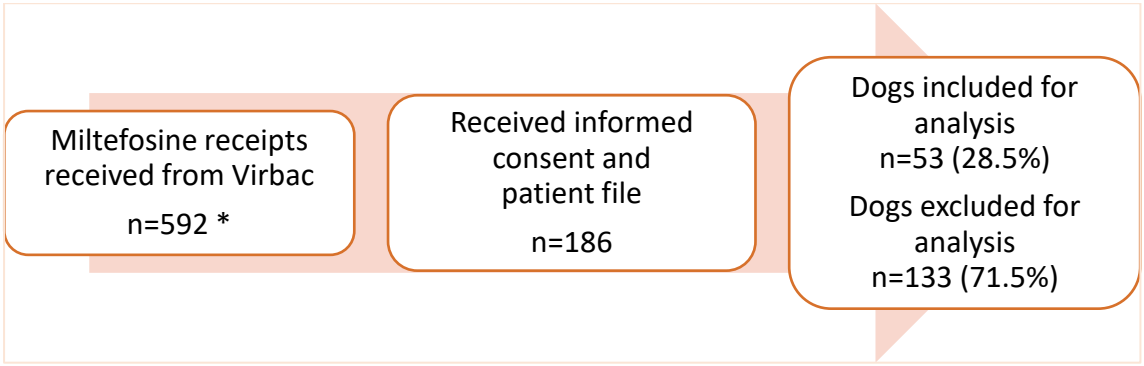

Figure 1: Flowchart of data

The following exclusion criteria were applied on 133 dogs: 1) immunosuppressive treatment (n=21), 2) too much missing information in patient file (n=41), 3) original diagnosis was made in country of origin (n=41), 4) no confirmed diagnosis of canine Leishmaniasis (n=8), 5) diagnosed with one or more other CVBD (n=13), 6) did not receive miltefosine treatment (n=3), 7) pretreatment with meglumine antimoniate (n=3) and 8) no clinicopathological parameters at diagnosis (n=3).

The complete dataset consisted of 53 dogs: 25 dogs were included in the subset of data "Inclusion T0-T1" and 28 dogs were included in the subset of data "Exclusion T0-T1", because of the following reasons: 1) checkup more than four months after commencement of first miltefosine treatment (n=11), 2) checkup more than one month before commencement of first miltefosine treatment (n=2) 3) no checkup after miltefosine treatment (n=3), 4) no clinicopathological parameters at checkup after treatment (n=4), 5) duration of therapy less than 20 consecutive days (n=1) and 6) combination of reasons (n=7).

Complete dataset

Signalment

As mentioned in figure 1, 53 dogs were included for analysis. Most of the 53 included dogs were crossbreeds (40/53), also a Bodeguero (1/53), Breton Spaniel (1/53), Dobermann (1/53), Épagneul Breton (1/53), German Shepherd (1/53), Podenco Ibicenco (2/53), Boomer (1/53), Dutch Shepherd (1/53), Galgo Español (1/53), Greyhound (1/53), Miniature Pinscher (1/53) and Pug (1/53) were represented. Only two dogs (3.8%) were born in the Netherlands and had travelled to Spain, France and/or Sweden, the other 51 dogs were imported from Spain (71.7%), Portugal (1.9%), Greece (20.8%) and Turkey (1.9%). Four dogs were male intact, 27 dogs were male neutered, one dog was female intact, and 21 dogs were female neutered. The dogs had a [median ± IQR] age at diagnosis of 3.4 ± 4.0 years and [median ± IQR] bodyweight at diagnosis of 18.4 ± 14.9 kg. Unfortunately, bodyweight at diagnosis of eight dogs was unavailable.

Diagnostics

Diagnosis of canine Leishmaniasis was confirmed by clinical signs, clinicopathological parameters and only serology (84.9%), only histology (1.9%), only cytology (1.9%), combination of serology and

cytology (5.7%) or combination of serology and PCR (5.7%). Hence, different diagnostics techniques were used and performed by the laboratories IDEXX VML, Laboklin, UVDL and AML.

Concurrent other CVBD

A subset of the 53 dogs was tested for concurrent other CVBD by serology and/or PCR. Twenty-seven dogs tested negative for Ehrlichia canis, four dogs tested negative for Anaplasma platys, 12 dogs tested negative for Borrelia burgdorferi, 14 dogs tested negative for Dirofilaria immitis, and 20 dogs tested negative for Babesia canis. Four dogs with a borderline or positive serology test result for Babesia canis or Ehrlichia canis were included in the study, which does not correspond to previously mentioned criteria. However, they were tested negative by PCR and/or showed no clinical signs related to the concerning disease which suggests that anti-Babesia canis and anti-Ehrlichia canis antibodies remained in the blood after a previous recovered infection [28 29]. The remaining dogs were not tested for concurrent other CVBD.

Clinical signs and clinicopathological abnormalities at diagnosis

Table 1 summarizes all 32 clinical signs and a selection, based on the most common abnormalities found in canine Leishmaniasis [1 2], of clinicopathological parameters at diagnosis. Unfortunately, not every dog was tested for each clinicopathological parameter and therefore only the tested values are shown in table 1.

In conclusion, the distribution of clinical signs was widely spread with general skin involvement, scaling of the skin, scaling of the skin and crustae head as cutaneous signs, and lymphadenomegaly documented most often. Anemia and the five variable protein spectrum parameters illustrated in table 1, were described in more than 50% of dogs. Luckily, renal azotemia was present in less than 20% of dogs, which deteriorates the prognosis [2]. Comparable laboratory findings are described in the literature [1]. Only thrombocytopenia was reported more often (44.7%) than the study by Ciaramella et al. (1997) [30] and Shaw et al. (2009) [31], 29% and 22%, respectively. However, this may be explained by the influence of aggregates in the thrombocytes value shown in table 1 and 3 (spurious thrombocytopenia) [32]. For urine laboratory analysis, all 14 UPC results at diagnosis were not useful because sediment of urine was not available or showed leucocytes and epithelial cells.

Therapy

Figure 2 shows the total individual treatment of canine Leishmaniasis from commencement of first antileishmanial therapy until censor date. Many variations were seen in the choice, duration and order of treatment, and period without treatment. However, the distribution of time between diagnosis and commencement of an antileishmanial treatment was quite equal between dogs ([median ± IQR] 6 ± 7 days). In the following paragraphs, an overview of the figure and information on dosages and side effects are given.

Table 1: Complete dataset with all 32 clinical signs and a selection of clinicopathological parameters at diagnosis. The specific location of crustae on the head, was seen around ears, eyes, and nose. Because of variable laboratories, the result of each clinicopathological parameter was compared to its own reference range. Whenever biochemical parameters total protein and albumin were examined, but a globulins result was absent; the formula 'total protein – albumin' was used for the calculation of globulins and compared to the reference range of another patient from the same laboratory. Unfortunately, analyzer Spotchem has no reference range for globulins and therefore five available values* were noted as not available. The A/G-ratio was calculated by dividing albumin with total protein - albumin. **One available value of thrombocytes was noted as not available because of platelet clumping. ***A urea value below reference range was seen in one dog and a creatinine value below reference range was seen in another dog, however these values have no clinical relevance for this study and therefore notes as nondeviant. ****Six dogs had increased creatinine and urea values, while three dogs had a creatinine value within reference range but an increased urea value. Two dogs had a urea value within reference range, but an increased creatinine value and one dog showed an increased creatinine value, but urea was not determined.

Clinical signs	n/total	%	Clinicopathological abnormalities	n/total	%
General signs			**Hematological parameters**		
Lethargy	16/53	30.2	Anemia	23/43	53.5
Anorexia	15/53	28.3	Thrombocytopenia **	17/38	44.7
Fever	3/53	5.7	Leukocytosis	6/42	14.3
Vomiting	7/53	13.2	Leukopenia	9/42	21.4
Diarrhea	14/53	26.4			
Sneezing	1/53	1.9			
Polyuria and polydipsia	10/53	18.9			
Weight loss	12/53	22.6			
Pale mucous membranes	5/53	9.4			
Ulcerative mucosae lesions	0/53	0			
Epistaxis	3/53	5.7			
Lymphadenomegaly	21/53	39.6			
Hepatomegaly	1/53	1.9			
Splenomegaly	0/53	0			
Cutaneous signs			**Protein spectrum**		
General skin involvement	29/53	54.7	Hyperproteinemia	34/53	64.2
Scaling skin	21/53	39.6	Hypoalbuminemia	28/51	54.9
Scaling skin and crustae head	26/53	49.1	Hyperglobulinemia *	38/46	82.6
Vasculitis	0/53	0	Gammaglobulinemia	19/21	90.5
Erythema	7/53	13.2			
Hypotrichosis	9/53	17.0			
Alopecia	13/53	24.5			
Seborrheic skin	1/53	1.9			
Nodular dermatitis	1/53	1.9			
Ulcerative dermatitis	1/53	1.9			

Long nails Onychogryphosis	2/53 2/53	3.8 3.8			
Locomotion signs			**Renal azotemia ******		
Stiff Lameness Overfilled joints Muscle atrophy	10/53 10/53 6/53 2/53	18.9 18.9 11.3 3.8	Increased Creatinine *** Increased Urea ***	9/50 9/49	18.0 18.4
Ocular signs	8/53	15.1	**Increased liver enzymes**		
			ALP ALT	6/46 4/44	13.0 9.1
Neurological signs	2/53	3.8			

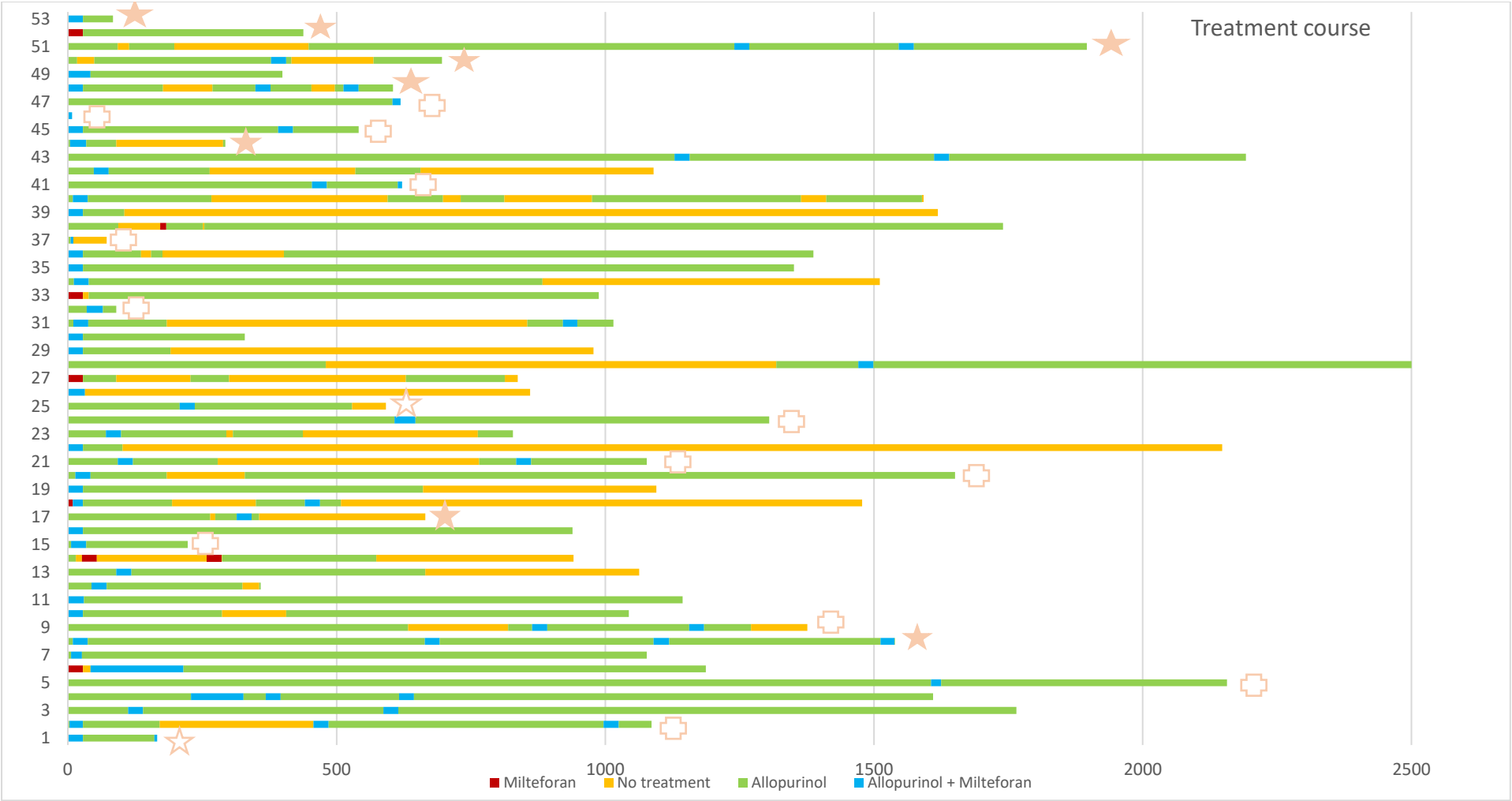

Figure 2: The total individual treatment of canine Leishmaniasis is illustrated from commencement of first antileishmanial therapy (x-axis = 0) until censor date (in days). The different (no) treatment options are shown in the legenda. Censor date could be the start of corticosteroids ☆ or meglumine antimoniate ★ treatment, dead by Leishmaniasis ✚ or still alive/lost to follow up. *After the third allopurinol as monotherapy treatment period, dog #23 received no treatment for a total of 326 days and allopurinol for a total of 65 days. However, for 13 months, this was administered in the following repetitive schedule: five days a month of allopurinol as monotherapy, followed by no treatment for the rest of the month and so on. **After the first no treatment period, dog #51 received allopurinol for a total of 84 days and no treatment for a total of 250 days. However, this was administered in the following repetitive schedule: seven days of allopurinol as monotherapy, followed by 21 days of no treatment for 11 months, and seven days of allopurinol as monotherapy and 19 days of no treatment for one month.

In 22 out of 53 dogs, miltefosine was administered as the first therapy after diagnosis with [median ± IQR] 14 ± 113 days in between. Seventeen out of 22 dogs received miltefosine combined with allopurinol and in five out of 22 dogs miltefosine was given as a monotherapy. The other 31 dogs received allopurinol alone as the first therapy after diagnosis, followed by a combined treatment with miltefosine (24/31) or no treatment (7/31). 69.8% of dogs received miltefosine once, while 22.6% twice and 5.7% three times, and miltefosine was administered four times in 1.9% of dogs. Treatment may be repeated based on relapses of clinical signs and/or clinicopathological parameters. Relapses cannot be biased by reinfections in this study.

The range of duration of the first miltefosine therapy cycle was 5 to 98 days, with a median of 28 days. In 39 dogs (73.6%), miltefosine was administered according to the user instructions in a 28-day course. For the other 14 dogs, the duration of therapy was 5, 8, 11, 15, 19, 20, 25, 30, 30, 30, 32, 39, 42 and 98 days. Two dogs died of canine Leishmaniasis during the treatment period and received miltefosine for only 8 and 15 days. Three other dogs showed side effects such as anorexia, lethargy, vomiting and/or diarrhea when miltefosine was administered and is the reason for discontinuing the treatment at 5, 11 and 19 days. One dog received miltefosine treatment for 98 consecutive days because of insufficiently improvement of clinical signs and laboratory values. At last, no explanation was provided for deviating from recommended duration of therapy in eight other dogs.

Most dogs (90.6%) received a daily dose of 2.0 mg/kg bodyweight once a day, while three dogs (5.7%) received 1.8 mg/kg bodyweight once a day, one dog (1.9%) received a daily dose of 2.1 mg/kg bodyweight once a day and another dog (1.9%) received 2.0 mg/kg bodyweight three times a day.

All 53 dogs received allopurinol as part of their therapy protocol, with a duration of [median ± IQR] 591 ± 814 days. The administered dosage of allopurinol was [median ± IQR] 19.9 ± 6.7 mg/kg bodyweight/day.

Besides receiving miltefosine and allopurinol therapy, a period without administration of antileishmanial drugs until censoring was documented in 32 dogs (60.4%). The duration of this “no antileishmanial treatment period” was [median ± IQR] 81 ± 434 days. However, this was temporarily in 24 out of 32 dogs (figure 2).

Side effects

When evaluating side effects of miltefosine treatment for all dogs, all 74 miltefosine treatments (30.2% of dogs received more than one treatment) were considered. Side effects were noted in 23% of treatments (17/74), which consisted of anorexia/hyporexia (12/17), lethargy (6/17), diarrhea (7/17) and/or vomiting/nausea (9/17). Patient files of 15 dogs reported side effects, with a side effect rate of 33%, 50% or 100%. A side effect rate of 50% means side effects were documented in one of two miltefosine therapy cycles or in two of four miltefosine therapy cycles.

Data analysis

There were no significant differences for comparison of signalment, individual frequency of clinical signs, clinical score and clinicopathological abnormalities (hematological and biochemistry analysis) at diagnosis, course of total treatment for canine Leishmaniasis, duration of the first cycle of miltefosine therapy, incidence of side effects and Kaplan-Meier survival probability curve in the group “Inclusion T0-T1” versus “Exclusion T0-T1” (data not shown). Therefore, all results are given for the complete dataset, with exception of the therapy efficacy results which is described for the subset “Inclusion T0-T1”.

Survival analysis

As described in statistical analysis, date of diagnosis, date of commencement of first miltefosine treatment, date and reason of censoring, were necessary for graphing a Kaplan-Meier survival probability curve.

Dogs were followed during a time course of [median ± IQR] 1055 ± 873 days from diagnosis to censor date. At censor date 26/53 and 4/53 of dogs were still alive or lost to follow up, respectively, 13/53 were dead due to canine Leishmaniasis, 2/53 and 8/53 of dogs were censored because of the use of glucocorticoids and meglumine antimoniate, respectively. The median survival probability from diagnosis and commencement of first miltefosine treatment was 2157 days (95% CI: 1651-NA days) and 1637 days (95% CI: 1637-NA days), respectively. However, in this dataset, the median survival time with a wide accompanying 95% CI, is quite unreliable due to insufficient events occurring at this timepoint [33] (figure 3). Therefore, it was preferred to use the survival probability of 78% and 71%, three-years from diagnosis and commencement of the first miltefosine therapy cycle, respectively.

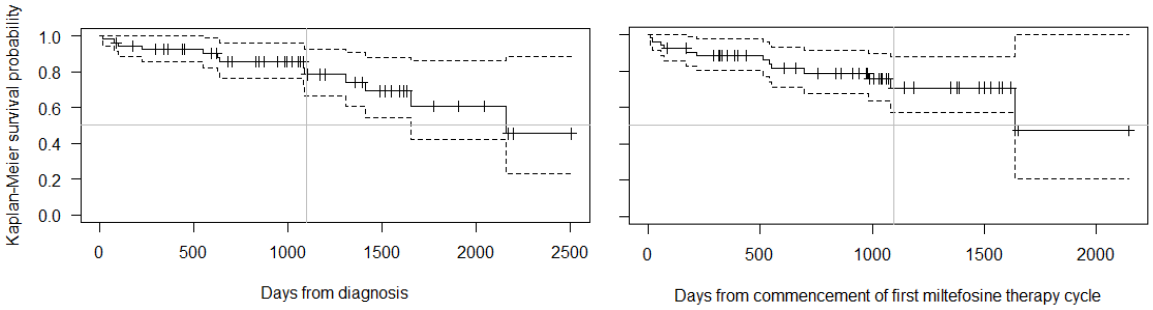

Figure 3A and 3B: Kaplan-Meier survival probability curves of complete dataset with corresponding 95% confidence intervals. Hatch marks indicate censored dog. The horizontal line at 0.5 illustrates the median survival probability and the vertical line at three-years illustrates the corresponding survival probability with an acceptable 95% confidence interval.

Efficacy of the first miltefosine therapy cycle

As described above, clinical signs and clinicopathological abnormalities, before and after treatment, were compared for the subset “Inclusion T0-T1”. For this group of 25 dogs, checkup before treatment was [median ± IQR] 10 ± 6 days and posttreatment examination date was [median ± IQR] 60 ± 49 days after commencement of the first miltefosine therapy cycle. The range of duration of first treatment with miltefosine was 20-39 days, with a median of 28 days.

Clinical signs

The clinical signs lymphadenomegaly and scaling skin improved significantly after therapy compared to before, while the *p*-value of the clinical signs lethargy, anorexia, scaling skin and crustae head, and stiff were almost below 0.05. The McNemar’s test was not applicable for sneezing, ulcerative mucosae lesions, hepatomegaly, vasculitis, seborrheic skin, nodular and ulcerative dermatitis, and neurological signs because these clinical signs were absent at pre- and posttreatment examination date and therefore not shown in table 2.

Besides statistically analyzing the efficacy of therapy for each clinical sign separately, it was useful to visualize each dog’s clinical symptom improvement and promising results are displayed in the column of ‘Return to normal values at T1, table 2. A 100% recovery rate was reported for 13 clinical signs. This table also presents the inevitable outcome of dogs showing no clinical sign

improvement at posttreatment examination date or showing a clinical symptom at posttreatment examination date which was not present before therapy.

Clinicopathological abnormalities

Before treatment the hematological parameters hematocrit and thrombocytes were below merged reference range and increased significantly after treatment. On the other hand, at pretreatment examination date total protein and globulins were above merged reference range and decreased significantly at posttreatment examination date. Even though, the median of albumin was within merged reference range before and after treatment, a significant increase was seen. The A/G-ratio also increased significantly.

Table 2: Clinical signs at pre- and/or posttreatment examination date, calculated *p*-value of therapy efficacy using the McNemar's test and return to normal values at posttreatment examination date. **P*-value <0.05.

Clinical signs	Present at T0 (n=25)		Present at T1 (n=25)		*P*-value	Return to normal values at T1	
General signs	Number	%	Number	%		Number	%
Lethargy	9	36	3	12	0.08	7	77.8
Anorexia	9	36	3	12	0.08	7	77.8
Fever	2	8	0	0	NA	2	100
Vomiting	4	16	1	4	0.25	3	75
Diarrhea	3	12	0	0	NA	3	100
Polyuria and polydipsia	5	20	1	4	0.22	5	100
Weight loss	4	16	0	0	NA	4	100
Pale mucous membranes	4	16	3	12	1.00	4	100
Epistaxis	1	4	0	0	NA	1	100
Lymphadenomegaly	8	32	2	8	0.04 *	6	75
Splenomegaly	1	4	0	0	NA	1	100
Cutaneous signs							
General skin involvement	11	44	5	20	0.11	8	72.7
Scaling skin	11	44	3	12	0.01 *	8	72.7
Scaling skin and crustae head	11	44	6	24	0.07	5	45.5
Erythema	3	12	1	4	0.48	2	66.7
Hypotrichosis	2	8	2	8	1.00	1	50
Alopecia	5	20	1	4	0.13	4	80
Long nails	1	4	0	0	NA	1	100
Onychogryphosis	1	4	0	0	NA	1	100
Locomotion signs							
Stiff	7	28	1	4	0.08	7	100
Lameness	4	16	2	8	0.62	3	75
Overfilled joints	2	8	1	4	1.00	2	100
Muscle atrophy	2	8	0	0	NA	2	100
Ocular signs	2	8	0	0	NA	2	100

Table 3: Median and IQR of clinicopathological parameters at pre- and posttreatment examination date. *P*-value was calculated using two-sample paired t-test[a] or Wilcoxon signed rank test[b]. Due to aggregates an available value of thrombocytes was eliminated from analysis. **P*<0.05

Clinicopathological parameters	**Merged reference range**	**Median ± IQR T0**	**Median ± IQR T1**	***P*-value**
Hematology				
Hematocrit (n=16)	39.2-56.9 (%)	33.6 ± 10.9	41.1 ± 5.7	0.005 [a] *
Leukocytes (n=15)	5.3-15.2 ($x10^9$/L)	7.9 ± 2.8	8.2 ± 2.4	0.53 [b]
Thrombocytes (n=14)	161.2-489.6 ($x10^9$/L)	138.5 ± 66	253 ± 119	0.002 [a] *
Biochemistry				
Total protein (n=21)	52.6-76.4 (G/L)	81 ± 15	68 ± 15	0.002 [b] *
Albumin (n=17)	24.8-41.2 (G/L)	25 ± 9.7	29 ± 11.7	0.002 [a] *
Globulin spectrum (n=15)	24.2-45.9 (G/L)	57 ± 18.1	43.5 ± 18	0.009 [b] *
A/G-ratio (n=16)	NA	0.44 ± 0.35	0.58 ± 0.40	0.0002 [a] *
Gamma globulin (n=6)	3.3-9.2 (G/L)	36.7 ± 53.8	20.8 ± 15.8	0.059 [b]
Urea (n=17)	2.7-9.9 (mmol/L)	6.6 ± 6.6	6.9 ± 3.3	0.33 [b]
Creatinine (n=19)	31.4-134.7 (µmol/L)	81 ± 46.9	84 ± 21.4	0.54 [b]
ALP (n=14)	12.0-170.8 (U/L)	43.5 ± 53	34 ± 55	0.75 [b]
ALT (n=13)	4.5-114.7 (U/L)	25 ± 10	33 ± 24	0.21 [b]

Discussion

A good clinical improvement of dogs with canine Leishmaniasis was shown in six clinical trials, which evaluated the therapeutic efficacy of miltefosine alone or in combination with allopurinol [16 25 26 34 35 36]. This study also demonstrates improvement of clinical signs when treated. Administration of allopurinol, in combination with a first cycle of miltefosine, was very common (21/25). An in vivo study by Farca et al. (2012) [37] reported efficacy of miltefosine alone or in combination with allopurinol, however a combination therapy was significantly more efficacious than administration of miltefosine alone. Similar results were described in a clinical trial by Dias et al. (2020) [38]. Even though, dosages and administered duration of allopurinol were variable in this study, falsely better therapy efficacy results may be expected compared to the efficacy of miltefosine treatment alone.

The clinical signs lymphadenomegaly and scaling skin improved significantly after receiving a first miltefosine therapy cycle, while the *p*-value of four other clinical signs was almost significant. If the sample size of this study increases, a significance could possibly be calculated for these parameters as well [39 40].

The method of measuring clinical improvement was similarly approached in various studies, however the clinical grading system was different. In comparison to the study of Manna et al. (2009) [35] and Nogueira et al. (2019) [16], clinical signs were graded by absent or present, because severity classified as absent, mild, moderate, and severe, was not reported in this study. Instead of measuring clinical improvement as the total clinical score, it was chosen to statistically analyze therapy efficacy for each clinical sign separately and therewith frequencies of present clinical signs before and after treatment were displayed. In addition, individual information on clinical signs returning to normal values at posttreatment examination date was visualized. Equally to table 1, the clinical signs lethargy, anorexia, lymphadenomegaly, and three cutaneous signs, were seen most often (≥32% of dogs) and were described in the literature as well [1].

Not entirely unexpected, some dogs in this study showed deterioration of clinical signs or no clinical symptom improvement after treatment. Contributing factors could be 1) failure of therapy, 2) expectation of course of the disease, 3) consultation after therapy was performed by another veterinarian which resulted in inconsistent judgement of the disease progression, 4) failure of the clinical grading system, 5) incomplete physical examination or documentation before and/or after therapy or 6) posttreatment examination date was too short after commencement of miltefosine treatment to improve a specific clinical symptom. Similar findings on failure to achieve complete clinical cure were reported by prospective studies [16 25 26 34 35 36] as well.

Besides assessment of clinical signs, clinicopathological parameters were also evaluated. These parameters increase the objectivity of the study; hence these results are more independent and valuable. The hematological parameters hematocrit and thrombocytes, and the biochemical parameters total protein, globulins, albumin, and A/G-ratio improved significantly after treatment. Unfortunately, only the study by Woerly et al. (2009) [26] and Miró et al. (2009) [34] performed statistical analysis on similar clinicopathological parameters before and after treatment, but luckily reported the same results.

Despite the possibility of a type I error (rejecting the null hypothesis that is true) occurring due to the small study sample size and the calculation of a six-fold greater total study sample size by a priori analysis test [40 41 42], the results in this study are similar to available literature [16 25 26 34 35 36] and suggests a real miltefosine therapy effect.

Unfortunately, only the efficacy of a first miltefosine therapy cycle was analyzed because of insufficient number of dogs receiving a second cycle of miltefosine and the variability of time between examination date before and after treatment. However, it would have been interesting to evaluate and compare the efficacy of a second miltefosine therapy cycle (due to relapse without re-

infection) to dogs receiving only one miltefosine treatment. Manna et al. (2009) [35] described a significant reduction of parasite load in lymph nodes one month after the first and the second miltefosine (in combination with allopurinol) therapy cycle compared to before therapy. Also, Woerly et al. (2009) [26] reported that 92.6% of dogs with recurrent diseases were considered to have been treated with an equal or higher efficacy compared to 77.7% in the fist-time canine Leishmaniasis group. Therefore, it would be useful to implement and evaluate the efficacy of repeated miltefosine therapy cycles in future research. In addition, more insight into the short- and long-term efficacy of miltefosine and the occurrence of relapses, can be gained if different posttreatment periods of one, three, six, nine and 12 months, are evaluated and compared. Also, the outcome can be used to make an optimal canine Leishmaniasis monitoring protocol. At the moment, checkups for clinically diseased dogs are recommended after the first month of treatment and every two to four months during the first year [2 43 44].

Besides efficacy of therapy, side effects were evaluated in this study. The gastro-intestinal signs as side effects in this study are comparable to other studies. This also applies to the incidence of side effects (approximately 28% of dogs) [25 26]. Besides the similarities with other studies, the value and reliability of this study parameter should be critically looked at because an under- or overrepresentation could have occurred. Examples are the absence of documentation of side effects if owner did not contact the veterinarian or unjustified reported as a miltefosine side effect, but in fact caused by the ongoing progress of canine Leishmaniasis itself or other simultaneous events happening.

In this study, it was chosen to represent two Kaplan-Meier survival probability curves in contemplation of providing information to a larger target group. At diagnosis, owners are interested to know the consequences of canine Leishmaniasis on the lifetime of their animal. With the available study data, survival of treated diseased dogs at three-years from diagnosis was 78%. After diagnosis, more than 50% of dogs received allopurinol alone as the first antileishmanial treatment, miltefosine was prescribed [median ± IQR] 90 ± 405.5 days after commencement of allopurinol and possibly prescribed to more severe diseased dogs, which suggests the survival time from commencement of first miltefosine treatment to be shorter. Therefore, it would be useful to provide information on survival time from commencement of miltefosine therapy. Figure 3 illustrates the expected outcome (survival probability of 78% and 71%, three-years from diagnosis and commencement of the first miltefosine therapy cycle, respectively).

It was chosen to censor dogs treated with meglumine antimoniate because only the influence of miltefosine and allopurinol on the survival time was thereby considered, in some cases insufficient information on dosage and duration of meglumine antimoniate was provided and possibly the data may be used for future research comparing different treatments. However, if dogs treated with meglumine antimoniate were not censored, the survival probability, three-years from diagnosis and commencement of first miltefosine treatment does not change much (80% and 74%, respectively). Possibly, the use of benazepril (n=5) may positively influence the survival time because it was described to significantly reduce proteinuria in dogs with chronic kidney disease [45].

To the author's knowledge only one research on the survival and prognostic factors of dogs treated with miltefosine in combination with allopurinol, was conducted. Remarkably, the study by Pereira et al. (2020) [46] reported a median survival time from diagnosis of only 514.5 days for the treatment of miltefosine and allopurinol (n=50). This difference can possibly be explained by the fewer number of repeated miltefosine treatments per dog, shorter duration and dosage of allopurinol treatment, selection of dogs evidencing more clinical signs and altered laboratory values and considering only events for the survival analysis which may result in bias and loss in sample size [47]. Luckily, a beneficial outcome on the survival of treated dogs with canine Leishmaniasis was described by many studies [46 48 49 50]. The presence of proteinuria, hypoalbuminemia, renal azotemia,

lymphopenia, was significantly correlated with the survival time and therefore may be useful prognostic parameters [46 50 51]. In follow-up research, Cox proportional hazards regression analysis should be performed to investigate the effect of previously described prognostic values upon the time of death by canine Leishmaniasis. In addition, like the study by Pereira et al. (2020) [46], it would be useful to study the long-term efficacy of different treatment protocols for dogs with canine Leishmaniasis and therewith compare survival and prognostic factors.

By assessing survival, the efficacy of miltefosine therapy and side effects, the use of miltefosine in Dutch veterinary first-line practice was evaluated. Remarkably, there was a lot of variation in posttreatment examination date (physical examination and laboratory work) and choice of clinicopathological parameters to be tested. It ranged from immediately after therapy to 951 days and from only hematology bloodwork to the recommended combination of hematology, biochemistry with protein electrophoresis and urinalysis. Unfortunately, UPC-values were not useful in this study because of incomplete performed urinalysis. Inflammatory conditions in the urinary tract increases protein and therefore urine sediment must be analyzed to interpret an UPC-value [52].

Because of the challenging canine Leishmaniasis management, it is understandable that Dutch first-line veterinarians seems to have difficulty treating and monitoring their patients. However, it is quite concerning because of 1) available resources such as the guidelines formulated by the UKG, the LeishVet Group and Canine Leishmaniasis Working Group, 2) the complexity of the disease, 3) the possible resistance of the parasite to miltefosine (published in human medicine), 4) the limited referrals to specialists, 5) the increasing numbers of canine Leishmaniasis in the Netherlands due to increased number of imported dogs and 6) the climate change which may cause the sandfly to move to northern non-endemic areas [2 15 43 53 54 55 56]. Therefore, it may be helpful to investigate the need of first-line veterinarians in the Netherlands through a questionnaire in future research.

Two types of biases should be taken into consideration for this retrospective study [57]. Patient files were only shared after receiving a written informed consent when the veterinarian agreed to contact the owner. Some veterinarians were not enthusiastic for research participation or were not comfortable contacting the owner if the patient had moved to another city or veterinary clinic, or if the patient died. Unfortunately, this contributes to selection bias. Also, information bias could have occurred. Students could have interpreted patient files differently or left out important information and therefore it is not recommended to view patient files individually. However, patient files were systematically evaluated, documented in a standardized template, and discussed with the supervisor, which suggests this bias to be negligible. The contribution of veterinarians on information bias was expected to be greater. For the evaluation of patient files, it is assumed that at every visit a complete physical examination was performed, and that documentation was complete. However, underrepresentation of clinical signs could have occurred due to lack of documentation or incompleteness of anamnesis and physical examination. Also, if more than one veterinary clinic was consulted at the same time, patient information was possibly missing.

In the following paragraphs, for this retrospective study some unavoidable but possibly contributing factors are discussed. The use of various drugs such as locally applied corticosteroids, maropitant, metoclopramide, omeprazole, cimetidine, meloxicam, amoxicillin/clavulanic acid, ephedrine, and triamcinolone ear ointment, before, during or after the first miltefosine treatment period, was inevitable and mostly necessary for symptom control. It does decrease the interval validity of the study; however, it is not expected to modulate the immune status of the dog, change the course of the disease, or interfere with miltefosine.

Twenty-five out of 53 dogs were not tested for concurrent other CVBD at all, while the other dogs were tested for at least one CVBD. Possibly, co-infection with another CVBD played a role in the clinical picture of included dogs. To increase the internal validity of the study and to exclude concurrent other CVBD, it is preferred that negative testing of the five CBVD mentioned above is an

inclusion criterion. However, this was not feasible in the study and low contribution of bias was expected.

Unfortunately, four different laboratories (IDEXX VML, UVDL, Laboklin and AML) were used to analyze clinicopathological parameters. Blood results from different laboratories were quite similar but not completely the same because of variable equipment and reference ranges used in the laboratory. Luckily, in this study clinicopathological parameter units were the same at each laboratory and reference range values were not widely distributed. Therefore, a merged reference range (average lower and upper reference value) was calculated and used to interpretate grouped blood results (table 3). Categorizing values of clinicopathological parameters based on severity (classified as no deviation, mild, moderate, and severe) was another option for resolving the inequality of blood results. However, by doing so, continuous data would be transferred to categorical data, which results in loss of essential information [58]. Hence, this statistical approach was not preferred nor used.

Conclusion

This study provided an accurate overview of the use of miltefosine in Dutch veterinary first-line practice. Valuable and promising results on the outcome of treatment with a first cycle of miltefosine were presented. The clinical signs lymphadenomegaly and scaling skin, and the laboratory parameters hematocrit, thrombocytes, total protein, globulins, albumin, and A/G-ratio improved significantly after treatment. A survival probability of 78% and 71%, three-years from diagnosis and commencement of the first miltefosine therapy cycle, respectively, was shown in two Kaplan-Meier survival probability curves. Future research suggestions may contribute to improvement of the challenging canine Leishmaniasis management.

Competing interests

The author declares that the paper was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The author gratefully acknowledges the provision of informed consent by veterinarians and owners, Drs. C. van Esch, DVM, Product Manager Companion Animals of Virbac Netherlands, for providing prescriptions for miltefosine and the participation of three students on data collection. Furthermore, the author is grateful for the statistical expertise of J.C.M. Vernooij, MSc and dr. C.J. Piek, PhD, ECVIM-CA for providing introduction to this topic, expertise and who greatly assisted the paper.

Abbreviations

%	Percentage	AML	Algemeen Medisch Laboratorium
#	Patient number in database	CVBD	Companion Vector-Borne Diseases
N	Number of dogs	ELISA	Enzyme-linked Immunosorbent Assay
n/total	Subset of total number of dogs	DAT	Direct Agglutination Test
NA	Not available	IFAT	Immunofluorescence Antibody Test
IQR	Interquartile range	PCR	Polymerase Chain Reaction
T0	Pretreatment examination date	A/G-ratio	Albumin/Globulin-ratio
T1	Posttreatment examination date	ALP	Alkaline phosphatase
95% CI	95% confidence interval	ALT	Alanine aminotransferase
UKG	Universiteitskliniek voor Gezelschapsdieren	UPC	Urine Protein/Creatinine-ratio
UVDL	University Veterinary Diagnostic Laboratory		

References

1. Noli C, Saridomichelakis MN. An update on the diagnosis and treatment of canine leishmaniosis caused by leishmania infantum (syn. L. chagasi). *Vet J*. 2014;202(3):425-435. doi: 10.1016/j.tvjl.2014.09.002 [doi].
2. Solano-Gallego L, Miro G, Koutinas A, et al. LeishVet guidelines for the practical management of canine leishmaniosis. *Parasit Vectors*. 2011;4:86-86. doi: 10.1186/1756-3305-4-86 [doi].
3. Solano-Gallego L, Koutinas A, Miro G, et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet Parasitol*. 2009;165(1-2):1-18. doi: 10.1016/j.vetpar.2009.05.022 [doi].
4. Alvar J, Canavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. *Adv Parasitol*. 2004;57:1-88. doi: S0065308X0457001X [pii].
5. Kaszak I, Planellas M, Dworecka-Kaszak B. Canine leishmaniosis - an emerging disease. *Ann Parasitol*. 2015;61(2):69-76.
6. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: Part one. *Trends Parasitol*. 2008;24(7):324-330. doi: 10.1016/j.pt.2008.04.001 [doi].
7. Carrera L, Fermin ML, Tesouro M, et al. Antibody response in dogs experimentally infected with leishmania infantum: Infection course antigen markers. *Exp Parasitol*. 1996;82(2):139-146. doi: S0014-4894(96)90018-1 [pii].
8. Pasa S, Toz SO, Voyvoda H, Ozbel Y. Clinical and serological follow-up in dogs with visceral leishmaniosis treated with allopurinol and sodium stibogluconate. *Vet Parasitol*. 2005;128(3-4):243-249. doi: S0304-4017(04)00592-8 [pii].
9. Cantos-Barreda A, Escribano D, Martinez-Subiela S, Pardo-Marin L, Segarra S, Ceron JJ. Changes in serum anti-leishmania antibody concentrations measured by time-resolved immunofluorometric assays in dogs with leishmaniosis after treatment. *Veterinary Immunology and Immunopathology*. 2018;198:65-69. doi: //dx.doi.org/10.1016/j.vetimm.2018.03.003.
10. Koutinas AF, Saridomichelakis MN, Mylonakis ME, et al. A randomised, blinded, placebo-controlled clinical trial with allopurinol in canine leishmaniosis. *Vet Parasitol*. 2001;98(4):247-261. doi: //doi.org/10.1016/S0304-4017(01)00399-5.
11. Solano-Gallego L, Di Filippo L, Ordeix L, et al. Early reduction of leishmania infantum-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. *Parasit Vectors*. 2016;9(1):235-0. doi: 10.1186/s13071-016-1519-0 [doi].
12. Saridomichelakis MN. Advances in the pathogenesis of canine leishmaniosis: Epidemiologic and diagnostic implications. *Vet Dermatol*. 2009;20(5-6):471-489. doi: 10.1111/j.1365-3164.2009.00823.x [doi].'
13. Oliva G, Scalone A, Foglia Manzillo V, et al. Incidence and time course of leishmania infantum infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. *J Clin Microbiol*. 2006;44(4):1318-1322. doi: 44/4/1318 [pii].
14. Oliva G, Roura X, Crotti A, et al. Guidelines for treatment of leishmaniasis in dogs. *J Am Vet Med Assoc*. 2010;236(11):1192-1198. doi: 10.2460/javma.236.11.1192.
15. Costagnaro M, Crotti A, Fondati A, et al. Part 1: Diagnostic approach and classification of the patient affected by leishmaniasis and management of dogs with proteinuria. *Rivista ufficiale della SCIVAC*. 2007;21:19-+.

16. Dos Santos Nogueira F, Avino VC, Galvis-Ovallos F, et al. Use of miltefosine to treat canine visceral leishmaniasis caused by leishmania infantum in brazil. *Parasit Vectors*. 2019;12(1):79-0. doi: 10.1186/s13071-019-3323-0.
17. Croft SL, Seifert K, Duchêne M. Antiprotozoal activities of phospholipid analogues. *Mol Biochem Parasitol*. 2003;126(2):165-172. doi: S0166685102002839 [pii].
18. Marinho Fde A, Gonçalves KC, Oliveira SS, et al. Miltefosine induces programmed cell death in leishmania amazonensis promastigotes. *Mem Inst Oswaldo Cruz*. 2011;106(4):507-509. doi: S0074-02762011000400021 [pii].
19. Lux H, Hart DT, Parker PJ, Klenner T. Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkyl phospholipid analogues. *Adv Exp Med Biol*. 1996;416:201-211. doi: 10.1007/978-1-4899-0179-8_33.
20. Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of leishmania donovani. *Antimicrob Agents Chemother*. 2004;48(8):3010-3015. doi: 48/8/3010 [pii].
21. Bianciardi P, Brovida C, Valente M, et al. Administration of miltefosine and meglumine antimoniate in healthy dogs: Clinicopathological evaluation of the impact on the kidneys. *Toxicol Pathol*. 2009;37(6):770-775. doi: 10.1177/0192623309344088.
22. Torres M, Pastor J, Roura X, et al. Adverse urinary effects of allopurinol in dogs with leishmaniasis. *J Small Anim Pract*. 2016;57(6):299-304. doi: 10.1111/jsap.12484.
23. Dorlo TP, Balasegaram M, Beijnen JH, de Vries PJ. Miltefosine: A review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J Antimicrob Chemother*. 2012;67(11):2576-2597. doi: 10.1093/jac/dks275.
24. Manna L, Reale S, Vitale F, Picillo E, Pavone LM, Gravino AE. Real-time PCR assay in leishmania-infected dogs treated with meglumine antimoniate and allopurinol. *Vet J*. 2008;177(2):279-282. doi: S1090-0233(07)00159-1 [pii].
25. Mateo M, Maynard L, Vischer C, Bianciardi P, Miró G. Comparative study on the short-efficacy and adverse effects of miltefosine and meglumine antimoniate in dogs with natural leishmaniosis. *Parasitol Res*. 2009;105(1):155-162. doi: 10.1007/s00436-009-1375-3.
26. Woerly V, Maynard L, Sanquer A, Eun HM. Clinical efficacy and tolerance of miltefosine in the treatment of canine leishmaniosis. *Parasitol Res*. 2009;105(2):463-469. doi: 10.1007/s00436-009-1404-2.
27. RStudio Team. RStudio: Integrated development environment for R. 2022. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.
28. Krause PJ, Auwaerter PG, Bannuru RR, et al. Clinical practice guidelines by the infectious diseases society of America (IDSA): 2020 guideline on diagnosis and management of babesiosis. *Clin Infect Dis*. 2021;72(2):e49-e64. doi: 10.1093/cid/ciaa1216.
29. Northern Territory Government. Infection with Ehrlichia canis (Ehrlichiosis). Guidelines for veterinarians. 2021. Retrieved September 1, 2022, from https://nt.gov.au/__data/assets/pdf_file/0007/979720/e-canis-guidelines-for-vets.pdf.
30. Ciaramella P, Oliva G, De Luna R, et al. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by leishmania infantum. *Vet Rec*. 1997;141:539-43. doi: 10.1136/vr.141.21.539.
31. Shaw SE, Langton DA, Hillman TJ. Canine leishmaniosis in the United Kingdom: A zoonotic disease waiting for a vector? *Vet Parasitol*. 2009;163(4):281-285. doi: 10.1016/j.vetpar.2009.03.025.
32. Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: A review. part I: Platelets. *International Journal of Laboratory Hematology*. 2007;29(1):4-20. doi: 10.1111/j.1365-2257.2006.00870.x.

33. Pocock SJ, Clayton TC, Altman DG. Survival plots of time-to-event outcomes in clinical trials: good practice and pitfalls. *The Lancet*. 2002;359(9318):1686-1689. doi: 10.1016/S0140-6736(02)08594-X.
34. Miró G, Oliva G, Cruz I, et al. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. *Vet Dermatol*. 2009;20(5-6):397-404. doi: 10.1111/j.1365-3164.2009.00824.x.
35. Manna L, Vitale F, Reale S, et al. Study of efficacy of miltefosine and allopurinol in dogs with leishmaniosis. *The Veterinary Journal*. 2009;182(3):441-445. doi: 10.1016/j.tvjl.2008.08.009.
36. Iarussi F, Paradies P, Foglia Manzillo V, Gizzarelli M, Caratozzolo MF, Navarro C, Greco B, Rubino GTR, Oliva G & Sasanelli M. Comparison of two dosing regimens of miltefosine, both in combination with allopurinol, on clinical and parasitological findings of dogs with leishmaniosis: A pilot study. *Frontiers in Veterinary Science, 7*. 2020. doi:https://doi.org/10.3389/fvets.2020.577395.
37. Farca AM, Miniscalco B, Badino P, et al. Canine leishmaniosis: In vitro efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of leishmania infantum. *Parasitol Res*. 2012;110(6):2509-2513. doi: 10.1007/s00436-011-2792-7.
38. Dias ÁFdLR, Ayres EdCBS, de Oliveira Martins DT, et al. Comparative study of the use of miltefosine, miltefosine plus allopurinol, and allopurinol in dogs with visceral leishmaniasis. *Exp Parasitol*. 2020;217:107947. doi: 10.1016/j.exppara.2020.107947.
39. Leppink J, Winston K, O'Sullivan P. Statistical significance does not imply a real effect. *Perspectives on Medical Education*. 2016;5(2):122-124. doi: 10.1007/s40037-016-0256-6.
40. Kang H. Sample size determination and power analysis using the G*Power software. *Journal of Educational Evaluation for Health Professions, 18, 17.* 2021. doi:https://doi.org/10.3352/jeehp.2021.18.1
41. Faul F, Erdfelder E, Lang A, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*. 2007;39:175-191. doi: 10.3758/BF03193146.
42. Ford C. Post hoc power calculations are not useful. 2021. Retrieved October 30, 2022, from https://data.library.virginia.edu/post-hoc-power-calculations-are-not-useful/.
43. Roura X, Fondati A, Lubas G, et al. Prognosis and monitoring of leishmaniasis in dogs: A working group report. *The Veterinary Journal*. 2013;198(1):43-47. doi: 10.1016/j.tvjl.2013.04.001.
44. Piek CJ. Behandeling en monitoring leishmaniasis UKG. *Faculty of Veterinary Medicine Utrecht University*. 2018. Retrieved September 11, 2022, from https://www.uu.nl/sites/default/files/20190621_protocol_behandeling_en_monitoring_leishmaniasis_ukg.pdf.
45. King JN, Font A, Rousselot JF, et al. Effects of benazepril on survival of dogs with chronic kidney disease: A multicenter, randomized, blinded, placebo-controlled clinical trial. *J Vet Intern Med*. 2017;31(4):1113-1122. doi: 10.1111/jvim.14726.
46. Pereira MA, Santos R, Oliveira R, et al. Prognostic factors and life expectancy in canine leishmaniosis. *Vet Sci*. 2020;7(3):128. doi: 10.3390/vetsci7030128. doi: 10.3390/vetsci7030128.
47. Leung K, Elashoff RM, Afifi AA. Censoring issues in survival analysis. *Annu Rev Public Health*. 1997;18(1):83-104. doi: 10.1146/annurev.publhealth.18.1.83.

48. Slappendel RJ, Teske E. The effect of intravenous or subcutaneous administration of meglumine antimonate (glucantime) in dogs with leishmaniasis. A randomized clinical trial. *Vet Q*. 1997;19(1):10-13. doi: 10.1080/01652176.1997.9694729.
49. Slappendel RJ, Teske E. A review of canine leishmaniasis presenting outside endemic areas. R. K. In: Killick-Kendrick R, editor. Canine Leishmaniasis: An Update. 1999; Hoechst Roussel Vet, Wiesbaden, pp. 54-59.
50. Geisweid K, Mueller R, Sauter-Louis C, Hartmann K. Prognostic analytes in dogs with leishmania infantum infection living in a non-endemic area. *Veterinary Record*. 2012;171(16):399. doi: //dx.doi.org/10.1136/vr.100637.
51. Baumgartner F, Boretti FS, Gerber B. Prognostic factors in dogs with common causes of proteinuria. *Schweiz Arch Tierheilkd*. 2022;164(7):525-533. doi: 10.17236/sat00362.
52. Beatrice L, Nizi F, Callegari D, et al. Comparison of urine protein-to-creatinine ratio in urine samples collected by cystocentesis versus free catch in dogs. *J Am Vet Med Assoc*. 2010;236(11):1221-1224. doi: 10.2460/javma.236.11.1221.
53. Faculteit Diergeneeskunde Utrecht. FelCan hondendag. 2018. Retrieved September 11, 2022, from http://felcan.nl/wp-content/uploads/2020/02/Handout-Hondendagt-2018-totaal.pdf.
54. Menn B, Lorentz S, Naucke TJ. Imported and travelling dogs as carriers of canine vector-borne pathogens in germany. *Parasites & Vectors*. 2010;3(1):34. doi: 10.1186/1756-3305-3-34.
55. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev*. 2006;19(1):111-126. doi: 19/1/111 [pii].
56. Radstake C. Immigratie van buitenlandse (zwerf) honden-(zwerf) katten naar nederland: De cijfers 2016. *Stray Animal Foundation Platform*. 2017. Retrieved September 11, 2022, from https://www.stray-afp.org/nl/wp-content/uploads/sites/2/2017/11/immigratie-van-buitenlandse-zwerf-honden-en-katten-naar-Nederland-de-cijfers-2016.pdf.
57. Ramirez-Santana M. Limitations and biases in cohort studies. In: R. Mauricio Barría, ed. *Cohort studies in health sciences.* Rijeka: IntechOpen; 2018:Ch. 3.https://doi.org/10.5772/intechopen.74324.10.5772/intechopen.74324.
58. Kotu V, Deshpande B. *Data science concepts and practice.* Second ed.; 2018:Chapter 3: Data Exploration. https://asolanki.co.in/wp-content/uploads/2019/04/Data-Science-Concepts-and-Practice-2nd-Edition-3.pdf