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Short-term efficacy of miltefosine (Milteforan®, Virbac) in the therapy of canine Leishmaniasis in first-line veterinary practice, a retrospective cohort study.

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Abstract

Background Canine Leishmaniasis (CanL) is a zoonotic, vector-borne disease caused by the intracellular protozoa Leishmania infantum. Despite the various therapeutic options and available guidelines, CanL persists to be a difficult and challenging disease to effectively treat and cure. Miltefosine, an alkyl-phospholipid (hexadecylphosphocholine), appeared to be safe and effective against CanL in several studies executed in endemic countries, and is nowadays widely used in the veterinary field as oral treatment against CanL.

Aim The aim of this retrospective study is to examine the efficacy of miltefosine (Milteforan, Virbac) in the therapy of CanL in first-line veterinary practice in the Netherlands, a non-endemic country.

Materials and methods Patient files of dogs with confirmed CanL diagnosis treated with Milteforan in 2016 were retrospectively reviewed. Diagnosis of CanL was confirmed by the detection of a high level of antibodies using the quantitative serological techniques ELISA or DAT in combination with clinical signs and clinicopathological abnormalities related to CanL. Clinical outcome measured as difference between clinical signs and clinicopathological abnormalities compatible with CanL at the time of pre-examination (T0) and time of check-up after treatment (T1), recovery rates, Kaplan-Meier estimated survival time, and incidence of side effects was used to determine the efficacy of Milteforan.

Results A total of seventeen dogs was included. No significant reduction of clinical signs and no significant improvement of clinicopathological abnormalities were observed after Milteforan treatment. Nevertheless, a complete clinical recovery after treatment was found in 29.4% of the patients. The parameters diarrhea, weight loss, lymphadenomegaly, pale mucous membranes, muscle atrophy, splenomegaly, ulcerative dermatitis, long nails, overfilled joints, neurological signs and leucocytosis showed a 100% recovery rate. The Kaplan-Meier estimated survival time was three years, and side effects occurred in 17,7%.

Conclusion This study was performed as part of a larger study into the efficacy of milteforan treatment. These preliminary results give a tentative indication of efficacy of Milteforan in the therapy of CanL in first-line veterinary practice in the Netherlands. For more certainty it would be worth repeating this study with a larger patient group.

Keywords *Canine leishmaniasis, miltefosine, efficacy, survival, clinical signs, clinicopathological abnormalities, treatment*

Introduction

Canine Leishmaniasis (CanL) is a zoonotic, vector-borne disease caused by the intracellular protozoa Leishmania infantum[1]. CanL is endemic in more than 80 countries in the world, including regions of Europe, Asia, America and Africa[1–4].

Two hosts are necessary to complete the lifecycle of Leishmania. Phlebotomine sand flies are the biological vectors of this protozoal disease and transmit the flagellated infective promastigote form. The intracellular amastigote form develops and replicates in the mammal [1,2]. The domestic dog is the main reservoir and the biggest threat for spreading the zoonotic disease to humans and therefore a risk for both veterinary and public health [1,2,5,6].

In endemic areas, climate conditions permit the presence of vectors required for transmission of the disease [5]. In non-endemic areas, such as northern Europe, infection occurs in dogs that have travelled to an endemic (Mediterranean) area or were imported from that region [2,7]. Other proven methods of transmitting Leishmaniasis without vectors are blood transfusion, organ transplantation, transplacental and venereal transmission[2,5].

Clinical manifestations of a CanL infection can be subclinical, self-limiting, or severe illness. Clinical CanL infection can be defined as presence of clinical signs and/or clinicopathological abnormalities with a confirmed CanL infection. Subclinical CanL infected dogs present neither clinical signs nor clinicopathological abnormalities but do have a confirmed CanL infection[4]. The different clinical manifestations can be partially explained by the difference in host immune response. The immune response of subclinical dogs is characterized by mild or no humoral response and strong cell-mediated response, while severe illness is characterized by an exaggerated humoral response and mild or no cell-mediated response[1,4,8,9]. The macrophage is the target cell of the parasite and therefore CanL is found in all tissues in which large numbers of monocytic-macrophagic cells occur[8]. CanL is thus a multisystemic disease with variable signs due to the different tissues affected, the numerous pathogenic mechanisms of the disease process and the diversity of immune responses by the host[1,10].

Major clinical signs of CanL are cutaneous lesions, generalized lymphadenomegaly, progressive weight loss, exercise intolerance, muscle atrophy and polyuria and polydipsia. Due to the divergent and different clinical signs, clinical classification of infected dogs is not well standardized[11]. A standard classification system is important for convenient management, treatment and prognosis of CanL. An attempt has been made to find markers that indicate the clinical status and severity of disease, for instance Reis et al. (2009) demonstrated a correlation of clinical status with degree of parasite load in bone marrow and spleen and Manna et al. (2009) described a positive relationship between parasite load and clinical signs in dogs[12,13]. In both studies it applies that the more serious the clinical symptoms, the higher the parasite load.

Due to the different immune responses and variable and non-specific signs, CanL is often difficult to diagnose [4]. The presence of clinical signs is an essential and important characteristic in the diagnostics. In a patient with clinical and/or clinicopathological abnormalities, the diagnosis can be confirmed with a high antibody titer[2]. The most frequently used quantitative serological techniques to detect specific serum antibodies (IgG) are immunofluorescence antibody test (IFT), enzyme-linked immunosorbent assay (ELISA) or direct agglutination test (DAT). Other diagnostic methods are based on the detection of the parasite. For example, detection of Leishmania DNA in tissues by PCR or the detection of amastigotes by cytology or histology can be used[14].

There are many different drugs which can be used in the treatment of CanL[15,16]. The current therapy for CanL, recommended in the guidelines that have been published by two different specialist

groups, the Leishvet group and the Canine Leishmania Working Group, is either allopurinol alone or in combination with meglumine antimoniate (Glucantime) or miltefosine (Milteforan)[2,8,17]. However, therapy is not always effective, and dogs often relapse.

Miltefosine, an alkyl-phospholipid (hexadecylphosphocholine), was developed as an oral anticancer drug, but due to its gastrointestinal side effects, it was only used for the topical treatment of cutaneous metastases[18–20]. Later, it appeared that phospholipid analogues also have an antiprotozoal activity[21]. In the 1980's, the first studies on antiprotozoal activity against Leishmania donovani appeared, which showed that the intracellular amastigotes were affected[22–24]. Later, the oral activity of miltefosine against L. infantum was confirmed in mice[25]. It is presumed that the signalling pathway, phospholipid metabolism and membrane biosynthesis of the parasite is perturbed by miltefosine, eventually leading to apoptosis-like cell death[21,23,26,27]. The major advantage of Miltefosine over meglumine antimoniate (Glucantime) is that it is not excreted renally and it has no effect on the kidneys[28,29]. Miltefosine is consequently the suggested choice for dogs with renal insufficiency, since long-term treatment with allopurinol can cause renal mineralisation and xanthine urolithiasis and meglumine antimoniate is contraindicated due to nephrotoxicity[2,30]. Teratogenicity is a risk for both the pregnant dog and owner, and use of miltefosine during pregnancy is strictly contraindicated[29]. Miltefosine appeared to be safe and effective against CanL, making it widely used nowadays in the veterinary and human field as an oral treatment for CanL[31–36].

CanL is nowadays often diagnosed in non-endemic countries, such as the Netherlands, due to the increased travel and import of dogs to and from endemic areas[1]. However, the guidelines published by the aforementioned working groups are mainly based on information from endemic countries. A major advantage of examining the efficacy of CanL therapy in a non-endemic country is that relapses cannot be biased by reinfections. This study provides an insight into the approach of CanL patients, the total treatment prescribed and information on the prescription and use of Milteforan by the Dutch veterinarian.

Aim of the study

The aim of this retrospective study is to examine the efficacy of treatment with miltefosine (Milteforan®, Virbac) of clinical Leishmaniasis in a cohort of dogs diagnosed and treated with canine Leishmaniasis in both first-line and referral veterinary practice in the Netherlands in 2016. The primary outcome parameters to assess the efficacy of Milteforan will be difference between clinical signs and clinicopathological (hematologic and biochemical) parameters pre and post treatment. Secondary outcome parameters will be survival analysis and incidence of side-effects.

Materials and methods

For this study a cohort of dogs treated with Milteforan in 2016 was examined.
Milteforan is not registered for veterinary use in the Netherlands but may be prescribed according to the cascade regulation for veterinary medicine as it is registered elsewhere in Europe for use in the dog. According to the user instructions, Milteforan should be administered at 2 mg/kg once a day for 28 days by oral route. The weight of the dog should be measured prior to and during treatment to avoid under-dosing and to decrease the risk of developing resistance. It is recommended to use Milteforan in combination with allopurinol for 28 days for a leishmanicidal induction, after which allopurinol can be continued alone as leishmanistatic therapy. This combination happened to be safe and effective[37].
Contact information on the veterinarians that ordered Milteforan for their clients was made available by Virbac Netherlands and Virbac consented to share this information. Information on diagnosis and outcome of treatment of these cases were evaluated, provided both veterinarian and owner

consented with being contacted and that this information from the patient file was shared with us for a scientific study.

Dogs of all breeds, sex and age, with a confirmed diagnosis of CanL and treated with Milteforan in 2016 by veterinary clinicians in the Netherlands were included. The diagnosis of Leishmaniasis was based on the combination of clinical signs of leishmaniasis and a positive serology test and/or the proven presence of Leishmania amastigotes (observed on cytological, or histological specimen, or PCR).
In order to be included in the study the files had to contain the following information. Results of the physical examination and laboratory blood test performed at the start and after treatment and the applied therapy, including time of administration, dosage and duration. The pre-examination should have been performed maximum 2 months before the start of treatment and the first check-up must have taken place within 6 months after the start of treatment.

Dogs were excluded if files were incomplete for the information mentioned above, received other drugs or treatments that may interfere with Milteforan, modulate the immune status of the dog or change the course of the disease. Dogs that were also diagnosed with another canine vector-borne disease (CVBD), such as Ehrlichia canis, Borrelia burgdorferi or Babesia canis, were also excluded. Cases in which the original diagnosis was made in the country of origin were excluded, because information about which diagnostic method was used and information about the possibly already initiated therapy is often not available and may influence the results.

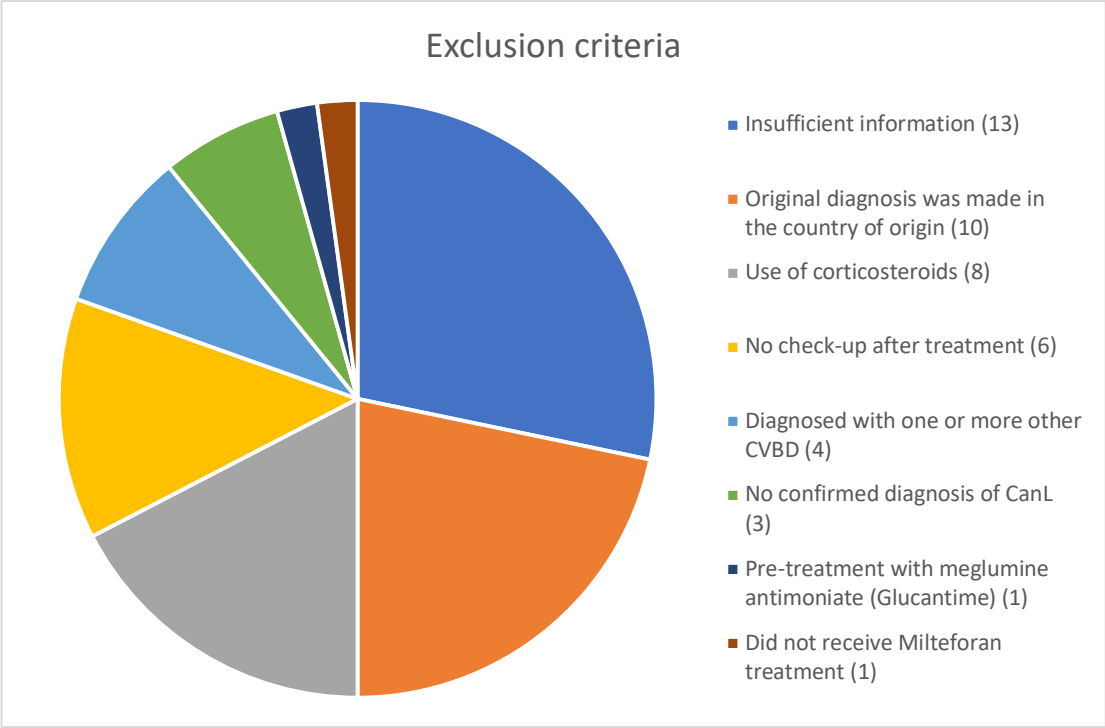
Statistical analysis

To analyse the difference between clinical signs pre- and post treatment, the McNemar's test for comparing paired observations with nominal data has been used. The clinical signs reported at the time of pre-examination before treatment with Milteforan (T0) and time of check-up after treatment (T1) were compared. The null hypothesis is that no significant change has occurred, thus assumes that Milteforan has had no impact on disease.
To analyse the difference between clinicopathological parameters, the Wilcoxon signed rank test has been carried out to compare these paired numeric data. The null hypothesis is that the de medians are the same and the data has identical distributions.
Survival analysis with Kaplan-Meier was performed to quantify the time to occurrence of the event. Time is defined as the time from diagnosis till last contact and event is defined as death due to CanL.

Results

A total of 131 veterinary practices were approached by e-mail and phone to obtain the files of the patients who were treated with Milteforan in 2016. A total of 63 patient files were received at the end of the recruitment phase. Among these 63 dogs, 17 dogs met the inclusion criteria. The other 46 dogs were excluded based on different exclusion criteria shown in figure 1.

Figure 1. The different exclusion criteria met by 46 dogs.



As a result, 17 dogs were included in the study. The 17 dogs were imported from the following countries: 10 from Spain, 6 from Greece and 1 from Portugal.
The dogs had a [median ± interquartile range (IQR)] age at diagnosis of 3.2 ± 2.9 years and bodyweight of 18.4 ± 16.7 kg. 8 were female neutered, 8 male neutered and 1 male intact. 12 were crossbreeds, 1 Breton Spaniel, 1 Boomer, 1 Podenco Ibicenco, 1 crossbreed Vizsla and 1 crossbreed Basset.

In all dogs, diagnosis of CanL was confirmed by the detection of a high level of antibodies using the quantitative serological techniques ELISA or DAT in combination with clinical signs and clinicopathological abnormalities related to CanL. ELISA performed by IDEXX VML was used as the serological technique in 16 patients, with a titer [median ± IQR] of 73.7 ± 18.8. In the other patient DAT was performed by the Universitair Veterinair Diagnostisch Laboratorium (UVDL) resulting in a positive titer of 5120. In two patients, amastigotes were identified as well by cytology of respectively lymph node and spleen aspirates.

Some dogs were tested negative on other CVBDs, i.e. Ehrlichia canis (35.3%), Babesia canis (23.5%), Borrelia burgdorferi (11.8%) and Macrofilaria Dirofilaria immitis (11.8%). The remaining patients were not tested for other CVBDs.

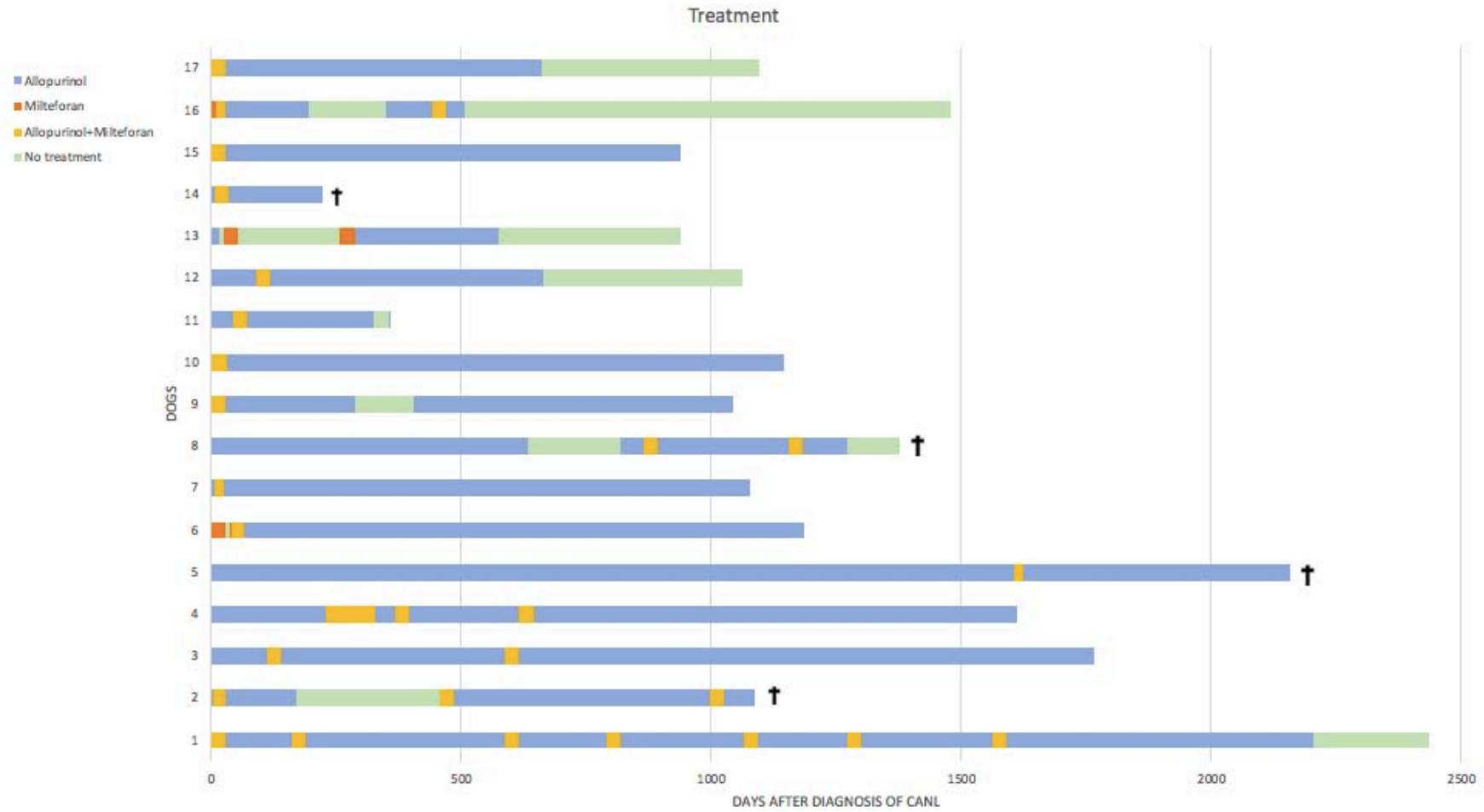
The clinical signs at diagnosis reported by the attending veterinarian and the clinicopathological abnormalities of the included dogs are summarized in table 1.

Clinical signs	Present (nr/n)	%	**Clinicopathological abnormalities**	Present (nr/n)	%
General signs			Hematologic parameters		
Lymphadenomegaly	7/17	41.2	Thrombocytopenia	9/14	64.3
Anorexia	7/17	41.2	Non-regenerative anaemia	7/15	46.7
Diarrhea	5/17	29.4	Decreased MCHC	6/14	42.9
Weight loss	5/17	29.4	Increased MCV	3/14	21.4
Lethargy	5/17	29.4	Leukopenia	3/15	20.0
Polyuria and polydipsia	3/17	17.7	Monocytopenia	2/11	18.2
Pale mucous membranes	3/17	17.7	Lymphocytopenia	2/15	13.3
Vomiting	2/17	11.8	Leucocytosis	1/15	6.7
Muscle atrophy	1/17	5.9			
Cutaneous signs			Protein spectrum		
Scaling skin and crustae head (ears, eyes, nose)	9/17	52.9	Hyperglobulinemia	16/16	100
General skin involvement	8/17	47.1	Decreased A/G ratio	16/16	100
Scaling skin	7/17	41.2	Increased Globulin γ	7/7	100
Alopecia	4/17	23.5	Hypoalbuminemia	11/16	68.8
Erythema	4/17	23.5	Hyperproteinaemia	11/17	64.7
Hypotrichosis	2/17	11.8	Increased Globulin β2	4/7	57.1
Long nails	1/17	5.9			
Ocular signs			Renal Azotaemia[1]		
Uveitis	1/17	5.9	Increased Urea	4/15	26.7
			Increased Creatinine	4/16	25.0
Other signs			Increased liver enzymes		
Neurological disorders (ataxia, tremor)	2/17	11.8	AST	3/9	33.3
Stiffness	2/17	11.8	ALT	2/14	14.3
Lameness	1/17	5.9	ALP	1/15	6.7
Overfilled joints	1/17	5.9			

Table 1. Clinical signs and clinicopathological abnormalities at diagnosis
Not all biochemical values have been tested in all 17 patients, therefore the number of patients for whom the value have been tested is shown in the middle column. ALP Alkaline phosphatase, ALT Alanine aminotransferase, AST Aspartate aminotransferase.
[1]Renal Azotaemia in 3 patients (serial nr) urea; creatinine: (2) 17.3; 232, (7) 11.3; 245, (15) 15; 167 and increased creatinine and increased urea alone both in one patient (6) NA; 165, (17) 24.4; 101.

Treatment

Figure 2. Entire therapy from the moment of diagnosis until the last contact*



The total therapy from the moment of diagnosis of CanL until the last contact of all 17 included dogs is shown. Treatment with Milteforan took mostly place in 2016.

*† Death due to CanL *The last contact is the last date stated in the file.*

The total therapy from the moment of diagnosis till the moment of last contact is shown in figure 2.

In 10 out of 17 dogs, Milteforan was administered as the first therapy after diagnosis. In 9 of these cases, Milteforan was directly combined with allopurinol, one dog was first given Milteforan alone. The number of treatments with Milteforan varied from 1 to 7 courses. Dog number 1 received a Milteforan course a total of 7 times, Prednisolone was started around the second course of milteforan in this dog for an unknown period and dosage. In the dogs in which a second course of Milteforan was given, the file indicated that signs of relapse or clinicopathological abnormalities preceded this second course of Milteforan therapy.

All dogs received Allopurinol as part of their therapy, with a duration of [median ± IQR] 939 ± 484 days. The daily dose was [median ± IQR] 19.7 ± 6.0 mg/kg/day. In some cases, the dosage of the treatment with Allopurinol was not mentioned in the file, in this case the dosage was calculated with the average weight of the patient during the period of treatment. In one case, xanthine oxalate urolithiasis was reported as side effect after 622 days (1.7 years) of treatment with Allopurinol.

The follow-up period was [median ± IQR] 1101 ± 435 days for the 17 dogs in this study. During this therapy period, the therapy was discontinued in nine dogs. The no treatment period (defined as no treatment with either allopurinol, milteforan or glucantime) was [median ± IQR] 195 ± 238.3 days. A no treatment period suggests that the therapy could be stopped because there were no more symptoms present. Conversely, restarting therapy suggests a relapse of the disease. However, the reviewed files showed that this was not always the case, in some cases (5) symptoms were also mentioned during a no treatment period. It can also be seen that in some cases the treatment was continued with lifelong administration of Allopurinol. In some cases, this is because an active disease persists, but in some cases, it could also be that the veterinarian continued the treatment fearing a relapse.

In five dogs the treatment was paused for a period of [median ± IQR] 186 ± 48 days. Treatment was resumed if there was, through clinical symptoms or laboratory abnormalities, an indication of relapse of CanL. In one dog there was no clear indication for resuming the treatment. Six dogs that were still alive at the last contact moment, did not receive any treatment for CanL anymore and did well clinically.

Milteforan

There were [median ± IQR] 12 ± 84 days between confirming the diagnosis of CanL and the first treatment with Milteforan.

Figure 3. First treatment with Milteforan

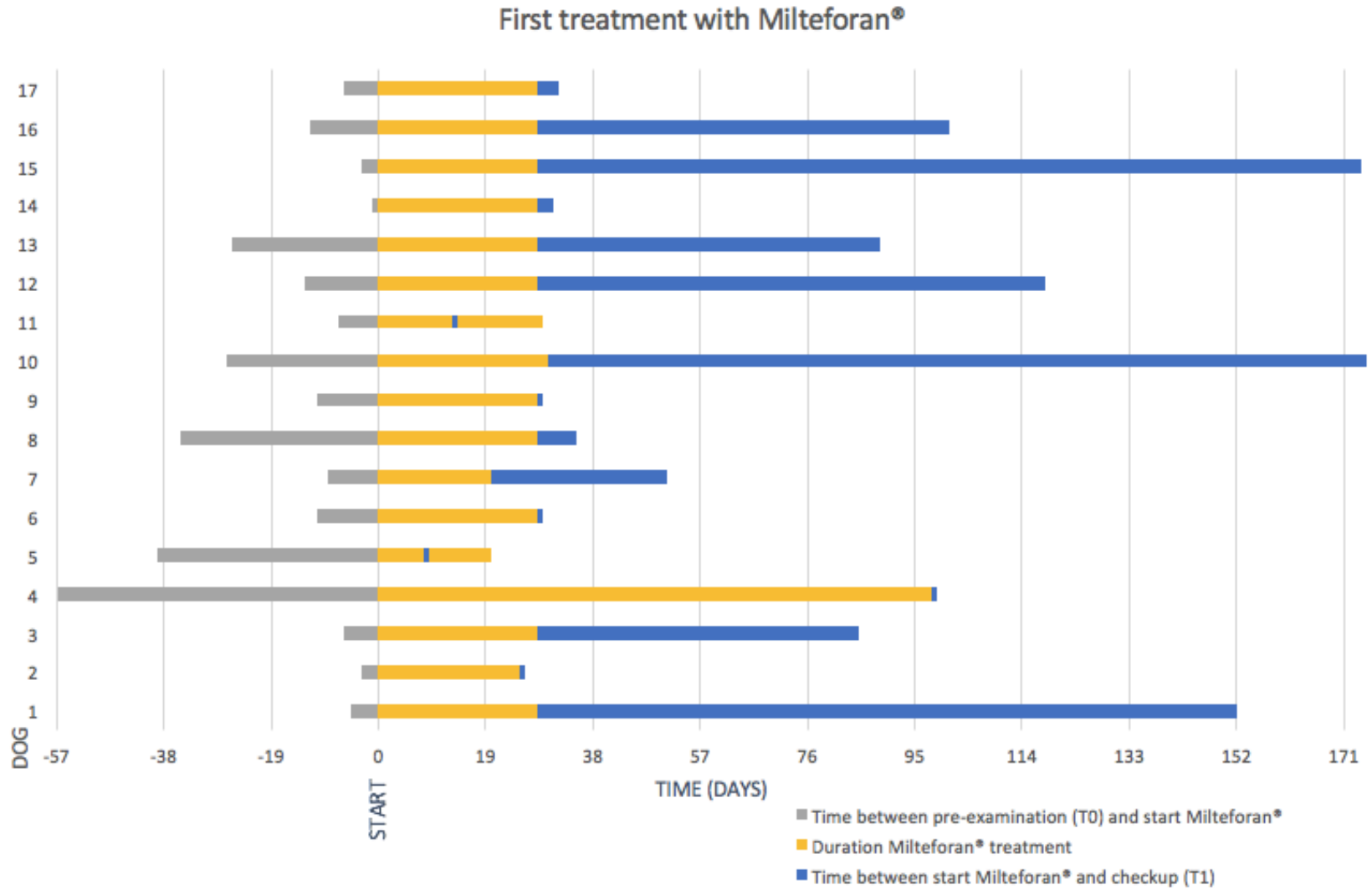


Figure 3 shows the duration of the first course of Milteforan treatment and both the time between pre-examination and start Milteforan (T0) and time between start Milteforan and the first check-up (T1).

Figure 3 shows the first treatment with Milteforan. The time between pre-examination and the start of treatment with Milteforan was [median ± IQR] 11 ± 20 days. A check-up was performed [median ± IQR] 51 ± 72 days after the start of treatment. In 5 dogs the therapy check-up was performed at the advised time-point of 3 months after start of therapy.

The range of duration of treatment with Milteforan was 19-98 days, with a median of 28 days. In 12 patients (70.6%), Milteforan was administered according to the user instructions in a 28-day course. For the other five patients, the duration of treatment was respectively, 19, 20, 25, 30 and 98 days. In one dog this was reported to be decided as a result of side-effects attributed to the Milteforan. The shortest duration of treatment was 19 days, due to anorexia and vomiting resulting in early cessation of Milteforan therapy. Mirtazapine therapy has been attempted to induce appetite in this dog, but this had no effect. After stopping Milteforan, the appetite improved. The longest duration of treatment was 98 days, in which Milteforan was administered continuously. The reason to continue the treatment for 98 days was because the symptoms (especially of the skin) had not completely disappeared. After the clinical symptoms had completely disappeared and the blood test showed no abnormalities, it was decided to stop Milteforan treatment.

All patients received a daily dose of 2,0 mg/kg/day, except for one patient who received a daily dose of 1.8 mg/kg/day.

Side effects were reported in three cases (17,7%), with vomiting reported in all three cases and anorexia in two of them. In two dogs, therapy with Maropitant (Cerenia) was started to prevent vomiting. Maropitant (Cerenia) was prescribed in the following dosage, 1.0-2.0 mg/kg/day and 1.3 mg/kg/day with a duration of respectively 11 and 2 days. In the other dog, vomiting resolved naturally after stopping the therapy with Milteforan.

To determine the efficacy of Milteforan, the clinical signs and clinicopathological abnormalities compatible with CanL at the time of pre-examination (T0) and time of check-up after treatment (T1) were compared.

The clinical signs at pre-examination (T0) and the check-up after treatment (T1) were compared with the McNemar's test. The results, including the return to normal values, are shown in Table 2.

Clinical sign	Present at T0		Present at T1		p-value	p-value[a] after Bonferroni correction[b]	Return to normal values at T1	
	N	%	N	%			N	%
General signs								
Lethargy	4	23.5	4	23.5	0.999	0.999	2	50.0
Anorexia	5	29.4	4	23.5	0.5637	0.999	0	0
Diarrhea	3	17.7	0	0	0.08326	0.999	3	100
Weight loss	3	17.7	0	0	0.08326	0.999	3	100
Vomiting	2	11.7	3	17.7	0.3173	0.999	0	0
Lymphadenomegaly	5	29.4	0	0	0.02535*	0.58305	5	100
Pale mucous membranes	3	17.7	0	0	0.08326	0.999	3	100
Muscle atrophy	1	5.9	0	0	0.3173	0.999	1	100
Polyuria and polydipsia	2	11.8	2	11.8	0.999	0.999	2	100
Splenomegaly	1	5.9	0	0	0.3173	0.999	1	100
Cutaneous signs								
General skin problems	9	52.9	7	41.2	0.3173	0.999	6	66.7
Scaling skin	6	35.3	3	17.7	0.1797	0.999	4	66.7
Scaling skin and crustae head (eyes, ears, nose)	7	41.2	4	23.5	0.1797	0.999	4	57.1
Erythema skin	4	23.5	1	5.9	0.08326	0.999	3	75.0
Hypotrichosis	3	17.7	1	5.9	0.1573	0.999	2	66.7
Alopecia	4	23.5	1	5.9	0.08326	0.999	3	75.0
Ulcerative dermatitis	1	5.9	0	0	0.3173	0.999	1	100
Long nails	2	11.8	0	0	0.1573	0.999	2	100
Other signs								
Epistaxis	1	5.9	1	5.9	NA	NA	0	0
Stiffness	1	5.9	1	5.9	0.999	0.999	1	100
Lameness	1	5.9	1	5.9	0.999	0.999	1	100
Overfilled joints	2	11.8	0	0	0.1573	0.999	2	100
Neurological signs	2	11.8	0	0	0.1573	0.999	2	100

Table 2. Evolution of clinical signs between the pre-examination (T0) and the check-up after treatment with Milteforan (T1).
[a]McNemar's test to compare presence of clinical signs between T0 and T1.
[b]Bonferroni correction was used because many tests of significance were performed within this study on the same study group. In total, the McNemar's test was applied to 23 different clinical signs. The probability that at least one out of 23 tests will be significant can be calculated by the following formula $1-(1-\alpha)^n$ where α is the level of significance of 0.05 and n is the number of tests. The probability that at least one finding will be significant with $\alpha = 0.05$ and n = 23 is 69,3%. Therefore, the Bonferroni correction was applied by multiplying the p-value by the number of tests[38].
**p <0.05*
NA Not applicable. Only one dog showed epistaxis at both T0 and T1. Because no change has occurred, the McNemar's test was not feasible.

After Bonferroni correction, there is no significant difference between clinical signs at T0 and T1. However, at the time of pre-examination all 17 dogs (100%) showed clinical signs but at the check-up after treatment 5 dogs (29,4%) had a complete recovery of clinical signs. Diarrhea, weight loss, lymphadenomegaly, pale mucous membranes, muscle atrophy, splenomegaly, ulcerative dermatitis, long nails, overfilled joints and neurological signs were no longer seen at the control moment and all dogs that did have these symptoms at T0 returned to normal. All dogs that showed polyuria and

polydipsia, stiffness and lameness at T0 became normal at T1, but some dogs that did not have these symptoms at T0 yet, developed it at T1. For anorexia, vomiting and epistaxis a 0% recovery rate was seen.

The presence of clinicopathological abnormalities before and after treatment with Milteforan are shown in table 3.

Clinicopathological abnormality	Present at T0		Present at T1		Return to normal values at T1	
	N	%	N	%	N	%
Hematologic parameters						
Non-regenerative anaemia	5/11	45.5	4/12	33.3	1	20.0
Increased MCV	1/11	9.1	2/12	16.7	0	0
Decreased MCHC	3/10	30.0	2/12	16.7	2	66.7
Leukopenia	2/11	18.2	2/13	15.4	0	0
Leukocytosis	2/11	18.2	0/13	0.0	2	100
Lymphocytopenia	3/12	25.0	1/12	8.3	2	66.7
Lymphocytosis	2/12	16.7	2/12	16.7	1	50.0
Thrombocytopenia	9/12	75.0	6/11	54.6	3	33.3
Biochemical parameters						
Hyperproteinaemia	9/15	60.0	3/11	27.3	5	55.6
Hypoalbuminemia	7/14	50.0	2/9	22.2	2	28.6
Hyperglobulinemia	12/14	85.7	4/9	44.4	4	33.3
Decreased A/G ratio	13/14	92.9	4/9	44.4	5	38.5
Increased Globulin β2	2/5	40.0	1/2	50.0	1	50.0
Hypergammaglobulinemia	5/5	100.0	1/3	33.3	1	20.0
Increased Urea	5/15	33.3	3/14	21.4	2	40.0
Increased Creatinine	5/16	31.3	3/15	20.0	2	40.0
Increased ALP	1/11	9.1	1/10	10.0	0	0
Increased ALT	2/11	18.2	0/10	0.0	0	0
Increased AST	3/7	42.9	0/5	0.0	0	0

Table 3. Presence of clinicopathological abnormalities at pre-examination (T0) and the check-up after treatment with Milteforan (T1). To determine an abnormality, the patient's own reference values were used.

All dogs with leukocytosis returned to normal at T1. The clinicopathological abnormalities decreased MCHC, lymphocytopenia, lymphocytosis, hyperproteinaemia and increased globulin β2 had a recovery rate of 50% or more.

The haematological and biochemical values between pre-examination (T0) and the check-up after treatment (T1) were compared with the Wilcoxon signed rank test. The results are shown in Table 4 and 5.

Parameter	Merged reference range		Median T0	Median T1	P-value	p-value after Bonferroni correction
Hematocrit	38.2-56.2	(%)	38.2	43.3	0.07556	0.999
MCV	59.9-76.2	(fl)	67.9	69.1	0.999	0.999
MCHC	31.5-36.7	(g/dL)	31.9	33.4	0.8336	0.999
MCH	19.5-25.1	(pg)	21.7	23.1	0.7792	0.999
Leukocytes	5.48-15.03	($x10^9$/L)	7.65	8.60	0.9102	0.999
Lymphocytes	0.97-4.54	($x10^9$/L)	2.29	3.05	0.375	0.999
Monocytes	0.09-1.05	($x10^9$/L)	0.62	0.54	0.4316	0.999
Segmented neutrophils	2,87-11.40	($x10^9$/L)	5.90	4.83	0.2969	0.999
Thrombocytes	152.8-479.3	($x10^9$/L)	128.5	148.0	0.07422	0.999
Eosinophils	0.04-1.06	($x10^9$/L)	0.16	0.31	0.9102	0.999

Table 4. Hematologic parameters
MCV mean corpuscular volume, MCHC mean corpuscular haemoglobin concentration, MCH mean corpuscular haemoglobin.
The haematology has been measured in different laboratories, therefore the reference ranges of these laboratories have been merged, the average lower reference value and the average upper reference value are displayed.

Parameter	Merged reference range		Median T0	Median T1	P-value	p-value after Bonferroni correction
Total protein	53-76	(G/L)	81	68	0.005077*	0.116771
Albumin	26-42	(G/L)	24.5	31.0	0.01403*	0.32269
Globulin spectrum	23-44	(G/L)	57.5	37.0	0.01277*	0.29371
Globulin $\beta1$	1.8-6.6	(G/L)	4.0	3.85	0.999	0.999
Globulin $\beta2$	5.1-13.0	(G/L)	12.9	12.1	0.999	0.999
Globulin γ	3.5-9.4	(G/L)	39.0	8.2	0.5	0.999
A/G ratio	<0.8	(G/L)	0.44	0.81	0.007812*	0.179676
Urea	2.7-9.5	(mmol/L)	6.7	7.0	0.06515	0.999
Creatinine	47-135	(μmol/L)	93	79	0.7148	0.999
ALP	21-159	(U/L)	55	31	0.293	0.999
ALT	11-119	(U/L)	31.0	35.5	0.3621	0.999
AST	14-159	(U/L)	42	26	0.375	0.999
GGT	1-13	(U/L)	2.0	3.5	0.05791	0.999

Table 5. Biochemical parameters
ALP Alkaline phosphatase, ALT Alanine aminotransferase, AST Aspartate aminotransferase, GGT Gamma-glutamyl transferase, A/G ratio Albumin/Globulin ratio.
The biochemical parameters have been measured in different laboratories, therefore the reference ranges of these laboratories have been merged, the average lower reference value and the average upper reference value are displayed.
**p<0.05*

After Bonferroni correction, there is no significant difference in median hematological and biochemical parameters between T0 and T1.

Survival

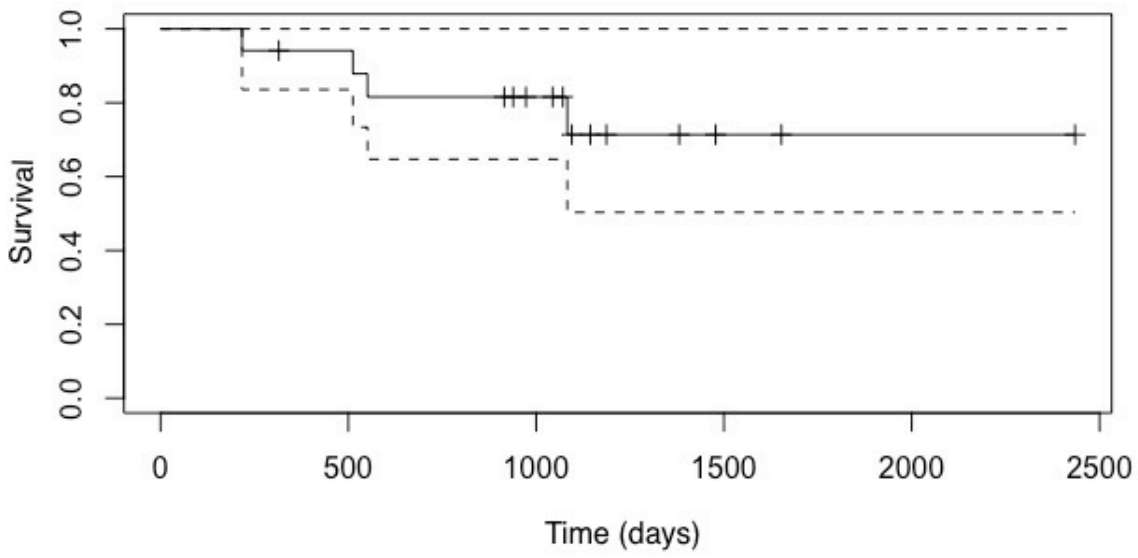


Figure 3. Overall survival in Kaplan-Meier curve. Hatch marks indicate censored dog.

Survival time was defined as time (in days) of confirmed diagnosis till time of death due to CanL (event). Dogs that were lost to follow up or were still alive at the last contact moment were censored. Four dogs died from CanL after respectively 217, 512, 551 and 1083 days after diagnosis. One dog was lost to follow up after 315 days. The remaining censored dogs were still alive at the last contact date, with a [median ± IQR; range] survival of 1120 ± 379 days. This means that in this study the Kaplan-Meier estimated survival time is three years after diagnosis of CanL.

Other treatment

In addition to the described therapy for CanL, some dogs received the following other medication for the following indications: (2) Fenylpropanolaminehydrochloride (Tensurin, Propalin) for urine incontinence, (3) Benazeprilhydrochloride (Benakor, Fortekor) for proteinuria, (3) Maropitant, Metoclopramide (Cerenia, Emeprid) against vomiting and nausea, (3) NSAIDs (Carprofen, Meloxicam) in lameness or other pain related complaints, (1) Doxycycline started awaiting the results of the Ehrlichia canis ELISA, (1) Mirtazapine to induce appetite, (1) Imaverol because a positive fungal culture was found and (1) sulfasalazine (Salazobactin) for an unknown indication. Assuming they have a different mechanism of action, it is not likely that these drugs have interfered with the Milteforan treatment.

Discussion

To determine the efficacy of Milteforan in the therapy of canine Leishmaniasis in first-line veterinary practice, the difference between clinical signs and clinicopathological (hematologic and biochemical) parameters at T0 and T1 were used as primary outcome parameters. In the evolution of clinical signs between T0 and T1, no significant difference was seen after Bonferroni correction. Nevertheless, 29.4% of the patients had a complete clinical recovery at T1. The clinical signs diarrhea, weight loss, lymphadenomegaly, pale mucous membranes, muscle atrophy, splenomegaly, ulcerative dermatitis, long nails, overfilled joints and neurological signs were no longer seen at the control moment and all dogs that did have these symptoms at T0 returned to normal. Additionally, diarrhea, weight loss,

lymphadenomegaly and pale mucous membranes showed a p-value <0.1 without Bonferroni correction. Therefore, these values may become significant in a follow-up study with a larger patient group. The improvement of clinicopathological abnormalities between T0 and T1 was, after Bonferroni correction, not significant either. Nevertheless, recovery rates of more than 50% were seen for the clinicopathological abnormalities leukocytosis, decreased MCHC, lymphocytopenia, lymphocytosis, hyperproteinaemia and increased globulin β2. Without Bonferroni, lymphadenomegaly, total protein, albumin, globulin and A/G ratio showed a significant difference between T0 and T1.

Several studies have investigated the (short- and long-term) efficacy, safety and use of miltefosine alone or in combination with allopurinol in dogs infected with CanL. Most studies have been performed prospectively with a comparable or larger patient group than this study. These studies examine the treatment with Milteforan or compare the Milteforan treatment, in combination with or without allopurinol, with allopurinol alone or allopurinol plus meglumine antimoniate. One study was conducted by the pharmaceutical company Virbac itself. Results of efficacy of miltefosine that emerge from these studies include progressive reduction of clinical signs and in some cases complete recovery, improvement of hematologic and biochemical parameters, a drastic reduction in parasite load in lymph node, skin and bone marrow samples and reduction of infectivity evaluated by xenodiagnoses[31–33,35,37,39,40]. Despite the progressive reduction, elimination or clearance of the parasite in lymph node aspirates and spleen and bone marrow smears was not seen after treatment[33,39,40]. In addition, miltefosine appeared more efficacious in vitro in combination with allopurinol than alone[34]. In vivo, the safety of the combination of miltefosine and allopurinol was confirmed by lack of effect on renal and hepatic parameters and other side effects[37].

In this study, an overall Kaplan-Meier survival time of three years is promising. It is known that treatment is associated with a longer survival, unpublished data of the faculty of Veterinary Medicine, Utrecht University showed a median survival of 6.4 years and Geisweid et al. (2012) found a significant survival benefit from anti-leishmanial treatment as well[41]. Survival of dogs that received a treatment with allopurinol alone or in combination with meglumine antimoniate has been investigated[9,41]. A 75% probability of survival for more than 4 years following treatment with Glucantime (100 mg/kg, IV or SC, for 3-6 weeks) was found in the Netherlands in dogs with CanL without serious renal insufficiency[42]. In dogs treated with allopurinol alone (20 mg/kg/day, for 6 months), a 78% chance of survival for more than 6 years was found[43]. Geisweid et al. (2012) showed that presence of proteinuria, hypoalbuminemia, renal azotaemia and lymphopenia was significantly correlated to survival time, and therefore could be useful parameters for predicting the survival of infected dogs. A study examining the survival and prognostic factors of a patient group treated with miltefosine alone or in combination with allopurinol is to the writer's knowledge not yet been carried out. Due to missing data and a small patient group in this study, prognostic factors for survival could not be determined. In follow-up research a multivariate analysis (e.g. cox regression proportional hazards model) should be performed for a better understanding of the prognostic value of the viewed parameters and their interaction. It would also be very useful to compare the survival and prognostic factors of patient groups receiving different treatments for CanL.

Various side effects due to Milteforan have been described in previous studies, in particular gastro-intestinal signs[4,31,32]. For vomiting and anorexia, a 0% recovery rate was seen. In the three cases were these side effects were mentioned in this study, the check-up examination (T1) was during or immediately after the end of treatment. Thus, it is unclear whether these symptoms are actually side effects of Milteforan or are caused by the ongoing progress of CanL. In one patient, Milteforan treatment had to be stopped earlier than the 28-course, because of the side-effects. This is in contrast to the mild side effects mentioned in the technical product profile of Virbac, in which it was stated that the side effects did not affect the efficacy of the treatment[44]. Finally, reporting of side-

effects in this study was subject to reporting bias since if side effects are not mentioned in the file, this does not necessarily mean that no side effects have occurred.

As shown in figure 2, a few patients have been treated with a second cycle of Milteforan. It would be interesting to examine the efficacy of a second cycle to review the difference between the group with one and the group with two cycles of Milteforan treatment. Manna et al. (2009) showed for example a major reduction of parasitic load of L. Infantum in lymph node aspirates after the first cycle of treatment with miltefosine in combination with Allopurinol, while after a second cycle the parasite was not completely eliminated from the lymph node aspirates[40]. Unfortunately, there were too few patients in this study group treated with a second cycle and furthermore there was too much difference in time between pre-examination before therapy and check-up afterwards, so they could not be properly compared. In future research this would be useful to implement and evaluate. In addition, it would be useful to review the difference in therapy efficacy between dogs presenting for the first time with CanL and dogs presenting with recurrent disease. Woerly et al. (2009) showed that both dogs with first time and recurrent disease responded almost just as well to Milteforan treatment[31].

The statistical methods performed can be discussed. The Bonferroni correction was applied, since multiple analysis were executed on the same sample. Opinions on the necessity of the Bonferroni correction are divided and it is not clear whether the correction should have been applied in this case[38]. It can be that the Bonferroni correction is too strict and is a limitation to this study, because without correction some values would be statistically significant. Hence, it is important to keep this in mind when evaluating the results. For the statistical analysis of reduction of clinical signs, the McNemar's test without Yates continuity correction was performed, since the sample size was very small[45,46]. Because the included dogs were treated by different practices, the blood tests were performed in different laboratories, each with their own reference values. The parameters compared in tables 4 and 5 are therefore not entirely the same. An option to equalise this is to categorize the parameters based on severity, for example (0) no deviation (1) mild (2) moderate (3) severe abnormalities. However, by doing so, the data was no longer continuous and therefore the Wilcoxon signed rank test could not be applied. On this categorical data a McNemar's test could be performed, but essential information would be lost. Because the reference values were not very far apart, it was decided not to apply any correction.

This study also provides an overview of the prescription and use of Milteforan in first-line veterinary practice in the Netherlands. Since there is no clear-cut guideline or protocol for the treatment of CanL, it is possible that the Dutch veterinarian has difficulty treating CanL patients. It appears that the guidelines formulated by the faculty of Veterinary Medicine from Utrecht University are frequently used, because they were often included in the viewed files[47]. Figure 2 has shown that there is a lot of difference in the approach and therapy of CanL patients. Therefore, it is unclear whether the currently available information is sufficient enough for the treating veterinarian. There may be need for more information and perhaps this should be provided by the faculty. It would be helpful to investigate the need of the Dutch veterinarian through a questionnaire in future research, because dogs diagnosed with CanL are likely to increase in the Netherlands and other non-endemic countries, since the number of imported dogs is already increasing over the years[48]. This increasing group can possibly form a reservoir which is a risk if future climate conditions will cause the sand-fly to move to northern non-endemic areas[7,49]. In addition, it is important to prevent misuse of Milteforan, because it can cause widespread resistance of the parasite in the future, making the therapy of CanL even more difficult[50].

It is notable that it was often seen that the dosage of the therapy with either Allopurinol or Milteforan was calculated on the basis of the current weight of the dog, where after the dosage was

not adjusted if the weight changed. It is not clear in which cases the continuation of allopurinol has really been necessary. In the results it appears that veterinarians have difficulty to determine when to discontinue Allopurinol treatment. This was to be expected, because the moment of discontinuation of allopurinol is not well defined. Solano-Gallego et al. (2009) have attempted to better define it by setting the following criteria: (1) complete recovery of clinical and clinicopathological abnormalities assessed during a full physical examination including blood and urine laboratory tests along with (2) negative or borderline antibody levels measured by a quantitative serological assay[4]. On the other hand, Ginel et al. (1998) concluded that intermittent administration of allopurinol could indeed be used for maintenance of remission. Allopurinol was effective in delaying a clinical relapse in dogs that had received initial therapy with meglumine antimonate and allopurinol, compared to a control group which only received the initial therapy[51].

Other discussion points that emerged during this research project are the following. The daily doses prescribed in the patient files have been used, however it is not checked if the owner actually administered this dose and so therapy compliance can be an unknown weakness of this study. Not all dogs have been tested for other CVBD, so it cannot be said with certainty that for dogs that have not been tested no other CVBD played a role in the clinical picture. In a follow-up study, the negative testing of other CVBD could be an inclusion criterion. For this research project, the patient files were reviewed by one investigator, which may cause the interpretation of the data from the patient files to be incorrect and is a risk for observer bias.

Despite the fact that no significant difference between before and after treatment with Milteforan was found, some parameters (clinical, hematologic and biochemical) did show improvement and thus gives a tentative indication of efficacy of Milteforan in the treatment of CanL. A follow-up study with a larger patient group to confirm the results and conclusion of this study is recommended. This study was conducted on a cohort without a control group. It is therefore difficult to say whether the outcome is due to the treatment with Milteforan and what the biological course and outcome without intervention would have been in this population. For instance, if the disease is self-limiting in a large proportion of these dogs, the 3-year survival found in this study can give a distorted picture for the efficacy of Milteforan. That is why, to be able to be more certain about the efficacy of Milteforan in the treatment of CanL, a prospective, randomized placebo-controlled follow-up study is necessary. For example, a study design could be the comparison of a cohort of dogs treated with allopurinol alone versus a cohort treated with allopurinol in combination with Milteforan.

Ideally, with a possible larger patient group, groups with check-ups after treatment at 1 month, 3 months and 6 months should be compared to provide more insight into the short- and long-term efficacy of Milteforan. An advice on the best time for a check-up after Milteforan treatment can emerge from this comparison and can save costs for the owner. In addition, survival analysis can be expanded when observing and combining data from multiple years at which prognostic factors for survival can be analysed as well. Finally, it would be useful to extend this research to a larger overview of the treatment of CanL in the Netherlands as non-endemic country, by comparing patient groups that have received different treatments.

Conclusion

A promising Kaplan-Meier estimated survival time of three years was found. In conclusion, 29.4% of the patients had a complete clinical recovery after treatment. Diarrhea, weight loss, lymphadenomegaly, pale mucous membranes, muscle atrophy, splenomegaly, ulcerative dermatitis, long nails, overfilled joints, neurological signs and leucocytosis showed a complete recovery after Milteforan treatment. No significant difference of clinical signs and clinicopathological abnormalities were observed after Milteforan treatment. However, this may be due to Bonferroni correction and

small group size. Nevertheless, without Bonferroni, lymphadenomegaly, total protein, albumin, globulin and A/G ratio showed a significant difference between T0 and T1. A follow-up study with a larger patient group to investigate or confirm the results and conclusion of this study is recommended. Despite the various therapeutic options and available guidelines, CanL persists to be a difficult and challenging disease to effectively treat and cure. Consequently, more information or education should be provided to the first-line veterinary practice, which will be even more necessary if the number of imported dogs increases in the future.

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Abbreviations

ALP	Alkaline phosphatase	GGT	Gamma-glutamyl transferase
ALT	Alanine aminotransferase	IFT	Immunofluorescence antibody Test
AST	Aspartate aminotransferase	IQR	Interquartile range
A/G ratio	Albumin/Globulin ratio	NA	Not available
CanL	Canine Leishmaniasis	NR	Number
CVBD	Canine Vector-Borne Disease	SD	Standard Deviation
DAT	Direct Agglutination Test	UVDL	Universitair Veterinair Diagnostisch Laboratorium
ELISA	Enzyme Linked Immunosorbent Assay		

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