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ANIMALES

Nº 1

- Meglumine Antimoniate and Miltefosine Combined With Allopurinol Sustain Pro-inflammatory Immune Environments During Canine Leishmaniosis Treatment

- Polyprenyl Immunostimulant Treatment of Cats with Presumptive Non-Effusive Feline Infectious Peritonitis In a Field Study

SUMARIO

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Meglumine Antimoniate and Miltefosine Combined With Allopurinol Sustain Pro-inflammatory Immune Environments During Canine Leishmaniosis Treatment

El antimoniato de meglumine y la miltefosina combinados con alopurinol para mantener entornos inmunes proinflamatorios durante el tratamiento de la leishmaniosis canina

Palabras clave: leishmaniosis canina, células mononucleares de sangre periférica, ganglio linfático, médula ósea, expresión génica de citoquinas, antimoniato de meglumina, miltefosina, alopurinol

Keywords: *canine leishmaniosis, peripheral blood mononuclear cells, lymph node, bone marrow, cytokine gene expression, meglumine antimoniate, miltefosine, allopurinol*

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La leishmaniosis canina (CanL) causada por Leishmania infantum es una enfermedad zoonótica de preocupación mundial. Las terapias farmacológicas antileishmaniales comúnmente utilizadas para tratar a los perros enfermos mejoran su condición clínica, aunque cuando se suspende pueden producirse recaídas. Por lo tanto, el estudio actual tiene como objetivo evaluar el efecto de los tratamientos con CanL en el perfil de citoquinas de sangre periférica, ganglios linfáticos y médula ósea asociados con la recuperación clínica.

Canine leishmaniosis (CanL) caused by Leishmania infantum is a zoonotic disease of global concern. Antileishmanial drug therapies commonly used to treat sick dogs improve their clinical condition, although when discontinued relapses can occur. Thus, the current study aims to evaluate the effect of CanL treatments in peripheral blood, lymph node, and bone marrow cytokine profile associated with clinical recovery. Two groups of six dogs diagnosed with CanL were treated with miltefosine combined with allopurinol and meglumine antimoniate combined with allopurinol (MT+A and MG+A), respectively. At diagnosis and after treatment, during a 3-month follow-up, clinical signs, hematological and biochemical parameters, urinalysis results and antileishmanial antibody titers were registered. Furthermore, peripheral blood, popliteal lymph node, and bone marrow samples were collected to assess the gene expression of IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β, and IFN-γ by qPCR. In parallel, were also evaluated samples obtained from five healthy dogs. Both treatment protocols promoted the remission of clinical signs as well as normalization of hematological and biochemical parameters and urinalysis values. Antileishmanial antibodies returned to non-significant titers in all dogs. Sick dogs showed a generalized upregulation of IFN-γ and downregulation of IL-2, IL-4, and TGF-β, while gene expression of IL-12, TNF-α, IL-5, and IL-10 varied between groups and according to evaluated tissue. A trend to the normalization of cytokine gene expression was induced by both miltefosine and meglumine antimoniate combined therapies. However, IFN-γ gene expression was still up-regulated in the three evaluated tissues. Furthermore, the effect of treatment in the gene expression of cytokines that were not significantly changed by infection, indicates that miltefosine and meglumine antimoniate combined therapy directly affects cytokine generation. Both combined therapies are effective in CanL treatment, leading to sustained pro-inflammatory immune environments that can

Abbreviations: *ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BID, bis in die; BUN, Blood urea nitrogen; CanL, Canine leishmaniosis; cDNA, Complementary DNA; CG, Control Group; CLWG, Canine Leishmaniasis Working Group; CPDA-1, Citrate phosphate dextrose adenine; CVBD, Canine vector-borne disease; DNA, Deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; IL, Interleukin; IFAT, Indirect Fluorescent Antibody Test; IFN-γ, Interferon gamma; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; MG+A, Meglumine antimoniate with Allopurinol; MPV, Mean platelet volume; MT+A, Miltefosine with Allopurinol; PBMC, Peripheral Blood Mononuclear Cell; PCA, Principal Component Analysis; PDW, Platelet distribution width; qPCR, Real-time PCR; RDW, Red cell distribution width; RNA, Ribonucleic acid; SID, semel in die; Th1, Type-1 T-Helper; Th2, Type-2 T-Helper; TGF-β, Transforming growth factor beta; TNF-α, Tumor necrosis factor alpha; Treg, Regulatory T-cells; UPC, Urine Protein Creatinine ratio.*

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compromise parasite survival and favor dogs' clinical cure. In the current study, anti-inflammatory and regulatory cytokines do not seem to play a prominent role in CanL or during clinical recovery.

Introduction

Leishmaniosis constitute a group of parasitic diseases of worldwide concern, that are considered by the World Health Organization as neglected tropical diseases[1]. Canine leishmaniosis (CanL) caused by the intracellular protozoan Leishmania infantum is a zoonotic disease endemic to several southern European countries, including Portugal. In CanL, a wide range of non-specific clinical signs can be present[2], posing difficulties to a correct diagnosis. Previous studies differentiated sick dogs into symptomatic, oligosymptomatic and polysymptomatic[3–6] although more recently it has been proposed an improved system to stage dog's clinical condition[7,8]. This classification system takes into account the physical examination, clinicopathological abnormalities, anti-Leishmania antibody titer, and the evaluation of renal function according to the International Renal Interest Society guidelines[9]. Other proposals also consider a first stage of exposed dogs as those living or that have lived in geographic regions in which the presence of vectors has been confirmed[10]. CanL conventional treatments improve the dog's clinical condition, reducing skin parasite load and consequently the risk of Leishmania transmission. Although it is not definitively proved that treatment completely eliminates the parasite[11], and relapses are common when therapy is discontinued[3,11,12] it remains crucial to improve the efficiency of protocols used for CanL treatment. The main protocols for dog treatment usually include meglumine antimoniate (N-methylglucamine antimoniate), miltefosine (1-O-hexadecylphosphocholine), and allopurinol. Meglumine antimoniate is a pentavalent antimonial-based drug whose precise mechanism of action is not yet well-understood, but being considered a multifactorial drug with probable activity on parasite molecular processes, and influence in macrophage microbicide activity[13,14]. Miltefosine is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely clear[15–18]. Allopurinol is a purine analog of adenosine nucleotide, which blocks RNA synthesis, inhibiting Leishmania growth[19]. Up to date, meglumine antimoniate in combination with allopurinol is considered the first line of treatment in Europe[2], while miltefosine plus allopurinol has been the second line of treatment. However, miltefosine therapy has been gaining more attention[3–6], being recently authorized in 2017 for CanL treatment in Brazil[20], a highly endemic country for both canine and human leishmaniosis. Nevertheless, with the arising of more reports of drug resistance that lead to either therapeutic failure, unresponsiveness or relapse, whether it be in humans or dogs, a deeper understanding of the usual therapies is imperative[13,15,21,22].

The immune response of dogs evidencing leishmaniosis clinical signs has been usually characterized by higher levels of specific antibodies, along with a type-2 T-helper (Th2) response associated with the expression of interleukin (IL)-4, IL-5, and IL-6[23–25]. On the contrary, protective immunity is thought to be dependent on a strong type-1 T-helper (Th1) response characterized by IL-2, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ production[23,25]. Furthermore, parasites may suppress host immunity by engaging regulatory T-cells (Treg) thus enabling the persistence of the infection[26], with one study showing clearance of Leishmania infection after depletion of Treg populations in mice[27]. Moreover, higher expression of regulatory cytokines (IL-10, tumor growth factor [TGF-β]) associated with high parasite burden observed in dogs presenting clinical signs[28] suggest a non-negligible role of these cytokines in disease progression. To the best of our knowledge, there is no study defining the ideal approach to

CanL treatment based on the knowledge of the immune response elicited by the different treatment protocols, and there is only one study analyzing more than one parasite target organ in non-treated CanL[29]. Therefore, further studies are essential to clarify how treatments affect dogs' ability to develop a protective immune response or, on the contrary, to elicit immune suppression of effector cells. In the present study, the influence of two different treatment protocols on disease evolution of naturally infected dogs and on immune response was evaluated by assessing the clinicopathological changes, and the gene expression of pro-inflammatory (IL-2, IL-12, TNF-α, IFN-γ), anti-inflammatory (IL-4, IL-5) and regulatory (IL-10, TGF-β) cytokines in blood, popliteal lymph node and bone marrow during a 3-month period.

Materials and Methods

Dog Selection

Twenty-three dogs with at least 1.5 years of age, weighing more than 5 kg, not having been vaccinated for CanL and diagnosed with CanL clinical stage I/II, according to the LeishVet Consensus Guidelines[7], and stage C in agreement to the Canine Leishmaniosis Working Group (CLWG) Guidelines[10] were selected from a total of 170 household dogs living in the zoonotic visceral leishmaniosis endemic area of the Metropolitan Area of Lisbon (Portugal). Twelve of those 23 dogs had not undergone any treatment in the last 8 months that could interfere with the immune response (such as antibiotic and corticosteroid therapy or administration of immunomodulators), and were negative for circulating pathogens potentially responsible of canine vector-borne diseases (CVBDs), were selected to partic-

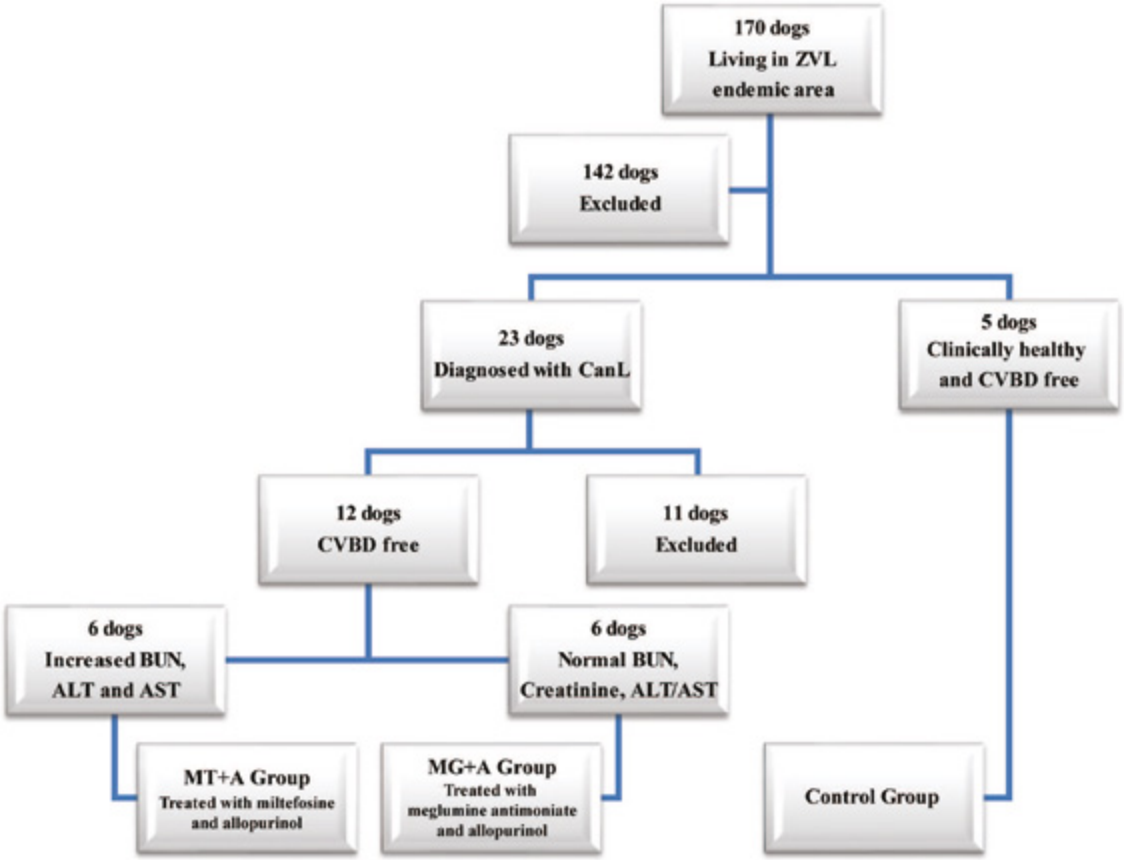

FIGURE 1. *Flowchart representing the dog's selection process used in the current study. From a total of 170 dogs, living in an endemic area of zoonotic visceral leishmaniosis, two groups of dogs with canine leishmaniosis (CanL) were constituted, and were treated with either miltefosine in combination with allopurinol (MT+A) or with meglumine antimoniate in association with allopurinol (MG+A) along with one group of clinically healthy dogs (Control Group–CG). These dogs were negative for Canine Vector-Borne Diseases (CVBD). ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood urea nitrogen.*

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ipate in the current study. Five clinical healthy dogs not having been vaccinated for CanL, negative for Leishmania antibody test and CVBDs were also included in the present study as a control group (**Figure 1**). All dog owners gave written consent after being informed about the objectives of the study and every procedure, ensuring that clinical results were made available. Selected dogs include 13 males and 4 females of various breeds with ages ranging between 2 and 9 years and weight between 7.6 and 32.1 kg. Animal handling and sample collection procedures were done by the Veterinary team of the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (Lisbon, Portugal). The present study followed the Council of the European Union Directive 86/609/EEC and was approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine, University of Lisbon.

Experimental Design

To reduce discomfort and ensure dog's well-being, the amount of sample collections and its periodicity were reduced to a minimum. Blood, popliteal lymph node, and bone marrow samples were collected from healthy (control group) and sick dogs prior the onset of treatment (Tp0) and then from sick dogs, 1 (Tp1), 2 (Tp2), and 3 months (Tp3) after the beginning of treatment.

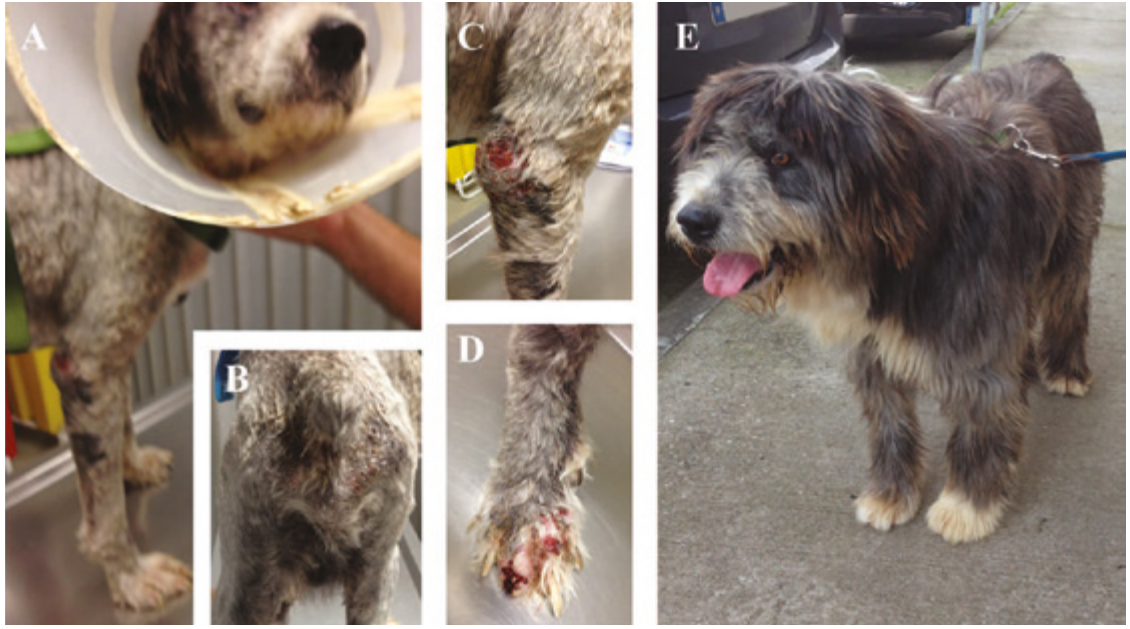

FIGURE 2. *Clinical manifestations of a dog naturally infected with Leishmania infantum. (**A,B**) Dog presenting evident loss of weight, lethargy, cutaneous alopecia, and exfoliative dermatitis; (**C**) Ulcerative and hyperkeratosis lesions in the elbow of the front limb; (**D**) Onychogryphosis with severe bleeding; (**E**) Dog from the MT+A group 6 months after the diagnosis with full remission of clinical signs. Photos by Marcos Santos.*

The samples collected from sick dogs at Tp0 were used, not only, to establish the baseline levels of cytokine mRNA accumulation, but also, for ethical reasons, to serve as controls of themselves, avoiding the need of an extra group of sick animals without any treatment. Treatment success was clinically and serological re-assessed 6 months after the initial diagnosis for each treated animal (**Figure 2**). Each dog was enrolled in one of the two treatment protocols (**Figure 1**), according to the following criteria:

- (i) Dogs presenting increased blood urea nitrogen (BUN), creatinine and/or alanine aminotransferase (ALT), aspartate aminotransferase (AST), and UCP between 0.2 and 0.6, pointing to the possibility of developing hepatic and renal lesion were treated with miltefosine (Milteforan®, Virbac S.A, France; 2 mg/kg per os, semel in die–SID–for 4 weeks) in association with allopurinol (Zyloric®, Laboratórios Vitória, Portugal; 10 mg/kg, per os, bis in die–BID–for at least 6 months) (MT+A);
- (ii) Dogs presenting changes in biochemical and hematological param-

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eters, serum proteins and UCP between 0.2 and 0.4 were treated with meglumine antimoniate (Glucantime®, Merial Portuguesa, Portugal; 100 mg/kg SID for 4 weeks) in association with allopurinol 10 mg/kg, per os, BID for at least 6 months (MG+A).

Deltamethrin-impregnated collars were applied to all dogs to prevent infections or re-infections during the current study and also in order to avoid Leishmania dissemination to sand flies. Blood samples were used for determination of hematological and biochemical parameters, and serological and molecular tests. Popliteal lymph node, bone marrow, and peripheral blood were used to examine cytokine gene expression. Urine samples were collected into sterilized containers for urinalysis and determination of protein/creatinine ratio (UPC).

Sample Collection, Hematological and Biochemical Analysis, and Serological Tests

Peripheral blood (20 ml) was collected into syringes containing citrate phosphate dextrose adenine (CPDA-1, Medinfar Sorológico, Portugal). Popliteal lymph node aspirates were collected into syringes containing 0.8 ml of saline solution (0.9% NaCl) in order to avoid cell disruption and were then transferred to ethylenediaminetetraacetic acid (EDTA) tubes to avoid coagulation. After cutaneous anesthesia with a Xylocaine 10% Pump Spray (AstraZeneca, UK), bone marrow aspirates were collected from the distal area of the costal ribs, between the 9th and the 11th, into syringes containing 0.8 ml of saline solution. An additional 4 ml of blood was collected in EDTA tubes and dry tubes to be used for hematological (complete blood count), biochemical analysis (serum measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, bilirubin, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium, chlorides), serum proteinogram electrophoresis, and CVBD screening. Peripheral blood samples were also used for the isolation of mononuclear cells. Popliteal lymph node and bone marrow were used for detection of Leishmania amastigote forms and isolation of mononuclear cells.

Leishmania Screening

Serum samples were used for detection of anti-Leishmania antibodies by IFAT assay (Leishmania-Spot IF, BioMérieux, France) using L. infantum promastigotes as antigen and following the manufacturer's instructions. Samples were screened using an Olympus DP10 microscope (model BX50F, wavelength of 425 nm) and classified as positive if fluorescence was observed in promastigote cytoplasm or membrane at a serum dilution of 1:80 or higher. According to LeishVet[7] and the Canine Leishmaniosis Working Group (CLWG) guidelines[10], IFAT is a gold standard test for canine leishmaniosis and to evaluate possible relapses.

To test for the presence of Leishmania DNA, total genomic DNA was extracted from 200 µl of peripheral blood using the DNeasy® Blood and Tissue kit (QIAGEN®, Germany) according to the manufacturer's instructions. DNA amplification by qPCR was done in a total volume of 20 µl, comprising 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems™, USA), 2 µl of ultra-pure water (Merck Millipore™ KGaA, Germany), 300 nM of forward and reverse primers for each set as well as 250 nM for each probe (**Table 1**) and 2 µl of target DNA. Reactions were carried out using the 7300 Real-Time PCR thermal cycler (Applied Biosystems™),

Target	Oligo	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
Leishmania (Kinetoplast)	Frw	GGAAGGTGTCGTAAATTCTGGAA	124	(30)
	Rev	CGGGATTTCTGCACCCATT		
	Probe[a]	AATTCCAAACTTTTCTGGTCCTCCGGGTAG		
Ehrlichia and *Anaplasma* (16S rRNA)	Frw	ACCTATAGAAGAAGTCCCGGCAA	100	(31)
	Rev	ACCTACGTGCCCTTTACGCCC		
	Probe[a]	GCAGCCGCGGTAATACGGAGGGGGC		
Babesia (18S rRNA)	Frw	ACCCATCAGCTTGACGGTAGGGT	97	(32)
	Rev	AGCCGTCTCTCAGGCTCCCT		
	Probe[b]	ACCGAGGCAGCAACGGGTAACGGGGA		
Rickettsia (OmpA)	Frw	AACCGCAGCGATAATGCTGAGTAGT	130	(33)
	Rev	CCCTGCAGAAGTTATCTCATTCCAA		
	Probe[b]	AGCGGGGCACTCGGTGTTGCTGCA		

TABLE 1. *Primers and TaqMan probes used for hemoparasite screening.*

with the following cycling conditions: 10 min at 95°C for AmpliTaq® Gold activation, followed by a total of 40 cycles of 15 s at 95°C and 1 min at 60°C. The positive control was constructed by cloning PCR fragments generated by the same primers into a pGEM®-T Easy Vector (Promega, USA), according to the manufacturer's instructions. Ligated fragments were transformed into JM109 competent cells and plasmid DNA was prepared using the QIAprep® Spin Miniprep Kit (QIAGEN®). The insert was sequenced using primers pUC/M13 (Promega) to ensure transformation stability. To exclude the presence of Leishmania amastigotes, lymph node and bone marrow slides were stained with Giemsa and observed by optical microscopy (Microscope Olympus CX31, using a 1,000× magnification).

CVBD Exclusion

Detection of antibodies against Babesia, Anaplasma, Ehrlichia, and Rickettsia was performed using commercial diagnostic tests (Megacor® MegaScreen, Austria, FLUOBABESIA canis–cut off 1:32; FLUOANAPLASMA phagocytophilum–cut off 1:50; FLUOEHRLICHIA canis–cut off 1:50; FLUORICKETTSIA conorii–cut off 1:40). The absence of Babesia, Anaplasma, Ehrlichia, and Rickettsia DNA was also evaluated by qPCR (**Table 1**) as previously described. To exclude the presence of Dirofilaria immitis microfilaria, blood samples were evaluated by Knott technique and parasite antigens were assessed by Witness® Dirofilaria kit (Zoetis, Portugal) according to the manufacturer's instructions.

Cell Isolation

Dog peripheral blood was re-suspended in PBS (1:1 v/v), overlaid onto a 1:2 Histopaque®-1077 solution (Sigma-Aldrich, Germany) and centrifuged at 400 g for 30 min at 18°C. Mononuclear cells were harvested and washed in cold PBS (300 g, 10 min, 4°C), re-suspended in PBS, and the total volume adjusted to 2 × 107 cells ml−1. Lymph node and bone marrow aspirates were centrifuged at 400 g (4°C) for 5 and 15 min, respectively, and re-suspended in 100 µl, with the total volume also adjusted for 2 × 107 cells.ml−1. Then, 200 µl of peripheral blood mononuclear cells (PBMCs) and 100 µl of lymph node and bone marrow cell suspensions were centrifuged at 400 g (4°C) for 5 min, re-suspended in 600 µl of RLT Buffer (QIAGEN®) supplemented with β-mercaptoethanol and stored at −80°C until further use.

mRNA Extraction and Reverse Transcription

Total RNA extracted from PBMCs, lymph node, and bone marrow cells, using RNeasy® Mini Kit (QIAGEN®) and QIAshredder® spin columns (QIAGEN®) was treated with DNase I Amplification Grade (Invitrogen™, USA) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of purified RNA, presenting a 260/280 absorbance ratio

ranging between 1.9 and 2.1 was denatured at 65°C for 5 min and reverse transcribed at 37°C for 60 min in a 30 µl final reaction mixture containing 6 µl of 5× M-MLV RT Buffer (Promega), 200 U/µl SCRIPT Reverse Transcriptase enzyme (Jena Bioscience, Germany), 500 µl dNTP Mix (Jena Bioscience), 1 µl of Oligo(dT)18 primers (Thermo Fisher Scientific Inc.™, EU), and 40 U/µl RiboLock RNase Inhibitor (Thermo Fisher Scientific Inc.™). cDNA samples were then heated at 95°C for 10 min for enzyme inactivation and stored at −20°C until further use.

Cytokine Gene Expression

To evaluate the effect of treatment in pro-inflammatory, anti-inflammatory, and regulatory cytokines, the accumulation of mRNA encoding for IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β, and IFN-γ was assessed by qPCR in PBMC, lymph node and bone marrow cell. cDNA amplification was conducted in a 20 µl final reaction mixture containing 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems™), 80 nM of forward and reverse primers for each cytokine and for housekeeping gene β-actin (**Table 2**), 4 µl of ultra-pure water (Merck Millipore™ KGaA) and 2 µl of canine cDNA. Each sample amplification was performed in triplicate, using the following conditions: 10 min at 95°C for AmpliTaq® Gold activation followed by a total of 40 cycles (thermal profile for each cycle: 15 s at 95°C, 1 min at 60°C). An extra dissociation step was added to confirm the specificity of amplification by melting point analysis, and absence of non-specific products. External cDNA standards for all target cytokines and internal control used in every reaction were constructed as previously described. The concentration of standards was determined by measuring the OD at 260 nm followed by calculation of the corresponding copy number, and serial dilutions of resulting clones were used as standard curves, each containing a known amount of input copy number[39,40]. Copy numbers of target genes were normalized to the housekeeping gene β-actin, therefore correcting for minor variations in mRNA isolation and reverse transcription. Final results were expressed as the copy number of each cytokine per 1,000 copies of the housekeeping gene. Amplification efficiencies were >90%.

Data Analysis

An exploratory multivariate statistical analysis, specifically the Principal Component Analysis (PCA), was performed per tissue, on all datasets, in order to identify principal components accounting for the majority of the variation and graphically assess the separation between the healthy control, sick (Tp0) and treated dogs (Tp1, Tp2 and Tp3). This statistical analysis was performed using JMP version 14.3.0 (SAS Institute). Likewise, a K-Means Cluster analysis was also used to complement the previous PCA analysis and confirm grouping separation. In order to reduce the number of irrelevant or redundant variables and present a more robust model, a feature selection method was employed. Using the Predictor Screening tool from JMP the individual contribution of each variable was obtained, and the selected features were considered in the final models.

Statistical analysis between treatment groups was performed using GraphPad Prism software package version 8.0.1. Data normality was assessed using the Kolmogorov-Smirnoff test. Wilcoxon signed rank test was used to compare hematological and biochemical results in each dog treatment group between Tp0 and Tp3, with differences being considered significant when $p < 0.05$. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used to evaluate differences in mRNA levels between treatment groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used to compare dogs at different time-points.

Target	Oligo	Oligonucleotide sequence (5′→ 3′)	Product size (bp)	References
IL-2	Frw	GCATCGCACTGACGCTTGTA	86	(34)
	Rev	TTGCTCCATCTGTTGCTCTGTT		
IL-4	Frw	CATCCTCACAGCGAGAAACG	83	(35)
	Rev	CCTTATCGCTTGTGTTCTTTGGA		
IL-5	Frw	GCCTATGTTTCTGCCTTTGC	106	(36)
	Rev	GGTTCCCATCGCCTATCA		
IL-10	Frw	CAAGCCCTGTCGGAGATGAT	78	(37)
	Rev	CTTGATGTCTGGGTCGTGGTT		
IL-12p40	Frw	CAGCAGAGAGGGTCAGAGTGG	109	(34)
	Rev	ACGACCTCGATGGGTAGGC		
TNF-α	Frw	AATCATCTTCTCGAACCCCAAGT	74	(38)
	Rev	GGAGCTGCCCCTCAGCTT		
TGF-β	Frw	CAGAATGGCTGTCCTTTGATGTC	79	(35)
	Rev	AGGCGAAAGCCCTCGACTT		
IFN-γ	Frw	TCAACCCCTTCTCGCCACT	113	(36)
	Rev	GCTGCCTACTTGGTCCCTGA		
β-actin	Frw	ACGGAGCGTGGCTACAGC	62	(38)
	Rev	TCCTTGATGTCACGCACGA		

TABLE 2. *Primers used for quantification of cytokine mRNA expression by qPCR.*

Results

Both Treatment Protocols Lead to Dog's Clinical Remission

Blood smears of dogs from MT+A, MG+A, and Control Group were all negative for CVBD causing agents. The dogs presented negative serology for Babesia, Anaplasma, Ehrlichia, and Rickettsia, and were negative for DNA detection of these parasites. Dogs were also negative in rapid immune migration for D. immitis antigen and microfilaria were absent in Knott technique.

Clinical signs observed in sick dogs at the beginning of the study (Tp0) included loss of body weight (**Figures 2A,B**), local/generalized lymphadenopathy, decreased/increased appetite, lethargy, mucous membrane pallor, polyuria/polydipsia, cutaneous alopecia, onychogryphosis (**Figure 2D**), hyperkeratosis, exfoliative-dermatitis, and erosive-ulcerative dermatitis (**Figure 2C**). Other clinical signs, such as epistaxis, lameness, and masticatory muscle myositis were also observed. No clinical signs were detected in dogs of the Control Group. Sick dogs showed also changes in hematological and biochemical parameters, including a mild decrease of hemoglobin values, mild erythropenia, lower hematocrit values, thrombocytopenia (**Table 3**), mild renal azotemia (**Table 4**), hyperglobulinemia with increased alpha 2 and gamma globulin fractions, and decreased values of albumin and albumin/globulin ratio (**Table 5, Figure 3**). Dogs of group MT+A presented higher BUN values and an accentuated AST and ALT while dogs of the MG+A group exhibited BUN normal values and a slight increase in ALT and AST values (**Table 4, Figure 3**). Three dogs of group MT+A also showed creatinine values inferior to 1.4 mg/dL and mild proteinuria, presenting a urine protein:creatinine ratio (UPC) of 0.6. Control group dogs exhibited normal hematological and biochemical parameters, serum proteins, and urinalysis values. Lymph node and bone marrow smears of dogs from both MT+A and MG+A groups presented amastigote forms inside macrophages associated with lymphoid hyperplasia. Dogs from both groups showed anti-Leishmania antibody titers ranging between 1:80 and 1:320. No antileishmanial antibodies were detected in dogs from the Control Group (**Table 6**). One month after treatment (Tp1) dogs of MG+A exhibited higher vivacity and energy than dogs from MT+A. Three months after treatment onset (Tp3), both groups exhibited a successful recovery, showing remission of all clinical signs. Dogs from the MT+A group presented a significant recovery ($p < 0.05$) of BUN values to normal levels. AST and ALT quickly recovered to normal values in dogs of group MG+A (**Table 4**). Although presenting higher AST and ALT values, combined

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Hemogram	MT+A group ($n = 6$)				Tp0 vs. Tp3	MG+A group ($n = 6$)				Tp0 vs. Tp3	Control group ($n = 5$)	Reference interval
	Tp0	Tp1	Tp2	Tp3		Tp0	Tp1	Tp2	Tp3			
RBC ($\times 10^6/\mu l$)	5.49 ± 1.32	5.30 ± 1.85	5.71 ± 1.07	5.87 ± 0.91	–	5.22 ± 0.55	5.54 ± 1.18	6.36 ± 0.65	6.82 ± 0.68	*	7.21 ± 1.05	5.5–8.5
Hemoglobin (g/dl)	12.32 ± 2.95	11.83 ± 3.55	13.08 ± 2.50	12.66 ± 3.24	–	11.24 ± 2.15	12.33 ± 2.59	14.25 ± 1.73	15.50 ± 1.26	*	16.26 ± 2.41	12–8
Hematocrit (%)	37.85 ± 10.89	35.45 ± 12.20	38.98 ± 7.60	37.44 ± 9.51	–	34.80 ± 6.09	37.38 ± 9.93	42.45 ± 5.86	45.27 ± 3.63	*	51.40 ± 7.29	37–55
MCV (μm^3)	68.48 ± 4.13	67.15 ± 2.95	68.24 ± 2.80	68.72 ± 2.13	–	66.38 ± 7.04	67.12 ± 9.17	66.63 ± 4.71	66.53 ± 3.46	–	71.40 ± 1.69	60–74
MCH (pg)	22.60 ± 1.53	22.67 ± 1.35	22.92 ± 0.89	23.26 ± 0.54	–	21.02 ± 1.94	22.30 ± 1.48	22.42 ± 1.07	22.75 ± 1.25	–	22.54 ± 0.65	19.5–24.5
MCHC (g/dl)	33.10 ± 2.95	33.83 ± 2.65	33.70 ± 2.42	33.84 ± 0.78	–	32.32 ± 1.60	33.60 ± 3.51	33.72 ± 1.49	34.22 ± 1.28	–	31.62 ± 1.63	31–36
RDW (%)	13.16 ± 0.94	13.78 ± 0.95	13.88 ± 1.09	13.46 ± 1.44	–	13.43 ± 0.99	14.45 ± 2.35	13.08 ± 1.07	13.00 ± 0.97	–	12.34 ± 0.54	12–18
Leukocytes ($\times 10^3/\mu l$)	8.17 ± 2.98	7.83 ± 2.38	9.22 ± 2.13	7.76 ± 3.82	–	7.50 ± 2.47	8.62 ± 3.83	9.03 ± 2.90	9.55 ± 3.42	–	10.24 ± 3.18	6–17
Lymphocytes ($\times 10^3/\mu l$)	1.67 ± 0.51	2.23 ± 0.86	3.18 ± 1.64	2.96 ± 2.29	–	1.56 ± 0.85	2.12 ± 1.11	2.37 ± 0.81	2.22 ± 0.87	–	2.80 ± 0.70	1–4.8
Monocytes ($\times 10^3/\mu l$)	0.66 ± 0.25	0.48 ± 0.19	0.48 ± 0.22	0.40 ± 0.23	–	0.68 ± 0.24	0.67 ± 0.50	0.50 ± 0.26	0.47 ± 0.23	–	0.48 ± 0.20	0.2–2
Neutrophils ($\times 10^3/\mu l$)	5.60 ± 2.27	4.38 ± 1.35	4.92 ± 1.41	3.86 ± 1.45	–	5.11 ± 1.45	5.40 ± 2.71	5.77 ± 2.40	6.43 ± 2.95	–	5.96 ± 2.14	3–11.8
Eosinophils ($\times 10^3/\mu l$)	0.27 ± 0.28	0.70 ± 0.57	0.58 ± 0.40	0.52 ± 0.64	–	0.14 ± 0.21	0.42 ± 0.23	0.35 ± 0.19	0.38 ± 0.21	*	0.98 ± 0.40	0.1–1.3
Basophils ($\times 10^3/\mu l$)	0.02 ± 0.04	0.03 ± 0.05	0.04 ± 0.05	0.00 ± 0.00	–	0.02 ± 0.04	0.03 ± 0.05	0.07 ± 0.08	0.03 ± 0.05	–	0.06 ± 0.05	0–0.5
Platelets ($\times 10^3/\mu l$)	280.67 ± 133.4	233.83 ± 130.8	254.00 ± 150.8	235.00 ± 43.62	–	212.80 ± 133.5	246.50 ± 125.1	227.17 ± 60.38	222.50 ± 58.32	–	217 ± 25.84	200–500
MPV (μm^3)	11.82 ± 2.94	12.82 ± 2.88	11.14 ± 2.27	11.82 ± 2.28	–	14.73 ± 3.14	11.82 ± 2.70	11.33 ± 2.56	10.73 ± 1.82	–	10.38 ± 1.28	5–15
Procalcitonin (%)	0.30 ± 0.14	0.28 ± 0.10	0.26 ± 0.13	0.26 ± 0.09	–	0.28 ± 0.15	0.23 ± 0.15	0.27 ± 0.08	0.25 ± 0.10	–	0.22 ± 0.04	0.2–0.5
PDW (%)	65.04 ± 11.24	64.33 ± 9.75	66.12 ± 11.74	64.62 ± 10.11	–	74.08 ± 3.95	57.98 ± 21.28	70.58 ± 8.48	73.87 ± 5.91	–	59.06 ± 5.03	40.6–65.2

At diagnosis time (Tp0), and 1 (Tp1), 2 (Tp2) and 3 (Tp3) months after the beginning of the treatment. Blood samples of sick (n = 12) and healthy dogs (control group [CG], n = 5) were used to evaluate hemogram parameters. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each treatment group.
**p < 0.05. MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; MCV, Mean Corpuscular Volume; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; RBC, Red Blood Cells; RDW, Red cell Distribution Width.*

TABLE 3. *Hemogram values exhibited by dogs of MT+A and MG+A groups.*

Biochemical parameters	MT+A group ($n = 6$)				Tp0 vs. Tp3	MG+A group ($n = 6$)				Tp0 vs. Tp3	Control group ($n = 5$)	Reference interval
	Tp0	Tp1	Tp2	Tp3		Tp0	Tp1	Tp2	Tp3			
BUN (mg/dl)	60.35 ± 22.86	45.27 ± 35.79	33.23 ± 8.49	25.25 ± 7.04	*	26.03 ± 5.09	31.92 ± 3.84	34.50 ± 7.11	34.60 ± 6.82	*	36.33 ± 3.64	15-40
Creatinine (mg/dl)	1.20 ± 0.88	1.08 ± 0.68	1.19 ± 1.04	0.88 ± 0.62	–	0.55 ± 0.08	0.64 ± 0.12	0.87 ± 0.26	0.82 ± 0.27	–	0.92 ± 0.24	0.4-1.4
Total bilirubin (mg/dl)	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.06 ± 0.04	–	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.02	0.04 ± 0.00	–	0.06 ± 0.02	0.04-0.4
Direct bilirubin (mg/dl)	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	–	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	–	0.04 ± 0.01	0-0.3
Indirect bilirubin (mg/dl)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.04	–	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	–	0.02 ± 0.02	0-0.3
AST (U/l)	62.80 ± 29.48	45.83 ± 12.12	46.17 ± 11.74	42.60 ± 17.62	–	49.25 ± 2.87	42.40 ± 13.79	58.50 ± 9.07	37.83 ± 17.36	–	40 ± 4.19	10-40
ALT (U/l)	93.83 ± 76.94	86.00 ± 42.70	78.33 ± 42.16	65.20 ± 40.53	–	44.17 ± 36.34	29.33 ± 16.19	30.33 ± 11.71	37.67 ± 16.11	–	40.25 ± 7.46	10-70
Alkaline phosphatase (U/l)	146.1 ± 166.4	131.78 ± 161.7	146.48 ± 243.8	30.05 ± 15.95	*	49.18 ± 15.34	38.95 ± 13.42	35.30 ± 7.10	35.28 ± 12.70	*	41.45 ± 29.56	20-200
Sodium (mmol/l)	145.60 ± 4.67	148.50 ± 8.76	146.50 ± 3.02	143.60 ± 3.21	–	142.25 ± 4.86	146.67 ± 2.34	146.67 ± 2.42	148.50 ± 3.56	–	146.75 ± 2.22	140-151
Potassium (mmol/l)	4.86 ± 0.79	4.70 ± 0.89	4.68 ± 0.49	4.54 ± 0.44	–	4.62 ± 0.34	4.59 ± 0.39	4.50 ± 0.27	4.40 ± 0.26	–	5.07 ± 0.53	3.4-5.4
Chloride (mmol/l)	112.80 ± 1.92	102.67 ± 13.94	113.50 ± 4.93	92.78 ± 51.56	–	108.00 ± 4.08	108.17 ± 9.35	104.17 ± 7.17	111.67 ± 6.02	–	113.00 ± 6.48	105-120
Calcium (mg/dl)	9.99 ± 0.51	9.54 ± 0.35	9.48 ± 0.72	9.08 ± 0.93	–	9.92 ± 0.42	9.93 ± 0.30	9.89 ± 0.32	10.14 ± 0.36	–	9.50 ± 1.57	9.5-12
Inorganic phosphorus (mg/dl)	4.96 ± 0.68	5.90 ± 2.87	4.35 ± 1.60	3.58 ± 0.92	–	3.95 ± 0.59	3.73 ± 0.67	3.35 ± 1.03	3.58 ± 1.25	–	4.60 ± 0.79	2.1-5
Biliary acids (μmol/l)	3.07 ± 2.11	3.22 ± 2.94	3.18 ± 3.34	3.36 ± 2.83	–	1.40 ± 0.25	3.54 ± 3.44	2.19 ± 1.28	1.72 ± 1.11	–	2.47 ± 1.34	1-10
Urinalysis												
Creatinine (mg/dl)	<1.4	<1.4	<1.4	<1.4	–	<1.4	<1.4	<1.4	<1.4	–	< 1.4	< 1.4
UPC	<0.2–0.6	<0.2–0.5	<0.2	<0.2	–	<0.2–0.4	<0.2–0.4	<0.2	<0.2	–	< 0.2	< 0.2

At diagnosis time (Tp0), and 1 (Tp1), 2 (Tp2), and 3 (Tp3) months after the beginning of the treatment. Blood and urine samples of sick (n=12) and healthy dogs [control group (CG), n = 5] were used to evaluate biochemical parameters and urinalysis. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each dog group.
**p < 0.05. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood Urea Nitrogen; UPC, Urine Protein Creatinine Ratio.*

TABLE 4. *Biochemical parameters and urinalysis results exhibited by dogs of MT+A and MG+A groups.*

Proteinogram	MT+A group ($n = 6$)				Tp0 vs. Tp3	MG+A group ($n = 6$)				Tp0 vs. Tp3	Control group ($n = 5$)	Reference interval
	Tp0	Tp1	Tp2	Tp3		Tp0	Tp1	Tp2	Tp3			
Total protein (g/dl)	9.58 ± 1.55	7.70 ± 0.60	7.56 ± 1.18	7.60 ± 1.30	*	8.43 ± 1.46	7.66 ± 0.89	7.92 ± 1.30	6.85 ± 0.56	–	6.28 ± 0.59	5.5–7.5
Albumin (g/dl)	2.46 ± 0.84	2.48 ± 0.53	2.72 ± 0.24	2.56 ± 0.58	–	2.14 ± 0.50	2.50 ± 0.40	3.23 ± 1.01	3.05 ± 0.36	–	3.03 ± 0.40	2.26–4.3
Alpha 1 (g/dl)	0.22 ± 0.04	0.20 ± 0.00	0.20 ± 0.00	0.18 ± 0.04	–	0.20 ± 0.00	0.22 ± 0.04	0.27 ± 0.08	0.25 ± 0.05	–	0.28 ± 0.05	0.1–0.31
Alpha 2 (g/dl)	1.40 ± 0.25	1.32 ± 0.40	1.30 ± 0.35	1.22 ± 0.23	–	1.46 ± 0.23	1.40 ± 0.10	1.47 ± 0.35	1.20 ± 0.11	–	0.95 ± 0.06	0.5–1.1
Beta (g/dl)	1.78 ± 0.29	1.46 ± 0.09	1.64 ± 0.26	1.72 ± 0.42	–	1.58 ± 0.33	1.76 ± 0.13	1.73 ± 0.14	1.32 ± 0.31	–	1.38 ± 0.29	0.93–2
Gama (g/dl)	3.32 ± 2.36	2.26 ± 1.43	1.70 ± 1.44	1.92 ± 1.22	–	2.74 ± 1.52	1.82 ± 0.91	1.28 ± 0.26	1.10 ± 0.43	–	0.65 ± 0.19	0.3–1
Albumin: globulin ratio (%)	0.44 ± 0.29	0.50 ± 0.19	0.60 ± 0.17	0.56 ± 0.30	–	0.38 ± 0.13	0.50 ± 0.19	0.68 ± 0.17	0.80 ± 0.13	*	0.95 ± 0.13	0.6–1.1

*At diagnosis time (Tp0), and 1 (Tp1), 2 (Tp2) and 3 (Tp3) months after the beginning of the treatment. Blood samples sick (n = 12) and healthy dogs [control group (CG), n = 5] were used to evaluate serum proteins. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each dog group. *p < 0.05.*

TABLE 5. *Serum proteins of dogs of MT+A and MG+A groups.*

treatment of miltefosine and allopurinol promoted the decrease of AST and ALT in dogs from the MT+A group, albeit slower, with urinalysis values returning to normal. Dogs in MG+A group exhibited a normalization of the albumin globulin ratio 2 months after the beginning of treatment (Tp2) and 1 month later (Tp3) total protein and gamma globulin were within reference values. However, in dogs of the MT+A group the total protein and gamma globulin remained high and alpha 2 globulin normalized 3 months after the beginning of the treatment (Tp3) (**Table 5, Figure 3**). Three months after treatment onset (Tp3), MG+A dogs were negative for anti-Leishmania antibodies and, with the exception of one dog that had a titer of 1:320 group MT+A dogs were also negative. When re-evaluated 6 months after the initial diagnosis this positive dog

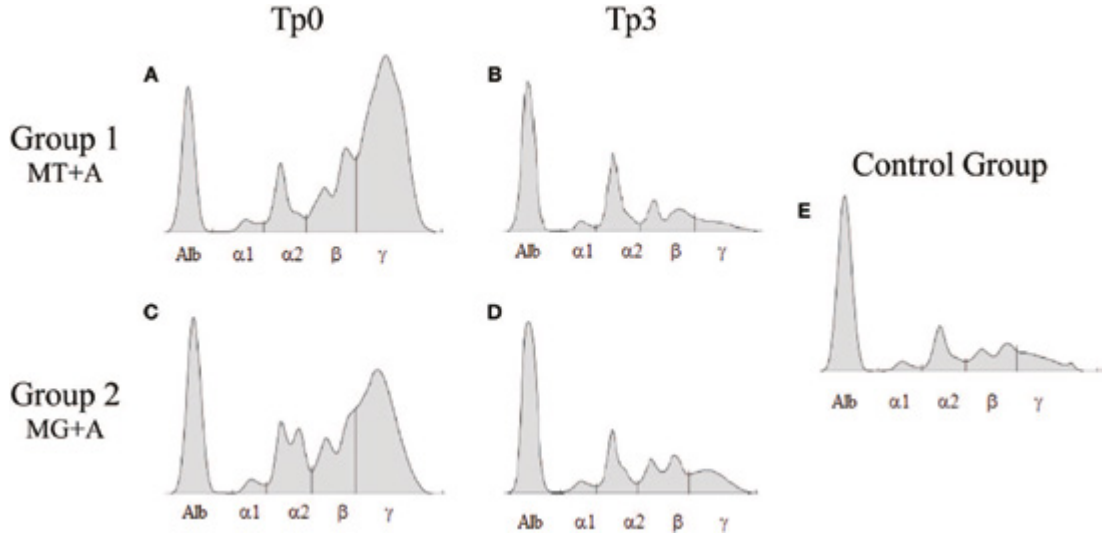

FIGURE 3. *Serum protein electrophoresis of sick and treated dogs. Representative proteinograms of sick [Tp0; (A) MT+A; (B) MG+A], treated [Tp3; (C) MT+A; (D) MG+A] and clinically healthy dogs (E) are shown. Alb, Albumin; α1, α1-globulin; α2, α2-globulin; β, β-globulin; γ, γ-globulin.*

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Leishmania antibody titer	Tp0	Tp1	Tp2	Tp3	Tp6
Group 1 ($n = 6$)	1:80–1:320	<1:80–1:320	<1:80–1:160	<1:80	<1:80
Group 2 ($n = 6$)	1:80–1:320	<1:80–1:320	≤1:80	<1:80–1:320	<1:80
Control group ($n = 5$)	<1:80	<1:80	<1:80	<1:80	<1:80

At diagnosis time (Tp0), and 1 (Tp1), 2 (Tp2), 3 (Tp3), and 6 (Tp6) months after the beginning of treatment, peripheral blood of sick (n = 12) and control dogs [control group (CG), n = 5] were collected and used to evaluated anti-Leishmania antibody titers by IFAT. A cut-off of 1:80 was used.

TABLE 6. *Anti-Leishmania antibody titers.*

was negative for antileishmanial antibodies (**Table 6**). Furthermore, amastigote forms were no longer observed in lymph node and bone marrow smears of dogs from both groups.

Principal Component and Cluster Analysis Enable the Distinction Between Healthy and Sick Dogs

Principal component analysis in PBMCs confirmed that healthy and sick dogs could be distinguished based on their expression of IFN-γ, IL-2, IL-4, IL-5, IL-12, and TGF-β along with IFAT results, with these features explaining 65.5% of the distribution (**Figure 4A**). In lymph node, PCA was also able to distinguish healthy and sick dogs based on the expression of IFN-γ, IL-2, and IL-10 along with IFAT results, with 63.4% of the distribution being explained by these variables (**Figure 4C**). For bone marrow the

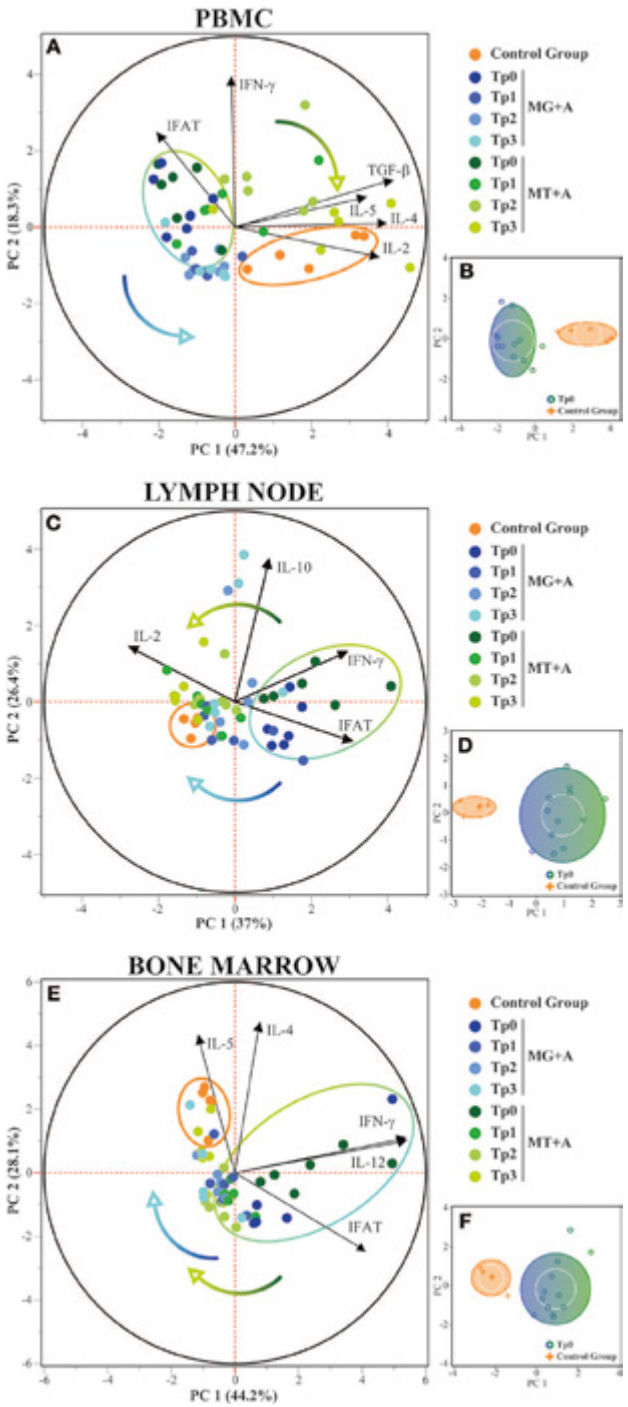

FIGURE 4. *Principal Component and Cluster Analysis of cytokine expression in PBMC, lymph node and bone marrow. Principal component analysis was used to identify the first two principal components which explain 65.5, 63.4, and 72.3% for each respective tissue, of the variation in the dataset. (A,C,E) Biplot of score and loading plots showing the variables which load on the respective principal components. Control, MG+A, and MT+A groups are presented by different colored dots along all time-points, with the control group and the sick dogs (Tp0) delimited by their respective halo. Colored arrows show the transition of treated dogs over time. (B,D,F) Cluster analysis confirming the separation of healthy and sick dogs (Tp0) using the selected variables. PC, Principal Component.*

expression of IFN-γ, IL-4, IL-5, and IL-12 along with IFAT results, enabled the distinction between healthy and sick dogs, with these features explaining 72.3% of the distribution (**Figure 4E**). These results are also supported by cluster analysis (**Figures 4B,D,F**), with the formation of two separate groups. Dogs from both treatment groups could not be distinguished based on the selected features, but the transition from the sick dogs cluster toward the healthy dog cluster along the time-points can be observed in PBMC, lymph node, and bone marrow.

Leishmania Infection Shapes Dogs' Cytokine Profile

Sick dogs (MT+A and MG+A) showed a significant accumulation of IFN-γ mRNA in cells of PBMC (pMT+A = 0.0057; pMG+A = 0.0425; **Figure 5J**), lymph node (pMT+A = 0.001; pMG+A = 0.0028; **Figure 5K**), and bone marrow (pMT+A = 0.0097; pMG+A = 0.0267; **Figure 5L**) when compared with clinically healthy dogs (CG). Bone marrow cells of dogs of MT+A showed a significant upregulation of IL-12 (p = 0.0059; **Figure 5F**) in comparison to control dogs. On the other hand, lymph node cells of sick dogs evidenced a significant reduction in IL-2 mRNA (pMT+A = 0.0365; pMG+A = 0.0068; **Figure 5B**). Dogs of MG+A group also showed a significant downregulation of IL-2 gene expression in PBMC (p = 0.0193; **Figure 5A**) and TNF-α in lymph node cells (p = 0.0186; **Figure 5H**). While dogs of MT+A group showed a significant upregulation of TNF-α gene expression in bone marrow cells (p = 0.0413; **Figure 5I**). No significant differences were found in gene expression of IL-12 by PBMC and lymph node cells, IL-2 by bone marrow cells and TNF-α by PBMC when compared to clinically healthy dogs.

PBMC (pMT+A = 0.0662; pMG+A = 0.0032; **Figure 6A**) and bone marrow (pMG+A = 0.0138; **Figure 6C**) of sick dogs evidenced a significant down regulation of IL-4 gene expression in comparison to the CG. In lymph node cells, no significant differences were observed in the IL-4 gene expression. PBMC of sick dogs from MT+A group (p = 0.0031; **Figure 6D**) and bone marrow cells of dogs of group MG+A (p = 0.0082; **Figure 6F**) showed a statistically significant downregulation of IL-5 gene expression. Additionally, lymph node cells of MT+A showed a significant accumulation of IL-5 mRNA (p = 0.0235; **Figure 6E**).

A significant IL-10 downregulation in PBMC of MG+A (p = 0.0153; **Figure 7A**) and an upregulation in lymph node cells of sick dogs (pMT+A = 0.0041, pMG+A = 0.0112; **Figure 7B**). No significant differences in IL-10 gene expression were observed in bone marrow cells of sick dogs when compared with control dogs.

A significant reduction in the accumulation of TGF-β mRNA was observed in PBMC (pMG+A = 0.0112; **Figure 7D**) and lymph node cells (pMT+A = 0.0425; pMG+A = 0.0057; **Figure 7E**) of sick dogs in relation to the CG. Bone marrow cells of MG+A group (**Figure 7F**) also showed a significant TGF-β downregulation (p = 0.0186).

Although there were differences between sick dogs, these results seem to indicate that Leishmania infection can shape the dogs' immune response by inducing IFN-γ upregulation while others pro-inflammatory (IL-2), anti-inflammatory (IL-4) and regulatory (TGF-β) cytokines were downregulated. Additionally, the modulation of TNF-α, a key player in macrophage activation, IL-5, which is involved in the differentiation of activated B lymphocytes into Ig-secreting plasma cells, and IL-10, a cytokine associated with immune regulation, seems to be tissue specific.

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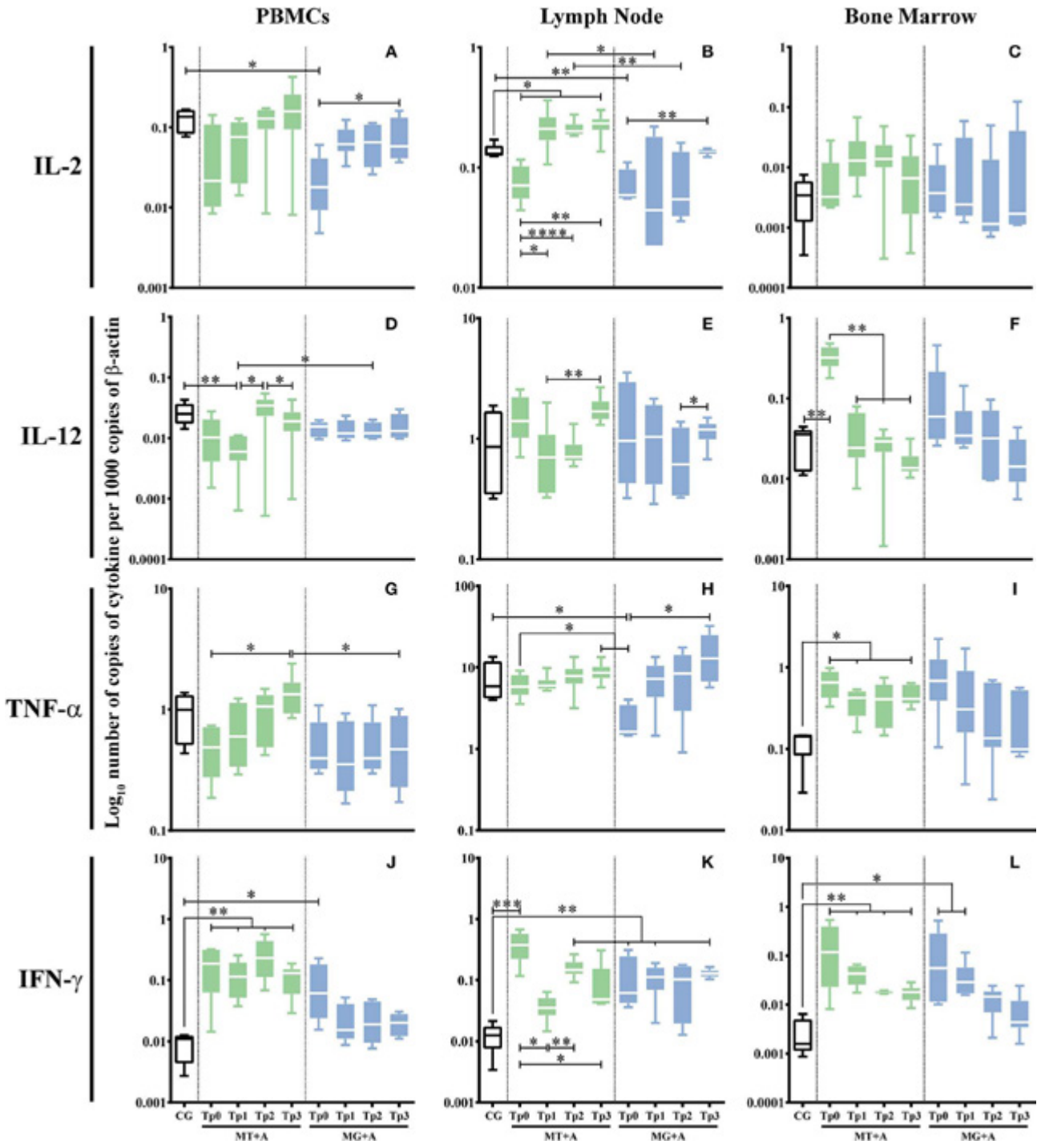

FIGURE 5. *Pro-inflammatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points. IL-2 (A–C), IL-12 (D–F), TNF-α (G–I), and IFN-γ (J–L) mRNA in PBMC (A,D,G,J), lymph node (B,E,H,K) and bone marrow (C,F,I,L) cells of dogs from MT+A, MG+A, and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum, and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001) indicate statistical significance.*

(R) evolution

PEQUEÑOS ANIMALES

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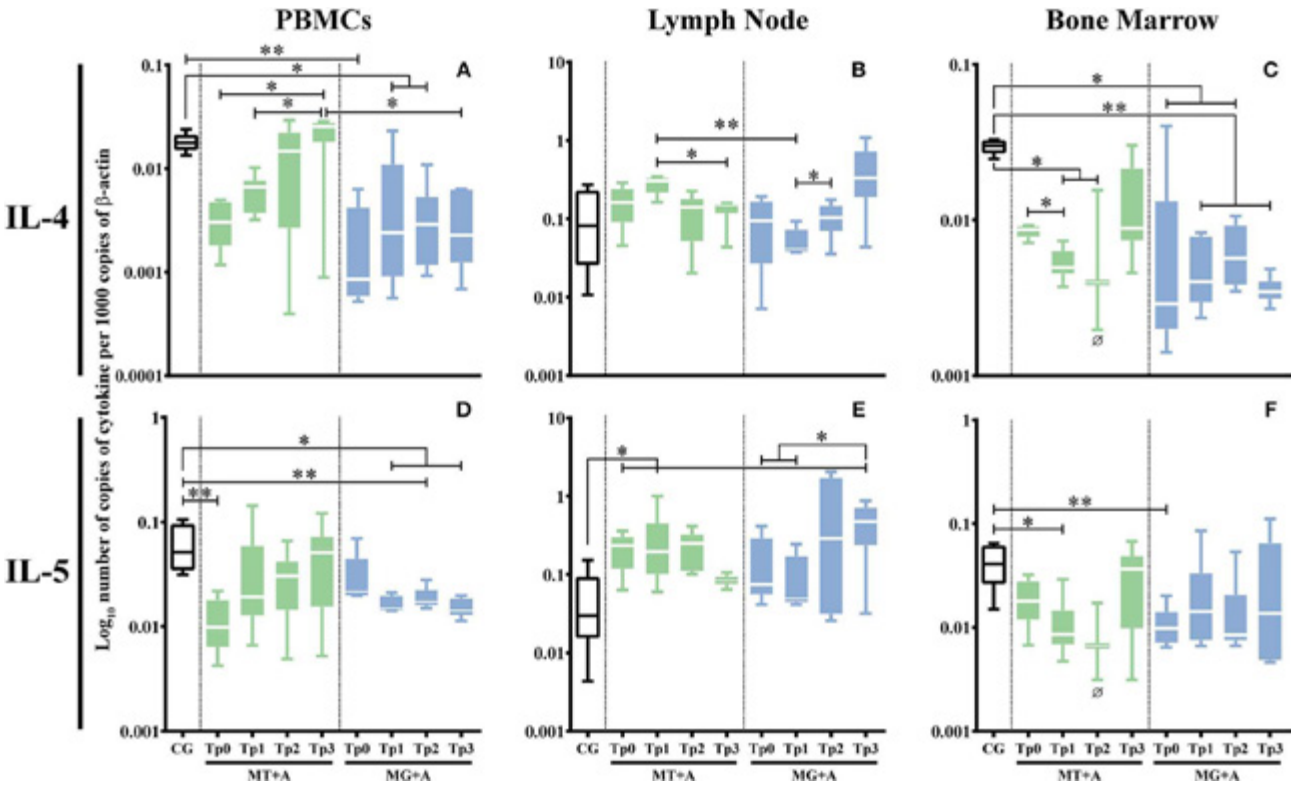

FIGURE 6. *Anti-inflammatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points. IL-4 (A–C) and IL-5 (D–F) mRNA in PBMC (A,D), lymph node (B,E), and bone marrow (C,F) cells of dogs from MT+A, MG+A, and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum, and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. *p < 0.05, **p < 0.01 indicate statistical significance. Ø shows mRNA expression values of only three dogs.*

(R) evolution

PEQUEÑOS ANIMALES

Tabla de contenido:

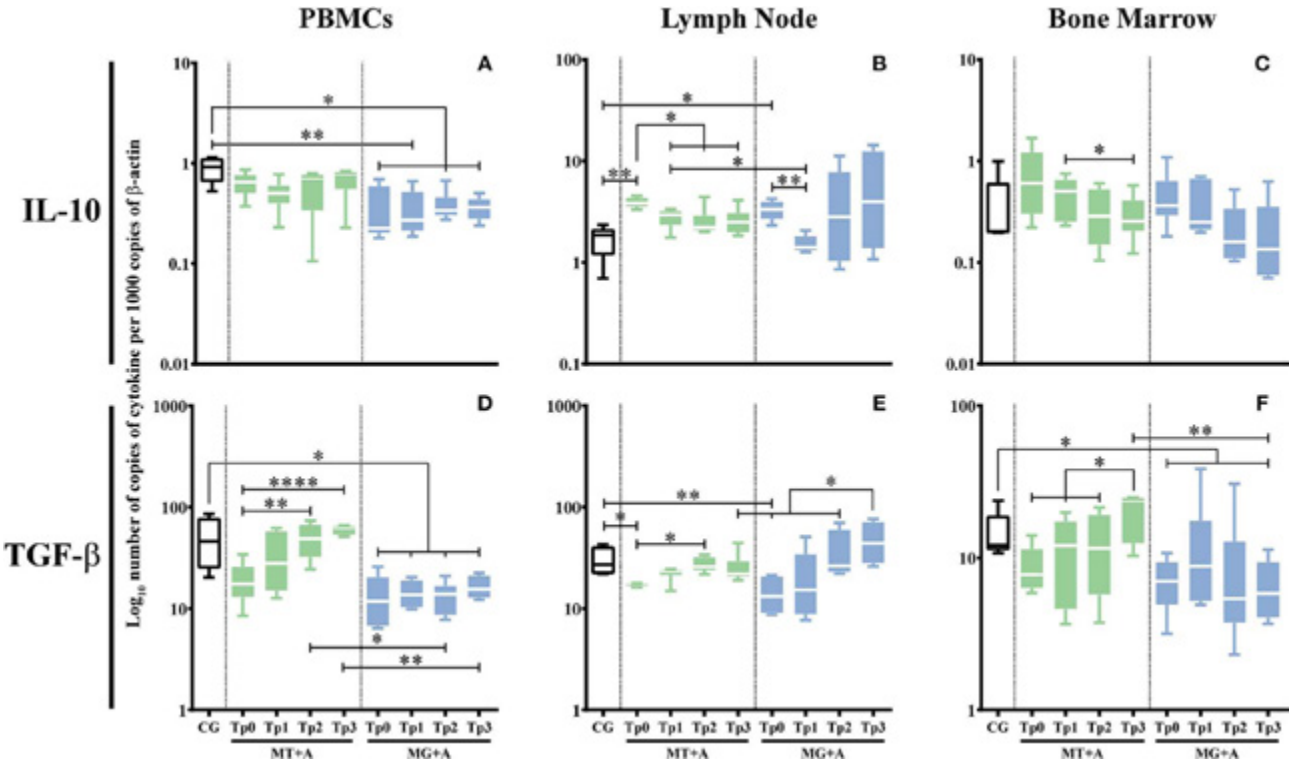

FIGURE 7. *Regulatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points. IL-10 (A–C) and TGF-β (D–F) mRNA in PBMC (A,D), lymph node (B,E), and bone marrow (C,F) cells of dogs from MT+A, MG+A, and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum, and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. *p < 0.05, **p < 0.01, and ****p < 0.0001 indicate statistical significance.*

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PEQUEÑOS ANIMALES

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Increased Gene Expression of Pro-inflammatory Cytokines Persists After Treatment With Miltefosine in Combination With Allopurinol

Gene expression of cytokines that were modified by infection was further evaluated along all time-points and studied tissues. In dogs treated with MT+A, bone marrow (**Figure 5F**) cells evidenced an IL-12 gene expression similar to CG 1 month after the beginning of treatment and throughout the study, pointing toward normalization. IFN-γ gene expression was still up-regulated in PBMC (pTp1 = 0.0027, pTp2 = 0.0023, pTp3 = 0.0013; **Figure 5J**), lymph node (pTp2 = 0.0061; **Figure 5K**) and bone marrow (pTp1 = 0.0057, pTp2 = 0.0061, pTp3 = 0.0047; **Figure 5L**) cells during the observation period. In lymph node cells, there was a slight increase in IFN-γ mRNA accumulation at Tp2 when compared to Tp1 (pTp1 = 0.0032). Nevertheless, a tendency to normalization was observed in bone marrow.

A significant high amount of IL-2 mRNA was found in lymph node cells at Tp3 ($p = 0.0143$) when compared to the CG (**Figure 5B**). In bone marrow cells, TNF-α gene expression persisted elevated (pTp1 = 0.0303, pTp3 = 0.0481) throughout all time-points (**Figure 5I**).

In PBMC, IL-4 gene expression recovered by Tp2 when compared with CG (**Figure 6A**), while in bone marrow a low accumulation of IL-4 mRNA (**Figure 6C**) was observed (pTp1 = 0.0365, pTp2 = 0.0420) throughout the study. Even so, at TP3 there was a slight upregulation of IL-4 gene expression, revealing a tendency to revert to normal values. Although a fluctuation of IL-5 gene expression was observed (**Figure 6D**), at Tp3 it reverts to values compared with CG. In lymph node cells, although IL-5 gene expression maintained increased at Tp1 (**Figure 6E**) when compared with CG ($p = 0.0124$), at Tp3 a trend to reduction in IL-5 mRNA accumulation points toward normalization.

IL-10 and TGF-β gene expression revealed a tendency to recuperation to normal values. Namely, IL-10 mRNA accumulation in lymph node (**Figure 7B**) was significantly decreased when compared to Tp0 (pTp1 = 0.0338, pTp2 = 0.0144, pTp3 = 0.0409), along with a significant increase of TGF-β mRNA accumulation in PBMC ($p < 0.0001$) at Tp3 (**Figure 7D**) similar to CG and in lymph node at Tp2 ($p = 0.0112$) when compared with Tp0 (**Figure 7E**).

Despite the generalized tendency of treated dogs to achieve normal levels, the upregulation of pro-inflammatory cytokines (IFN-γ and IL-2) together with the trend to the normalization of anti-inflammatory (IL-4 and IL-5) and regulatory cytokines (IL-10 and TGF-β) point toward a persistent inflammatory immune response during the 3 months of treatment.

Upregulation of IFN-γ Gene Expression Persists After Treatment With Meglumine Antimoniate Combined Therapy

Dogs treated with MG+A evidenced a normalization of IFN-γ gene expression in PBMC (**Figure 5J**). IFN-γ gene expression remained significantly higher (pTp1 = 0.0029, pTp3 = 0.0018) in lymph node cells in comparison to CG (**Figure 5K**). On the contrary, bone marrow cells (**Figure 5L**) showed a progressive decrease of IFN-γ mRNA. At Tp1 ($p = 0.0425$) the values were significantly increased when compared to the CG. However, by Tp2 and Tp3, IFN-γ gene expression lowered toward levels comparable to the CG. On the other hand, IL-2 (**Figure 5B**) and TNF-α (**Figure 5H**) gene expression in lymph node cells was similar to the CG. IL-2 at Tp1 in PBMC (**Figure 5A**) presented values similar to control dogs, with Tp3 having significant difference to Tp0 ($p = 0.0425$). The same was verified in lymph node, with IL-2 recovering to amounts comparable to CG by Tp3 ($p = 0.0098$). TNF-α (**Figure 5H**) in lymph node recovered to values similar to CG showing a significant difference when compared to Tp0 ($p = 0.0451$).

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During treatment follow-up, IL-4 gene expression remained downregulated in PBMC (pTp1 = 0.0219, pTp2 = 0.0297) (**Figure 6A**) and in bone marrow cells (pTp1 = 0.0068, pTp2 = 0.0229, pTp3 = 0.0013; **Figure 6C**) when compared with the CG. In PBMC, IL-5 gene expression was also downregulated (pTp1 = 0.0199, pTp2 = 0.0071, pTp3 = 0.0343; **Figure 6D)**. Despite a slight reduction in the accumulation of IL-5 mRNA in bone at Tp2, it was noticed a tendency to normalization (**Figure 6F**).

During treatment, accumulation of IL-10 (pTp1 = 0.0076, pTp2 = 0.0101, pTp3 = 0.0108; **Figure 7A**) and TGF-β (pTp1 = 0.0192, pTp2 = 0.0235, pTp3 = 0.0473; **Figure 7D**) mRNA was highly reduced in PBMC when compared to the CG. However, in lymph node cells, IL-10 (**Figure 7B**) and TGF-β (**Figure 7E**) gene expression was similar to control dogs. Nonetheless, at Tp3, a slight increase of TGF-β mRNA in lymph node was observed when compared to Tp2 (p = 0.0285). Despite bone marrow cells showed a normalization of TGF-β gene expression (**Figure 7F**), 2 months after treatment (Tp3) a significant decrease of TGF-β mRNA accumulation (p = 0.0453) was observed when compared to healthy dogs. Leishmania infected dogs treated with meglumine antimoniate in combination with allopurinol (MG+A) evidence a generalized tendency to achieve normal cytokine levels in the Leishmania host tissues evaluated in the current study. However, the persistent upregulation of IFN-γ gene expression associated with downregulation of IL-4, IL-5, IL-10, and TGF-β gene expression indicates the possible predominance of an inflammatory immune response. On the other hand, the slight increase of TGF-β at Tp3 in the lymph node can point toward the local activation of a regulatory immune response.

The Activity of Miltefosine and Meglumine Antimoniate Combined Therapies Can Influence Cytokine Gene Expression

To estimate the influence of the drugs in cytokine generation, the cytokines that were not significantly altered by Leishmania infection were analyzed by comparing gene expression of sick (Tp0) and treated dogs (Tp1-Tp3).

After the first month of treatment (Tp1) with miltefosine in association with allopurinol (MT+A), PBMC evidenced a downregulation of IL-12 (p = 0.0025; **Figure 5D**) and IL-10 (**Figure 7A**), and a slight upregulation of IL-2 (**Figure 5A**) and TNF-α (**Figure 5G**). During the second and third month IL-2, TNF-α, and IL-10 showed a progressive upregulation, with IL-12 having an accentuated increase at Tp2 (p = 0.0411) and a slight decrease by Tp3 (p = 0.0233). Regarding lymph node cells, it was observed a considerable upregulation of IL-12 (**Figure 5E**) after Tp1 (p = 0.0062), along with a slight overexpression of TNF-α (**Figure 5H**) at Tp2 and Tp3 (p = 0.0138) time-points and a considerable gene expression of IL-4 (**Figure 6B**) at Tp1 followed by downregulation by Tp2 and Tp3 (p = 0.0410). In bone marrow, the treatment caused accumulation of IL-2 (**Figure 5C**) mRNA that persisted until Tp2, along with an increase of TGF-β (**Figure 7F**) that peaked at Tp3 (p = 0.0106). IL-5 (**Figure 6F**) mRNA levels showed a downregulation by Tp1 (p = 0.0343) that persisted until Tp2. A progressive downregulation of IL-10 (**Figure 7C**) was evident from Tp1 to Tp3 (p = 0.0452).

PBMC of dogs treated with MG+A showed a progressive downregulation of IL-12 (**Figure 5D**) from Tp0 to Tp2, followed by an increase at Tp3, and a progressive TNF-α (**Figure 5G**) increase from Tp0 that reached maximum values by Tp3. Lymph node cells presented IL-12 (**Figure 5E**) mRNA levels increased by Tp3 (p = 0.0484), exhibiting a slight and transitory downregulation of IL-4 (**Figure 6B**) levels at Tp1 (p = 0.0293) followed by a progressive upregulation that peaked at Tp3. IL-5 (**Figure 6E**) was slightly downregulated at Tp1 but showed an accentuate increase when meglumine antimoniate was discontinued (Tp2), with a slight decrease by Tp3 (p = 0.0344). Regarding bone mar-

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row cells, a continuous decrease in IL-12 and TNF-α mRNA accumulation was noticed from Tp1 to Tp3. However, IL-2 gene expression presented an irregular pattern, suffering a downregulation at Tp2. When compared with Tp0, IL-10 presented a progressive downregulation until Tp3.

These findings indicate that MG+A directs the overexpression of cytokines in blood and lymph node and is possible that allopurinol plays a key role in enhancing cytokine generation. In the bone marrow, the drugs seem to downregulate cytokine gene expression. MT+A also seems to enhance cytokine gene expression. However, when miltefosine was discontinued, IL-4 in lymph node and IL-10 in the bone marrow became downregulated.

Discussion

Progression of L. infantum infection is mainly dependent on the competence of the dog's immune system, which is related to inherent characteristics such as genetic background. Thus, the spectrum of clinical manifestations can range from subclinical infection to severe disease. During active disease, dog's immune response has been mainly characterized by a marked humoral immune response and specific immunosuppression of T lymphocyte proliferation[41]. Despite being the major domestic reservoir of L. infantum, dogs have also intrinsic value, more notably a recognized social and affective role. Therefore, the use of therapies that can ensure a successful CanL treatment is highly required.

Several studies have pointed out the efficacy and faster recovery rate of dogs treated with meglumine antimoniate in combination with allopurinol[5,19,42,43]. Regarding the progress of hematological, biochemical, and urinary parameters, it is worth to emphasize that both combined therapies used in the current study were able to recover erythrocytes, hemoglobin, hematocrit, and UCP normal values, while leukocytes, neutrophils, creatinine, and albumin were within the reference intervals during the 3 months of treatment. Dogs evidencing less clinicopathological alterations, that were selected to be treated with meglumine antimoniate in combination with allopurinol, presented a fast recovery of hematological, biochemical and urinary parameters. Dogs showing more clinicopathological alterations, and which were treated with miltefosine in combination with allopurinol, took longer to reach normalization of those parameters.

Three months after CanL diagnosis (Tp3), both combined therapies were successful in promoting remission of clinical signs, recovering of hematological and biochemical normal values in all dogs and in restraining parasite infection since amastigotes were not found in the bone marrow and lymph node smears. Anti-parasite antibodies also diminished to non-significant titers in most of the dogs, with only one dog treated with MG+A taking more time to become negative (>3 months).

During CanL, L. infantum parasites are hosted in several organs of the reticuloendothelial system, having a widespread influence on the host's immune system. As previously reported[28,39,44], in CanL, IFN-γ gene expression is increased in parasite-host tissue prior to any treatment. Also in the current study PBMC, lymph node and bone marrow cells evidenced a pronounced generation of IFN-γ. Although such immune response is widely verified in many other studies, it also raised the question if this Th1 immune response is positively correlated with parasite control. Previous studies in experimentally infected hamsters and in humans suffering from visceral leishmaniosis have shown high parasite loads in Th1 environments, indicating an IFN-γ inability to confer protection[45,46]. Thus, the main consensus indicates that sick dogs express high levels of IFN-γ in Leishmania-target tissues, possibly directing a Th1 immune response against persistent infection.

The most studied tissue regarding cytokine expression during CanL is the peripheral blood, which in animals presenting clinical signs is characterized as having suppression of T cell mediated immunity and production of high levels of specific antibodies[24], as a consequence of a predominantly Th2 response with production of anti-inflammatory cytokines, such as IL-4 and IL-5[23]. In the present study, with the exception of high IFN-γ gene expression, peripheral blood IL-2, TGF-β, IL-4, and IL-5 of non-treated dogs were decreased, suggesting that Leishmania caused an overall lymphocyte deactivation, leading to unbalance of pro- and anti-inflammatory immune mediators. Still, taking into consideration that the peripheral blood is not the tissue of election for L. infantum replication and persistence[47,48], along with possible natural genetic variability between dogs, it may be the reason why there is so much divergence between studies regarding cytokine expression in this tissue.

Despite most of CanL studies being focused in only one tissue, usually the peripheral blood, more and more studies consider that every single tissue affected by this parasite presents its own immune response[28,39,49,50]. IL-10 is a key regulatory cytokine that prevents excessive pathology. This cytokine can negatively regulate innate and adaptive immune responses by impairing the production of pro-inflammatory (e.g., IL-12, IL-2, IFN-γ, and TNF-α) and anti-inflammatory (IL-4 and IL-5) cytokines, restraining T cell activity in lymph nodes and limiting tissue inflammation. In CanL, the lymph node is reported as having a predominantly Th1 immune response[39]. Besides this, a true consensus has not been established, with studies showing higher expression of Th1 cytokines, like IFN-γ and TNF-α[51], in pre-scapular lymph nodes of dogs without external clinical signs and lower parasite burden, pointing toward a possible role of these cytokines in controlling parasite replication. In contrast, dogs presenting clinical signs showed no expression of IL-4 and IL-12, but high levels of immunosuppressor cytokines like IL-10 and TGF-β[51], posing a role in disease progression. In the current study, the lymph node of dogs with CanL seems to evidence a mixed Th1/Treg immune response with low IL-2, but high IL-12 and IFN-γ, along with down expression of TGF-β but over expression of IL-10, pointing toward a balance between the differentiation of IFN-γ mediated inflammatory response and a regulatory immune response that could favor parasite persistence.

Considering the cytokine expression in bone marrow of dogs with CanL, to our best knowledge, there are only a few documented studies[28,39,44], which report this tissue as a predominantly Th1 environment that tends to develop high parasite loads, characterized by an increased expression of IFN-γ and TNF-α and low to no detection of IL-10, along with lower expression of IL-4[28,44]. In the current study, bone marrow cells of sick dogs also evidence IFN-γ overexpression and low expression of IL-4, IL-5, and TGF-β pointing to a predominantly pro-inflammatory immune response. Furthermore, the PCA and cluster analysis reinforce that each tissue presents a distinct cytokine pattern of response to infection, confirming previous reports[39]. Furthermore, infection level also seems to influence local cytokine gene expression, namely TNF-α, that points toward a diminished generation of this cytokine in lymph node cells of dogs presenting less clinicopathological signs (MG+A), and overexpression in bone marrow cells of dogs with severe clinicopathological signs (MT+A). TNF-α together with IFN-γ induce the upregulation of inducible nitric oxide synthase (iNOS) by macrophages, directing L-arginine oxidation and nitric oxide (NO) production[52]. NO is a powerful oxidative molecule that mediates parasite killing. Thus, the hypothesis that TNF-α can be a biomarker of CanL severity needs to be further investigated. Furthermore, IL-5, a cytokine linked to growth and differentiation of B cells, evidenced to be over-expressed in lymph node

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cells of dogs presenting more clinicopathological signs (MT+A). These findings point to a higher B cell activation in lymph node. The over expression of IL-12 in bone marrow cells of dogs exhibiting more clinicopathological signs (MT+A), a signaling pathway cytokine that prime naïve T cells to differentiate into Th1 cells, supports the possible establishment of a Th1 cell population.

By analyzing the peripheral blood, popliteal lymph node and bone marrow along the course of two of the most used CanL protocol treatments, the current study shows evidence of a higher IFN-γ generation during the 3 months of follow up of dogs treated with MT+A. Furthermore, lymph node cells also exhibited a TNF-α overexpression, suggesting that there are conditions for macrophage activation and parasite inactivation, and increased generation of IL-2, indicating a possible lymphocyte proliferation. These findings indicate that miltefosine associated therapy does not promote reduction of pro-inflammatory immune response, but, induces the normalization of anti-inflammatory IL-4 and IL-5 and of immune-suppresor TGF-β in mononuclear blood cells, of immune-suppressor IL-10 in lymph node and of IL-5, TGF-β and pro-inflammatory IL-12 in bone marrow.

MG+A lead to the normalization of the proinflammatory immune response, restoring IFN-γ and IL-2 expression levels in blood cells, IL-2, IL-12, and TNF-α in lymph node and IFN-γ in the bone marrow. Although showing some instability, IL-5 tends to normal values in the bone marrow. Treatment also seems to induce the normalization of immunosuppressor cytokines in the lymph node. However, the continuous overexpression of IFN-γ in lymph node cells points toward the maintenance of a local inflammatory response despite the drug activity in promoting the remission of clinical signs, and the rise of IFN-γ gene expression in mononuclear blood cells 1 month post-treatment suggests the predomination of a Th1 immune response. On the other hand, IL-4 and IL-5 stay downregulated in mononuclear blood cells as well as IL-10 and TGF-β indicating the inhibition of Th2 and Treg immune response even during dogs' clinical improvement. In bone marrow as well, treatment did not induce the normalization of IL-4 gene expression.

The effect of drug therapies used in the current study in cytokine gene expression was investigated in the cytokines that were not significantly affected by parasite infection (Tp0). Although combined therapies seem to have similar outcomes, it was not possible to find a distinctive pattern, exhibiting cytokine, and tissue dependent effects. The drug activity possibly empowered by free parasite antigens seems to favor mainly cytokine generation.

The current study enables a close overview of the effect of the two most used anti-leishmanial therapies, miltefosine, and meglumine antimoniate in association with allopurinol, in reversing CanL progression on naturally infected dogs, including clinical signs remission, normalization of hematological, biochemical and urinary parameters, and IFAT seroconversion. Both combined therapies are effective in CanL treatment, favoring clinical recovery of all dogs and the overexpression of pro-inflammatory cytokines, pointing toward the persistence of inflammatory immune environments that can direct parasite inactivation at least during the initial 3 months of treatment. The current study also demonstrates that anti-inflammatory and regulatory cytokines do not seem to play a key role in CanL immune response. Furthermore, the combined therapies also appear to play a direct role in cytokine generation. These are relevant findings, since both are two of the most used protocols in the treatment of this zoonotic parasitosis, the evolution of the cell-mediated immune response generated while under these specific treatments should be further studied. With the recent implementation of miltefosine for CanL treatment in Brazil, an extremely endemic country for canine and human leishmaniosis, it becomes a subject of ensuring the best for the reinforcement of Public Health protection.

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Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Ethics Statement

The animal study was reviewed and approved by Ethics Committee and Animal Welfare (CEBEA–Comissão de Ética e Bem-Estar Animal) of the Faculty of Veterinary Medicine, University of Lisbon. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author Contributions

GS-G, CM, MS, and IF conceived and designed the study. MS, CM, MP, JG, JC, AB, AR, JM, and IF collected samples. MS, LG, and IF processed samples and did subsequent microscopic, molecular, and serological tests. MS and CM conducted the experiments. MS, GS-G, IF, and MB analyzed the data. MS and GS-G conducted statistical analysis. MS, GS-G, and IF drafted the manuscript. GS-G, IF, GA-P, MB, AD, LT, AVR, MB, and DS-M made in depth reviews of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BID, bis in die; BUN, Blood urea nitrogen; CanL, Canine leishmaniosis; cDNA, Complementary DNA; CG, Control Group; CLWG, Canine Leishmaniasis Working Group; CPDA-1, Citrate phosphate dextrose adenine; CVBD, Canine vector-borne disease; DNA, Deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; IL, Interleukin; IFAT, Indirect Fluorescent Antibody Test; IFN-γ, Interferon gamma; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; MG+A, Meglumine antimoniate with Allopurinol; MPV, Mean platelet volume; MT+A, Miltefosine with Allopurinol; PBMC, Peripheral Blood Mononuclear Cell; PCA, Principal Component Analysis; PDW, Platelet distribution width; qPCR, Real-time PCR; RDW, Red cell distribution width; RNA, Ribonucleic acid; SID, semel in die; Th1, Type-1 T-Helper; Th2, Type-2 T-Helper; TGF-β, Transforming growth factor beta; TNF-α, Tumor necrosis factor alpha; Treg, Regulatory T-cells; UPC, Urine Protein Creatinine ratio.

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Polyprenyl Immunostimulant Treatment of Cats with Presumptive Non-Effusive Feline Infectious Peritonitis In a Field Study

Tratamiento inmunoestimulante de poliprenilo de gatos con peritonitis infecciosa felina presuntiva no efectiva en un estudio de campo

Palabras clave: peritonitis infecciosa felina, inmunoestimulante de poliprenilo, aumento de la supervivencia, enfermedad crónica, coronavirus felino, estudio de campo

Keywords: *feline infectious peritonitis, Polyprenyl immunostimulant, increased survival, chronic disease, feline coronavirus, field study*

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La peritonitis infecciosa felina (FIP) es una enfermedad mortal sin tratamiento clínicamente efectivo. Este estudio de campo evaluó el tratamiento con inmunoestimulante de poliprenilo (PI) en gatos con la forma no efusiva de FIP. Debido a que la inmunpresión es un componente importante en la patología de la FIP, planteamos la hipótesis de que el tratamiento con un estimulante del sistema inmunitario aumentaría los tiempos de supervivencia de los gatos con FIP seco.

Feline infectious peritonitis (FIP) is a fatal disease with no clinically effective treatment. This field study evaluated treatment with Polyprenyl Immunostimulant (PI) in cats with the non-effusive form of FIP. Because immune suppression is a major component in the pathology of FIP, we hypothesized that treatment with an immune system stimulant would increase survival times of cats with dry FIP. Sixty cats, diagnosed with dry FIP by primary care and specialist veterinarians and meeting the acceptance criteria, were treated with PI without intentional selection of less severe cases. The survival time from the start of PI treatment in cats diagnosed with dry FIP showed that of the 60 cats with dry FIP treated with PI, 8 survived over 200 days, and 4 of 60 survived over 300 days. A literature search identified 59 cats with non-effusive or dry FIP; no cat with only dry FIP lived longer than 200 days. Veterinarians of cats treated with PI that survived over 30 days reported improvements in clinical signs and behavior. The survival times in our study were significantly longer in cats who were not treated with corticosteroids concurrently with PI. While not a cure, PI shows promise in the treatment of dry form FIP, but a controlled study will be needed to verify the benefit.

Introduction

Feline infectious peritonitis (FIP) is considered to be one of the most devastating diseases of domestic cats with an incidence of 2–12%[1] in multi-cat environments. FIP has long been considered fatal[2–4] and a leading cause of mortality in young cats. No clinically effective treatments exist for FIP[5]. FIP has an effusive form with abdominal and thoracic fluid accumulations; a median survival time of 9 days was noted in 21 cats with effusive FIP[6]. Dry (non-effusive) FIP is often characterized by pyogranulomatous infiltrates in the liver, kidneys, lymph nodes, eyes, and central nervous system. The dry form of FIP has longer survival times within a range of 1–200 days noted in 59 cats[6–13]. Two cats with mixed dry and wet forms FIP treated with combinations of corticosteroids, human alpha interferon, and nelfinavir survived 181 and 477 days[7].

Mutation of the enteric coronavirus that induces a tropism for macrophages initiates the disease process[3,14]. Cell-mediated immunosuppression due to a decrease in CD4+ lymphocytes is commonly seen in cats with FIP[14]. Deficiencies in cell-mediated immunity promote an exuberant production of antibodies to the coronavirus, which results in deposition of immune complexes. With immunosuppression being

(R) evolution

PEQUEÑOS ANIMALES

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a major component of pathophysiology, treatment with an immune stimulant is a rational approach.

Polyprenyl Immunostimulant (PI) is a veterinary biologic licensed by the U. S. Department of Agriculture for the reduction of the severity of signs of feline herpesvirus and is safe in cats over 8 weeks of age. It was used in our pilot study to treat cats with the dry form of FIP and produced promising results[15]. It upregulates Th-1 type pathway via toll-like receptors[16] and may thus be of benefit in the diseases involving suppression of cellular immunity. In this field study, we tried to determine if PI treatment increases survival time and quality of life in cats diagnosed with dry FIP.

Materials and Methods

General Study Design

The field study had a single treatment arm, without a placebo control group, and was limited to cats with non-effusive or dry FIP. Only cats in the United States and Canada were accepted. Cats were diagnosed and treated by their primary care veterinarians in conjunction with, in many cases, veterinary specialists. The veterinarians' usual laboratories performed diagnostic tests. The study measured survival times from the start of PI treatment to death or euthanasia in terminal condition. The survival data from this study were compared to the historic data from a number of published articles. The study included cats of all signalments with clinical signs that represented the clinical spectrum of dry form FIP and were accepted and treated regardless of the severity of the disease or current treatments. Addition of appetite stimulants, antiemetics, antibiotics, vitamins, or special diets was not prohibited in our study, but the protocol advised against the use of corticosteroids because they cause immunosuppression. This study was carried out in accordance with the recommendations of the University of Tennessee Office of Laboratory Animal Care. The protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee Protocol #1946.

Case Recruitment

Preliminary findings of a prior pilot study were published in 2009[15]. The Veterinary Information Network site published a note of this trial in February 2010. Following the publication of the article and the note, practicing veterinarians with suspected or confirmed FIP cases contacted the Principal Investigator (AML) via e-mail or phone. All cats diagnosed with dry FIP by their veterinarian were considered and assigned a number (**Figure 1**). The initial diagnostics were done by veterinarians and reviewed and assessed for acceptance by AML based on sufficient data to support the diagnosis. In eight instances, AML accepted the cats into the study without all laboratory diagnostics if the diagnosis was made by invasive techniques [immunohistochemistry (IHC), histopathology of biopsied material, or cytology of aspirates].

The initial veterinarian diagnosis was supported using the diagnostic approach proposed by Addie et al.[2] as reflected by our data collection form (**Table 1**), which included questions about patient age, history, environment, and observations such as pyrexia, weight loss, lethargy, anorexia, presence of abdominal lesions (masses, enlarged mesenteric lymph nodes). Required laboratory tests included complete blood count, biochemistry, and antibody titers to feline coronavirus (FCoV) and to pathogens that may mimic FIP. Surgical biopsy or aspiration of suspected lesions was encouraged. Necropsy was offered at the University of Tennessee free of charge or veterinarians could provide results of necropsy done by their providers.

The accepted cats received PI at 3 mg/kg orally three times per week and were clinically assessed by their veterinarians initially and then on follow-up examinations (monthly was recommended), which collected the data for the analysis as shown in Table 2. Submission of the initial and follow-up lab-

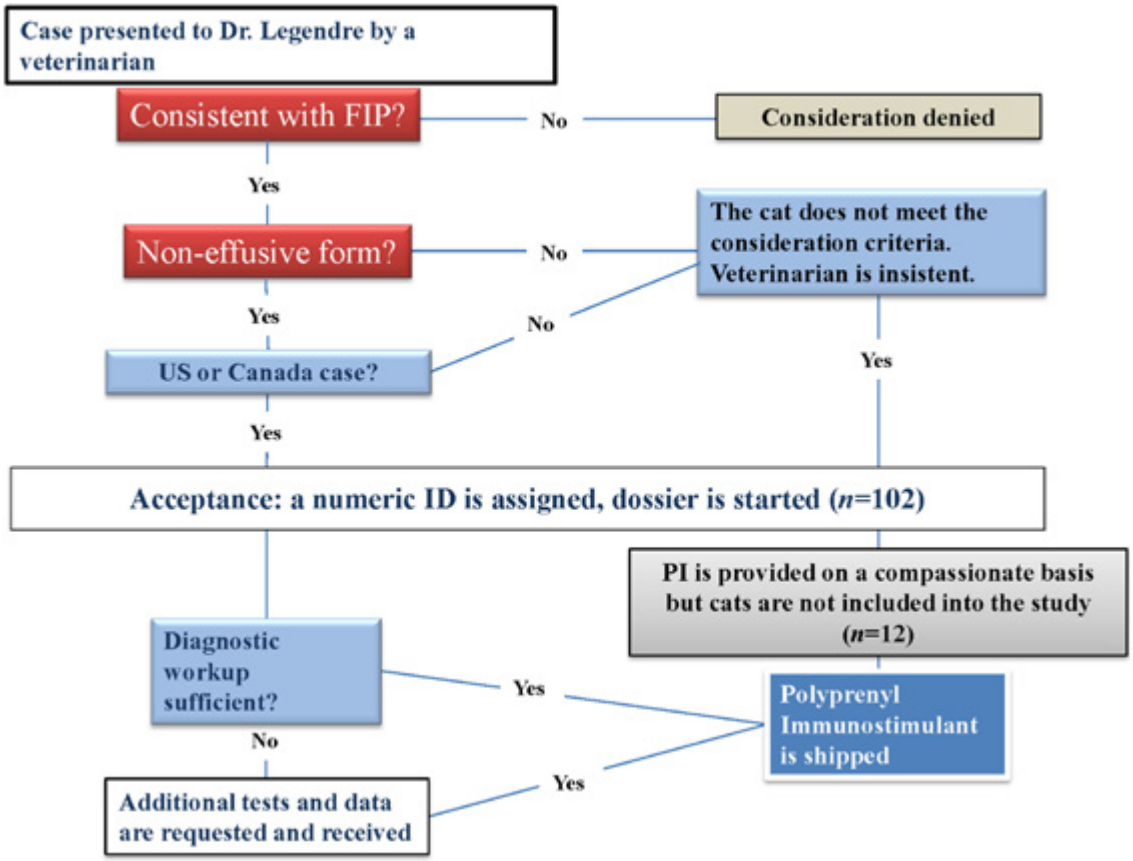

FIGURE 1. *Study decision-making tree at a glance.*

oratory test results was required. Refills of PI were shipped to collaborating veterinarians after the results of the follow-up evaluations were received. Monitoring of the progress of the cats accepted into the study was continued until death or euthanasia.

Veterinarians were advised to taper corticosteroid treatments if started before the study, but they were allowed to continue corticosteroid therapy at the lowest effective dose to maintain appetite and well-being.

Quality of life assessment was done using responses on the questionnaires and communications by the primary care veterinarians and owners. In many cases, more extensive comments were recorded in the cat's medical records, and all records were analyzed for the comments. We considered communications by veterinarians and owners regarding restoration of routines, activity levels, appetite improvement, etc. as indicators of the quality of life. Table 3 shows the questionnaire used to provide the data collected in the study.

Statistical Analysis of Study Results

Skewness and kurtosis statistics found non-normal distributions for all temporal variables associated with survival. Therefore, non-parametric statistics were employed to yield inferences based on the respective research questions. Between-subjects comparisons for age groups and disease groups were conducted using Kruskal–Wallis and Mann–Whitney U-tests. In addition to means and SD, medians and interquartile ranges were reported to give context to non-parametric statistical findings. Kaplan–Meier survival curves were used to display the cumulative survival of cats across time. t-tests were employed for the comparison of mean and SD. An alpha value of 0.05 assumed statistical significance, and all analyses were conducted using SPSS Version 21 (Armonk, NY, USA: IBM Corp.).

ID #	Date Polyprenyl Immunostimulant (PI) shipped		
Breed	Multi cat?	Origin	
Age now	Age at diagnosis		
Type of feline infectious peritonitis	Changes?	Histopathology?	
Date of Diagnosis	Date of PI start	Feline coronavirus (FCoV) titer	Necropsy

	Baseline	Follow-up								
Date		30 days	60 days	90 days	120 days	150 days	180 days	210 days		
Weight										
FCoV titer										
Total protein										
Globulins										
Albumin										
HCT										
WBC										
Neutrophils										
Lymphocytes										
ALT										
Temperature										
Uveitis										
Neuro										
Diarrhea										
Abdominal mass										
Steroids										

Survival, days (check)										
Date of death										

TABLE 1. *Data collection form.*

Case #/Name			
Breed			
Male/female			
Neutered			
Age			
Weight		Did not start Polyprenyl Immunostimulant (PI) (check)	

Clinical signs	**1 or 0**
Ocular	
Neuro	
Decreased appetite	
Fever 102.5+	
Lethargy/depression	
Abdominal lesions	
Weight loss	

1 = yes
0 = no

Diagnosis	**1 or 0**
Abdominal imaging	
Surgery	
Histopathology	
Cytology	
Elevated globulins	
Elevated total protein	
Decreased albumin	
Feline coronavirus titer	
1:400	
1:800	
1:1,600+	
Elevated WBC	
Decreased HCT	
Elevated neutrophils	
Decreased neutrophils	
Elevated lymphocytes	
Decreased lymphocytes	
Palpable/visible abdominal mass	
Enlarged mesenteric lymph nodes	
Colon involvement	

Weight loss	1 or 0 compared to start
@ 30 days	
@ 60 days	
@ 90 days	
@ 120 days	
@ 150 days	

Weight gain	1 or 0 compared to start
@ 30 days	
@ 60 days	
@ 90 days	
@ 120 days	
@ 150 days	

Weight, kg	
@ 30 days	
@ 60 days	
@ 90 days	
@ 120 days	
@ 150 days	
Status/survival	
@ 30 days	
@ 60 days	
@ 90 days	
@ 120 days	
@ 150 days	
>150 days	
Other	
Lost to follow-up	

Improvement (subjective owner/vet assessment)	
@ 30 days	
@ 60 days	
@ 90 days	
@ 120 days	
@ 150 days	
>150 days	

Treatment	
PI	
Steroids at start of PI	
Steroids at 1 month after start of PI	
Steroids at 2 months after start of PI	
Other	
Other	

Survived after PI start	days

TABLE 2. *Data analysis form.*

Results

Consideration and Acceptance

Consideration began March 1, 2010, and ended May 6, 2011. A total of 102 cats were considered for the study. All cats were diagnosed by their primary care veterinarians. There were 60 cats that met the qualification criteria and were therefore accepted into the study, and 23 of those had been referred to specialists, including veterinary ophthalmologists[12], internists[6], neurologists[3], ophthalmologist and a neurologist jointly[1], and a veterinary cardiologist[1]. The remaining considered cats were disqualified before or during the study for the following reasons: insufficient diagnostic information[10], died before PI arrived[10], wet form FIP(10; PI was provided on a compassionate basis), treatment was stopped after one to two doses[2], overseas cases (2; PI was provided on a compassionate basis), incorrect diagnosis (2; PI was provided to one of the two cats on a compassionate basis), cats that were lost to follow-up because the veterinarian never provided any information after PI was shipped[5] and one cat enrolled in error, i.e., started the treatment prior to March 1, 2010[1]. The accepted cats were evaluated and treated by veterinarians throughout the USA[58], and in Canada[2]

Physical exam findings				
Date of exam			Cat's name	
			Owner's name	
Initial exam or recheck exam (mark)				
Date of first dose of Polyprenyl Immunostimulant (PI)				
Current dosing schedule				
Please answer the following questions based on the current examination. If you check YES, give additional details including locations, duration, and severity as necessary				
			Comments	
Weight loss	Yes	No	Current weight:	
Weakness	Yes	No		
Appetite	Yes	No		
Vomiting	Yes	No		
Diarrhea	Yes	No		
Fever	Yes	No		
Lameness	Yes	No		
Neurologic signs	Yes	No		
Paraplegia	Yes	No		
Ocular signs	Yes	No		
Other	Yes	No		
Initial history/history since last exam				
PI Study Concomitant medications				
Start date	End date	Drug	Dosage	Route

TABLE 3. *Questionnaire for collection of patient information from the initial and follow-up examinations.*

in their practices. Fewer than 32% (19 of 60) of the cats were diagnosed at 10 days or less before the start of PI treatment. The remaining 41 cats were diagnosed with dry FIP 11 or more days before the beginning of treatment with PI. The mean time span between the diagnosis and the treatment was 22.97 ± 21.60 days. The cat that died after the administration of the first dose was diagnosed 161 days before the start of PI treatment.

Diagnostics

Signalment

There were 25 female, 1 hermaphrodite, and 34 male cats; 38 were non-purebred and 22 were purebred (5 Ragdoll, 4 Siberian, 3 Bengal, 3 Maine Coon, 2 Siamese, 2 Sphynx, 1 Tonkinese, 1 Manx, and 1 Birman). Data on the household were provided for 30 cats; 22 of the 30 were from multi-cat households and 8 from single-cat households. The age distribution of the 60 cats is shown in **Figure 2**. Seventy percent of the cats were under 24 months old, and 43% were under a year of age.

Clinical Signs

Fifty-nine of the 60 cats accepted into the study had clinical signs of FIP listed on the algorithm proposed by Addie et al.[2]. The presence of clinical signs caused the cats' owners to bring the cat to their primary veterinarian for examination. During this initial examination, the clinical signs were documented in medical records. **Figures 3A,B** show the clinical signs and their distribution in the cats accepted into this study. Fifty-four of 59 cats showed two or more clinical signs (**Figure 3B**) with five cats having one sign. Of the five cats with one sign, 2/5 were neurologic, 1/5 ocular, 1/5 was evaluated because of persistent vomiting (an abdominal mass in the ileocolic region was discovered at the examination), and no data of a comprehensive initial examination were provided for one whose abdominal mass was discovered at physical examination.

One cat was included in the study that had no clinical signs when seen for neutering. This 8-month-old, male Siberian cat had a 10.7 g/dL serum total protein on a pre-anesthesia screening. The albumin was 2.1 g/dL, and the globulin was 8.6 with an A/G ratio of 0.24. The serum electrophoresis was interpreted as polyclonal gammopathy.

The cat was anemic with a hematocrit of 23%. The coronavirus antibody titer was positive at 1:1,600, and the feline leukemia antigen was negative and the feline immunodeficiency antibody titer was negative. Thoracic radiographs showed an increase in cranial mediastinal density, and enlarged mesenteric lymph nodes were seen on abdominal ultrasound. Lymph node aspirates showed an increase in neutrophils on aspirate cytology.

Fifty-nine of the 60 cats on the study were categorized into one of five subforms of dry FIP based on the initial clinical signs, physical examination findings by primary veterinarians (and specialists where applicable) and diagnostic workup. One cat had no clinical signs. The subform categories were distributed as follows: mixed (18/59), gastrointestinal (16/59), non-localized (11/59), ocular (9/59), neurologic (5/59).

At the initial presentation, the cats in the gastrointestinal category had anorexia (15/16), diarrhea[4], and/or vomiting[3], which were the primary reasons for the veterinary visit. Abdominal masses were found in 23/59 cats in the study, and the cats were categorized into either the gastrointestinal or mixed subform depending on whether they had additional clinical signs more often associated with another subform.

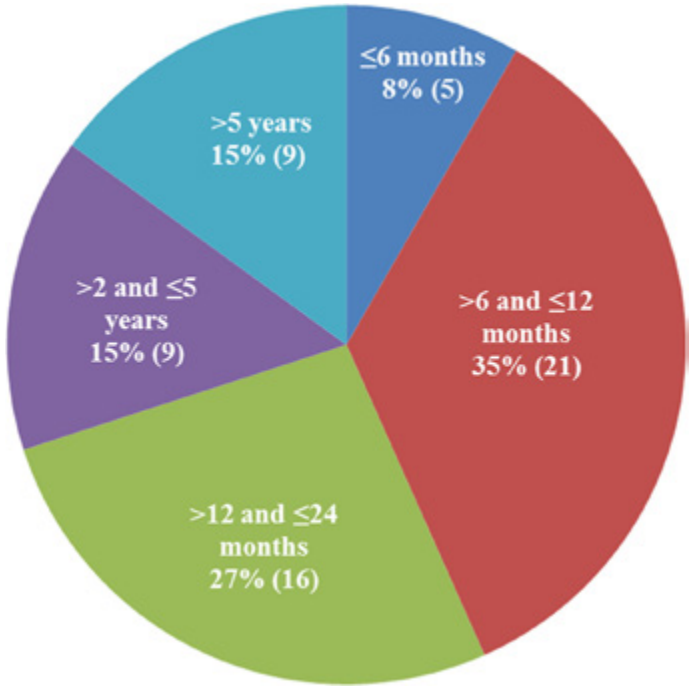

FIGURE 2. *Age distribution of the qualified patients. The majority of cats diagnosed with dry feline infectious peritonitis were under 2 years of age (70%).*

Ocular changes were reported in 17 cats and included anterior uveitis (17/17), retinal detachment (2/17), and keratic precipitates (4/17). One cat had a corneal ulcer. In 10/17 cats, ocular signs were the reason for the initial veterinary visit, and the cats were assigned to the ocular subform. The other 7 cats with ocular signs also had neurologic, abdominal, or non-localized signs and were classified as having mixed form. Neurologic signs, such as seizures, ataxia, and disorientation, were the main reason for the veterinary evaluation in 5/59 cats, and they were categorized as neurologic subform.

The "non-localized" category (11/59) included cats with persistent fever uncontrollable-with-antibiotics, lethargy, anorexia, and/or weight loss. The mixed subform cats had simultaneous signs from two or more subcategories, such as ocular combined with neurologic signs (e.g., uveitis and seizures).

Hematology, Serology, Differential Testing

Results of hematology tests from blood drawn during the initial examination are shown in **Table 4**. The blood testing data were unavailable for nine cats initially diagnosed based on histology

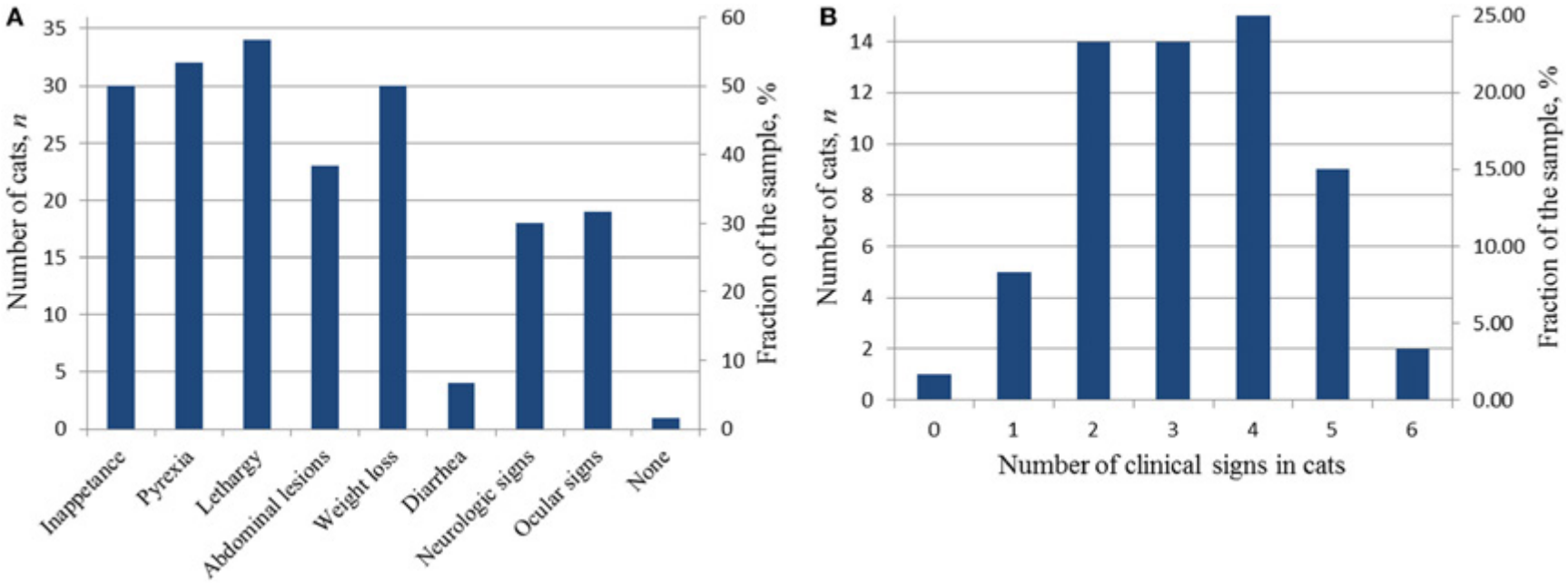

FIGURE 3. *Diagnostic clinical signs in the cats accepted into the study at the time of the initial presentation. (**A**) Distribution of clinical signs in the study sample; (**B**) distribution of the number of clinical signs in individual cats.*

and cytology findings consistent with FIP which were accepted as diagnostic. Two cats had serology and hematology tests done after the acceptance and the start of the treatment, and those later data are not included on the table. In some cases the testing did not include items of interest, thus the data sets are not complete.

Jaundice was observed in one cat whereas hyperbilirubinemia was noted in 8/50 cats. Anemia (HCT < 29%) was observed in 28 of 49 cats. Increased WBC counts were noted in 20/50, neutrophilia was observed in 24/44, and lymphopenia in 16/48 cats.

Hyperglobulinemia and/or an albumin/globulin (A/G) ratio ≤0.6 were noted in 48 of the 50 (96%) cats; two cats had A/G ratio equal to 0.8. The mean and SD of the value in the whole group was 0.37 ± 0.14, and the spread is shown in **Table 4**.

The antibody titers were tested by IFA in 49 cats and, on the scale proposed by Addie et al.[17], were ranked from high positive (400–1,280; n = 13) to very high positive (>1,280, n = 36); 10 of the 49 had titers > 12,800. In the two cats with low positive titers (100), the diagnosis was confirmed by immunostaining of biopsied lesions for the FCoV antigen.

The 7b ELISA was used in three cats. The 3c PCR test done in three cats and showed negative results in two; these two cats were retested by IFA with positive results and also had the diagnosis by cytology.

We collected data sets on differential testing on 50 cats; the tests included FeLV antigen[45], feline immunodeficiency virus (FIV)[45], and toxoplasma[18]. Except in one cat who was FIV positive, all other test results were negative. No serologic data were available for 10 cats. In the group with probable diagnosis with four missing data sets, one cat was diagnosed by neurologist

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based on the results of spinal tap and MRI, and another one had uveitis as a 3.3 lb 7-month Maine Coon kitten. In the group with the diagnosis confirmed by histologic, cytologic, and immunochemical methods, of the six cats without differential data sets, four were diagnosed by IHC and two by histology on the biopsied tissues.

Specialized Laboratory Testing

Specialized laboratory testing methods used to confirm the dry FIP diagnosis are summarized in Table 5. Histology and cytology were performed in 36 cats and were conclusive in 34, they were further validated by immunostaining for FCoV antigen in 13/36 cats. One cat with pyogranulomatous mesenteric lymphadenopathy on lymph node aspirate also had elevated FCoV transcripts in the RT-PCR test on the same sample.

Three cats with neurologic disease had the diagnosis confirmed by CSF tap, and two of those had MRI results consistent with the FIP diagnosis. For one cat with ocular form ocular centesis followed by quantitative PCR confirmed the presence of high titers of FCoV subgenomic mRNA of the M gene.

Necropsy was done of 15/60 cats. In 3/15, the necropsy was done of cats whose diagnosis was previously confirmed antemortem by histology or cytology. Histopathological analysis of the necropsied tissues was conclusive for FIP for 14/15 cats and inconclusive for the 965-day survivor.

Concurrent Treatments Used in the Study

Of the 60 cats accepted into the field study, 13 received PI as the only treatment; the other 45 cats received treatments before the enrollment and/or concurrently with the PI including one or more of appetite stimulants, antiemetics, antibiotics, corticosteroids, vitamins, and/or special diets. There were no data on concurrent treatments for two cats.

Measurement	Reference interval	Data sets received, *n*	Mean	WNL, *n* (%)	Below normal, *n* (%)	Above normal, *n* (%)
Albumin, g/dL	2.3–3.9	51	2.5 ± 0.5	32/51 (62.7)	19/51 (37.3)	0
Total protein, g/dL	5.9–8.5	51	9.8 ± 1.5	12/51 (23.5)	0	39/51 (76.5)
Globulins, g/dL	3.0–6.6	51	7.3 ± 1.6	18/51 (35.3)	0	33/51 (64.7)
A/G ratio	0.4–0.8	50[a]	0.37 ± 0.14	See the breakdown below:		
	<0.8			2/50 (4.0)[b]	48/50 (96.0)	0
	<0.6				36/50 (72.0)	N/A
	<0.4				32/50 (64.0)	N/A
Total bilirubin, mg/dL	0.0–0.4	50	0.5 ± 1.1	42/50 (84.0)	N/A	8/50 (16.0)
WBC, $10^3/\mu L$	4.2–15.6	51	15.6 ± 10.6	27/51 (52.9)	1/51 (2.0)	23/51 (45.1)
HCT, %	29–45	49	29.4 ± 6.9	20/49 (40.8)	28/49 (57.1)	1/49 (2.0)
Neutrophils	Varies	44	N/A	19/44 (43.2)	1/44 (2.3)	24/44 (54.5)
Lymphocytes	Varies	48	N/A	32/48 (66.7)	16/48 (33.3)	0

Seven animals were accepted based on the results of histopathology and cytology, and hematology results for two cats were received after the start of the treatment.
[a]*In one cat, concentrations of individual fractions were provided in lieu of the total globulin level and showed markedly elevated serum globulins and gammopathy. In another cat, no albumin levels were provided.*
[b]*A/G ratio was 0.8 in two cats; no cat had A/G ratio above 0.8.*
WNL, within normal limits.

TABLE 4. *Hematology test results at the first presentation considered in the feline infectious peritonitis diagnosis.*

Sixty-two percent of the cats (36/60) were prescribed corticosteroids orally at the time of the initial diagnosis (27/36), topically (ocular, 7/36), or both (2/36). In 4/36 cats; the corticosteroid treatment was stopped before or shortly after beginning the treatment with PI. Statistics on the use of corticosteroids are presented in **Table 6**. During the study, 31 cats received corticosteroids concurrently with PI (7 ocular topical and 25 systemic or systemic with ocular topical), and 27 cats were treated with PI without concurrent corticosteroids.

The four cats whose corticosteroid treatment was stopped in compliance with the requested test protocol before or at the start of PI treatment all had clinical signs consistent with FIP: weight loss (4/4), ataxia (1/4), abdominal masses (3/4), pyrexia (2/4), lethargy (2/4), anorexia (2/4) with the number of clinical signs from 2 to 5. Their laboratory tests also supported the diagnosis.

Two veterinarians started corticosteroid treatment when the cats deteriorated, one at 9 and at one 24 days before death; these cases were end-of-life care and were not counted as corticosteroid receivers. One cat was given one dose of nelfinavir the day before dying by her veterinarian owner.

Duration of Survival Post-Diagnosis and Clinical Progress

Duration of survival was determined as the time from the start of the PI treatment to death or euthanasia. Of the 60 cats treated with PI, 16 survived for over 100 days, 8 cats survived for over 200 days, 4 cats survived for over 300 days (one additional cat survived for 298 days and is not counted here), 2 for over 900 days, and 1 cat for 1,829 days.

The survival times of the cats in the three groups, i.e.,[1] treated with oral corticosteroids concurrently with PI,[2] treated with topical ocular corticosteroids concurrently with PI; and[3] treated with PI without concurrent use of corticosteroids were significantly different from each other ($p = 0.03$, Kruskal–Wallis test). Table 7 lists all statistical data, and the survival curves are presented in Figure 4. For cats who did not receive corticosteroids the median survival time was 73.5 days (n = 27). For the cats that received corticosteroids by any route concurrently with PI, the median survival time was 21.5 days (n = 31). The difference in survival times between the groups (corticosteroid receiver versus non-receiver) was significant ($p = 0.003$, Mann–Whitney U-test). There was no significant difference in the survival times of the cats treated with corticosteroids systemically or topically ($p = 0.57$, Mann–Whitney U-test). The four cats whose initial corticosteroid treatment was stopped before the treatment with PI survived for 79, 91, 279, and 1,829 days. No data on concurrent treatments were available for two cats diagnosed by histology[1] and histology with IHC[1].

For the cats receiving PI alone and whose diagnosis was not confirmed by cytology, histology (ante- or post-mortem) survival times were 261.1 ± 329.7 days (4–965 days, median 148 days, n = 7); the cats who were treated with PI and corticosteroids concurrently with PI survived 52.8 ± 74.2 days (3–298 days, median 22 days, n = 19). The difference in the survival between the cats treated or untreated with corticosteroids concurrently with PI was significant ($p = 0.03$).

Cats whose diagnosis was confirmed by any type of analysis of biopsied or necropsied samples and treated with PI without concurrent corticosteroids survived 180.5 ± 400.0 days (3–1,829 days, median 63 days, n = 20); the cats treated with PI and corticosteroids concurrently survived 38.9 ± 51.3 days (4–185 days, median 20.5 days, n = 12). Two cats with inconclusive results of cytology[1] and necropsy[1] were accounted for as unconfirmed by those methods. The difference in the survival between the cats treated or untreated with corticosteroids concurrently with PI was significant ($p = 0.04$). A subgroup in which

Test type	Other tests on the same cat	Total tests, *n*		Total histology, cytology, necropsy, *n*	Total CSF tap, *n*	Total ocular centesis, *n*	Total imaging, *n*
		By test	Total				
Cytology on fine needle aspirate		1[a]	24	36			
Histology	Only	8					
	+IHC	12					
	+Necropsy	2					
	+IHC+necropsy	1					
Necropsy	Only	12					
CSF tap cytology	Only	1			3		
	+MRI	2					
Q-PCR (m-gene mRNA) on aqueous humor		1				1	
Thoracic and abdominal imaging (X-rays and ultrasound)	Only	3					6
	+Histology or cytology or necropsy	3[c]					
Consistent with FIP				34/36	3/3	1/1	3/3[c]
Inconclusive				1/25[a]	0	0	0
Inconsistent with FIP				1/25[b]	0	0	0

TABLE 5. *Specialized tests used in support of the diagnosis.*

[a]*Low cellularity.*
[b]*Necropsy results inconsistent with FIP; details are provided in the text.*
[c]*The count is included into histology, cytology, necropsy total (n = 36).*

the diagnosis was also confirmed by IHC included eight cats treated with PI only (survived 8–1,829 days) and four cats treated with PI concurrently with corticosteroids (survived 7–185 days). No concurrent treatment data were available for one cat who survived one day. The size of those subgroups was insufficient to render statistical power to the analysis.

There was no significant difference in survival times between groups treated with PI only and diagnosed without biopsy-based tests (n = 20) versus those diagnosed with biopsy-based tests (n = 7; p = 0.27, Mann–Whitney U-test). Similarly, there was no difference in survival times between groups treated with corticosteroids concurrently with PI, which were diagnosed with tissue biopsies (n = 12) or without it (n = 19; p = 0.93 Mann–Whitney U-test).

No significant difference was found between the survival times for the cats receiving oral (n = 24) or topical ocular corticosteroids (n = 7, p = 0.57, Mann–Whitney U-test); and the median survival times were 16 and 30 days, respectively. No significant differences were noted between survival times of cats with different subforms of the disease regardless of the use of steroids (**Table 6**). Survival times of cats belonging to age groups under 6, 7–12, 13–24 months, and over 25 months did not differ significantly (p = 0.90, Kruskal–Wallis test).

After the beginning of treatment, the non-effusive form progressed to effusive in six cats (10%), and five of those died or were euthanized within 2 weeks thereafter. One cat (#31) whose initial corticosteroid treatment was stopped at the beginning of PI trial developed palpable abdominal masses and effusion after 3 months of the treatment, which resolved by the next monthly visit. After 6 months on the treatment,

Form	Treatment	*n*	Survival, days mean ± SD	Survival range, days
Ocular	PI	3	99.33 ± 109.77	32–226
	PI + ToCS	2	185.50 ± 159.10	73–298
	PI + SyCS	4	67.75 ± 80.19	6–184
Neurologic	PI + SyCS	5	38.80 ± 38.21	5–100
Gastrointestinal	PI	11[a]	252.45 ± 533.25	15–1,829
	PI + SyCS	5	53.20 ± 75.60	7–185
Non-localized	PI	6[b]	268.83 ± 363.00	4–965
	PI + ToCS	1[c]	60	60
	PI + SyCS	4	11.00 ± 13.47	7–31
Mixed	PI	8[b]	77.38 ± 94.87	1–131
	PI + ToCS	6	32.67 ± 28.49	4–39
	PI + SyCS	4	17.25 ± 16.46	6–77
No signs	PI	1	148	148

PI, treated with Polyprenyl Immunostimulant, no concurrent corticosteroid treatment; PI + SyCS, treated with Polyprenyl Immunostimulant and systemic corticosteroids concurrently (includes 1 combined systemic and topical ocular corticosteroid treatment); PI + ToCS, treated with Polyprenyl Immunostimulant and topical ocular corticosteroids concurrently.

[a]*Includes two cats whose corticosteroid treatment was stopped at the start of PI treatment.*

[b]*Each number includes one cat whose corticosteroid treatment was stopped at the start of PI treatment.*

[c]*The cat initially diagnosed with non-localized form started ocular topical corticosteroid treatment after uveitis developed.*

TABLE 6. *Survival time by the subform of the disease. No statistically significant differences were observed between any groups.*

a small mass was palpated and a small amount of fluid in mid-cranial abdomen was identified per the veterinarian's records. The mass remained unchanged for the next two monthly visits, and the cat developed a distended belly with palpable fluid by the eighth month of the PI treatment. The amount of the fluid decreased by the next monthly check. Between 1 and 8 months on the treatment, the cat was doing clinically well, gained weight, returned to normal routines, played, and had an appetite. The cat started declining in the ninth month of the PI treatment, lost weight, and there was an increased amount of abdominal fluid, and the cat died naturally after 279 days from the start of the treatment.

Three of the 17 cats with ocular disease survived for over 180 days. Their initial signs included anterior uveitis[3], keratic precipitate[1], discoloration[1], anisocortia[1]. In two of those cats the anterior uveitis was significantly improved or resolved after 2 months on PI treatment with no corticosteroids. The uveitis did not improve in the one cat who was receiving topical ocular corticosteroids concurrently with PI, and the eyes were enucleated.

In the 13/22 cats with palpable abdominal masses that survived over 30 days (life span 57–1,829 days), the reduction or resolution of the abdominal masses was noted in 6/13 during

Treatment	*n*	Survival statistics, days				*p*-value (Mann–Whitney *U*-test)
		Range	Median	Mean	SD	
All study cats (*n* = 60)						
No concurrent corticosteroids	27	3–1,829	73.5	201.4	378.6	0.003
Concurrent corticosteroids:	31	3–298	21.5	47.5	49.3	
systemic	24	3–185	16	40.5	71.4	0.57
topical	7	4–298	30	51.2	103.3	
No data on concurrent treatments	2	1–15	N/A	8	9.9	N/A
Diagnosis confirmed by specialized tests on biopsied tissues (*n* = 34)						
No concurrent corticosteroids	20	3–1,829	63	180.5	400.0	0.03
Concurrent corticosteroids	12	4–185	20.5	38.9	51.3	
No data on concurrent treatments	2	1–15	N/A	8	9.9	N/A
Diagnosed without confirmation on biopsied tissues (*n* = 26)						
Necropsy/cytology inconclusive, no concurrent corticosteroids (*n* = 2)	7	4–965	148	261.1	329.7	0.04
No concurrent corticosteroids (*n* = 5)						
Concurrent corticosteroids	19	3–298	22	52.8	74.2	

TABLE 7. *Survival of cats treated with or without corticosteroids concurrently with polyprenyl immunostimulant by the method of the diagnosis.*

the first or second monthly follow-up examinations, three of the six cats received corticosteroids together with PI. One of the six cats (#31, described above), whose initial neurologic signs resolved and palpable masses were no longer reported after 1 month of the treatment, redeveloped a small palpable abdominal mass and abdominal fluid at 6 months into the treatment and 3 months prior to her natural death at 279 days; the corticosteroid treatment of this cat was stopped before PI treatment began. In another cat who received no corticosteroids, the masses were initially resected and did not reappear until 1 month prior to euthanasia at 374 days. The masses remained unchanged on palpation in 4/13 cats; all four cats received no corticosteroid treatment. The findings were confirmed by ultrasound tests in one of those four, two cats died before the follow-up examination, and follow-up data were not available for one cat.

Four cats survived over 300 days and were considered long term survivors. Their records were scrutinized in considerable detail after death. The summary of the data for these cats is presented in Table 8 and Figure 5. All four cats were brought initially to their veterinarians with signs typical of dry FIP, including inappetance (3/4), lethargy (3/4), and weight loss (4/4). One cat had persistent diarrhea and vomiting; in two of the four cats, abdominal masses were detected by their veterinarians. The A/G ratios for all four cats ranged from 0.3 to 0.5. Three of the four cats were anemic, and all had moderate to high coronavirus antibody titers of 1:320 (K-ELISA), 1:800, 1:1,600 and 1:6,400 (all three by IFA). In two of the four cats, histopathology of biopsied tissues had pyogranulomatous inflammation, and the diagnosis was validated by immunostaining for FCoV antigen.

Cat 105, the longest survivor (1,829 days), initially received prednisolone to control the weight loss but the corticosteroid therapy was discontinued at the start of PI treatment. Based on reports by owners and veterinarians, all four cats returned to normal behavior by the first checkup visit, about 1 month from the beginning of the treatment, and the weight either stabilized or increased. The clinical signs of vomiting, diarrhea, lethargy, etc. also resolved. Three cats lost weight before death; data were unavailable for one. The A/G ratios increased after 2 months on the PI treatment in the two longest survivors, and reached >0.8, and anemia improved in one and resolved in another one. The borderline anemia, although somewhat improved, was present in the Cats 2 and 52 survivors and became more pronounced before death. The treatment was stopped after about 700 days in Cat 78, and no laboratory test data are available from that time until his death at 965 days when necropsy was performed. The PI treatment frequency was tapered first to twice weekly and then to once weekly in Cat 105. All cats were euthanized in extremis. The necropsies on the Cat 2 showed lesions consistent with FIP. The necropsy on Cat 78 showed lymphoplasmacytic interstitial nephritis and cystitis cystica without lesions of FIP. Toxicosis, azotemia, and ischemia probably associated with renal failure led to the euthanasia of Cat 105. The ultrasound showed no masses on the internal organs and the results were unremarkable; no necropsy was performed.

A number of veterinarians and owners voluntarily provided information on the quality of life in communications, and veterinarians commented from the first recheck on after 30 days of PI treatment using the questionnaire. More quality-of-life-related comments were recorded by veterinarians on medical records which were analyzed. We found improved quality-of-life comments for 32 of the 34 cats that survived for more than 30 days. All comments for those 32 cats indicated an

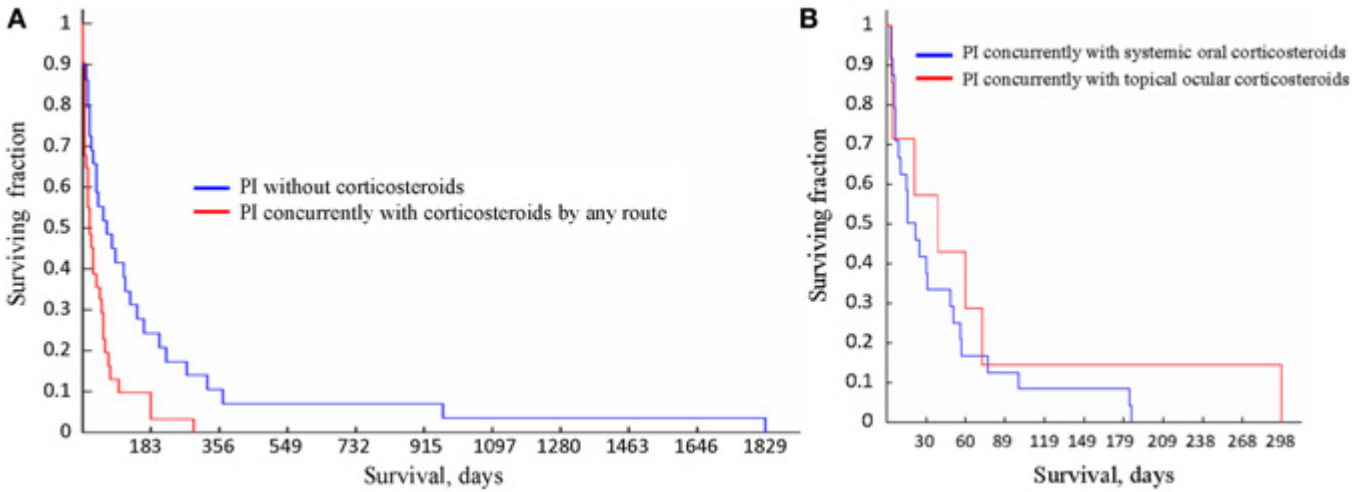

FIGURE 4. *Survival curves for the cats receiving different treatments. (**A**) Survival of the cats treated with Polyprenyl Immunostimulant (PI) without concurrent corticosteroids (blue) was significantly longer (p = 0.003, Mann–Whitney U-test) than of the cats treated PI with corticosteroid administered concurrently by any route (red). (**B**) Survival of the cats treated with PI with concurrent corticosteroid administration topically (red) or orally (blue) did not differ significantly (p = 0.57, Mann–Whitney U-test).*

Tabla de contenido:

	Cat #2 (A)	Cat #52 (B)	Cat #78 (C)	Cat #105 (D)
Age at Dx	11 years	12 months	6 months	3 years
Sex	FS	FS	MN	MN
Breed	DMH	Bengal	DSH	DLH
Housing density	2 cats	>3 cats	>20 cats	>3 cats
Household and FIP history	Not known	Previously lost 3 cats to FIP	Rescued by a rescue with FIP outbreak. Foster queen died of FIP	Not known
Keeping condition	Inside	Inside	Inside	Inside
Survival time, days	**375**	**334**	**965**	**1,829**
Initial presentation and diagnostics				
Days between initial visit and the start of PI	36	23	19	31
Diagnostic signs	Frequent vomiting, persistent diarrhea, weight loss. Not active. Palpable abdominal mass	Weight loss, vomiting, persistent diarrhea with occasional blood streak	URI, weight loss, weakness, possible diarrhea (too many cats to tell), poor appetite	Sudden weight loss, abdominal mass noted on ultrasound
Additional clinical signs	CRF (Dx 1 month after FIP)	None	Submandibular lymphoadenopathy, gingivitis, conjunctivitis	Mild gingivitis at the end of life
Differential	FIV-neg, FeLV-neg, toxoplasma-neg	FIV-neg, FeLV-neg, toxoplasma-neg	FIV-neg, FeLV-neg, toxoplasma-neg	FIV-neg, FeLV-neg, toxoplasma-neg
Albumin, g/dL	2.3	2.6	2.6	2.3
Tp, g/dl	9.3	10.8	8.3	8.8
Globulin, g/dl	7.3	8.2	5.7	6.5
A/G ratio	0.3	0.3	0.5	0.4
Bilirubin, mg/dL	0.1	0.1	0.1	0.4
WBC cells/uL	23,400	22,600[a]	20,300	11,700
HCT, %	30.9	31.0[a]	8.4	33 (N 32–49)

TABLE 8. *Conitue...*

Tabla de contenido:

	Cat #2 (A)	Cat #52 (B)	Cat #78 (C)	Cat #105 (D)
Neutrophilia	YES	YES[a]	YES	NO
Lymphopenia	YES	YES[a]	YES	YES
Monocytosis	YES	NO[a]	NO	NO
Feline coronavirus (FCoV) titer	1:6,400 (ELISA IFA)	1:320 (7B ELISA)	1:1,600 (ELISA IFA)	1:800 (ELISA IFA)
FIP subtype	GI	GI	Non-localized	GI
Specialized laboratory testing and findings	Ultrasound, resection, and anastomosis of ileocecal region. Biopsies had pyogranulomatous reaction, IHC was FCoV antigen-positive	Not done	Not done	Polyclonal gammopathy, FIP mRNA-, ultrasound, FNA, biopsy. Histopatholo revealed pyogranulomatous lymphoadenitis and pancreatitis. IHC positive for FCoV antigen
Concurrent medications	Calcitrol 10 mg orally daily, Sucralfate PRN, Metoclopramide PRN	Metoclopramide PRN	PI treatment stopped after about 700 days	PI was tapered to 2× weekly after 3 years and to once weekly after 4 yea
Progress on the PI treatment				
Hyperglobulinemia and A/G ratio	No major change	No major change	Changed to WNL after 2 months	Decreased to normal range after 1 mo
Anemia	No. Before death only	No. Last test 40 days before death	Hematocrit increased to >30% after 2 months on PI. No data after 700 days	Increased to >35% after 2 months on decreased before death
Diagnostic clinical signs	Resolved	Resolved	Resolved	Resolved
Life quality	Improved and returned to normal	Returned to normal	Improved, stable (records until the end of PI treatment). Cystitis Dx after 600 days on PI	Returned to normal

TABLE 8. *Conitue...*

	Cat #2 (A)	Cat #52 (B)	Cat #78 (C)	Cat #105 (D)
Cause of death	Euth: weight loss, lethargic, dehydrated, trouble breathing	Euth: inappetance, lethargy, weight loss, fever	Euth *in extremis*	Euth: anorexia, vomiting, severe azotemia indicating kidney failure or severe trauma from infection or toxin/ischemic injury. No abdominal mass or ultrasound before death
Necropsy	Pleural effusion, small mesenteric mass, close to the resection site. Multifocal granulomatous colitis and hepatitis consistent with FIP	Not done	Mild changes consistent with prior hepatic injury and nephritis, cystitis cystica	Not done

[a]*Tested 9 days after the start of the PI treatment.*

CRF, chronic renal failure, chronic renal insufficiency; Euth, euthanasia; FCoV, feline corona virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FNA, fine needle aspirate; IHC, immunohistochemistry; URI, upper respiratory infection; WNL, within normal limits.

TABLE 8. *Case summaries for the cats with feline infectious peritonitis (fip) surviving over 300 days on polyprenyl immunostimulant (pi) treatment.*

improvement in the perceived quality of life ("return to normal," "as before the disease," "appears healthy," got back to normal routines, and/or improvement in appetite, mobility, socialization, and responsiveness) during the preceding period, most commonly every month on the treatment. Weight stabilized or increased in 31/32 patients, while 1/32 continued to lose weight while showing improvement in behavior and appetite.

No toxicity or adverse events due to the administration of PI were reported by the veterinarians or owners.

Discussion

We report that of the 60 cats with presumed non-effusive FIP diagnosed using the recognized algorithm[2] and treated with PI 1 cat survived for 1,829 days, 2 cats for over 900 days, 4 cats survived over 300 days, 8 for over 200 days (one of those survived 298 days), and 16 lived over 100 days from the start of treatment. The 31 cats given oral corticosteroids or receiving topical ocular corticosteroids concurrently with PI survived a mean of 47. 5 ± 49.3 days (3–298 days, median 21.5 days), while the 27 cats treated with PI without corticosteroids survived a mean of 201.4 ± 378.6 days (3–1,829 days, median 73.5 days), which is significantly longer (**Table 7; Figure 4A**). Currently the most common therapy for FIP is corticosteroid[2,4].

Of the 35 cats started on corticosteroids at the time of diagnosis, the treatment was stopped in 4 cats before the start on PI, while 31 were continued on corticosteroids concurrently with PI. The four cats whose corticosteroid treatment was stopped survived a mean of 569.50 ± 844.65 days (79–1,829 days, median 91 days). These four cats had multiple signs of the disease, and none of the four cats at the time of diagnosis appeared less severely affected than the other cats in our study.

In the subgroups of FIP, there was a difference in the likelihood of corticosteroid use, with 31% (5/16) in the GI subform of FIP receiving corticosteroids to 100% (5/5) in neurologic subforms receiving corticosteroids. Both topical and ocular corticosteroids appeared to

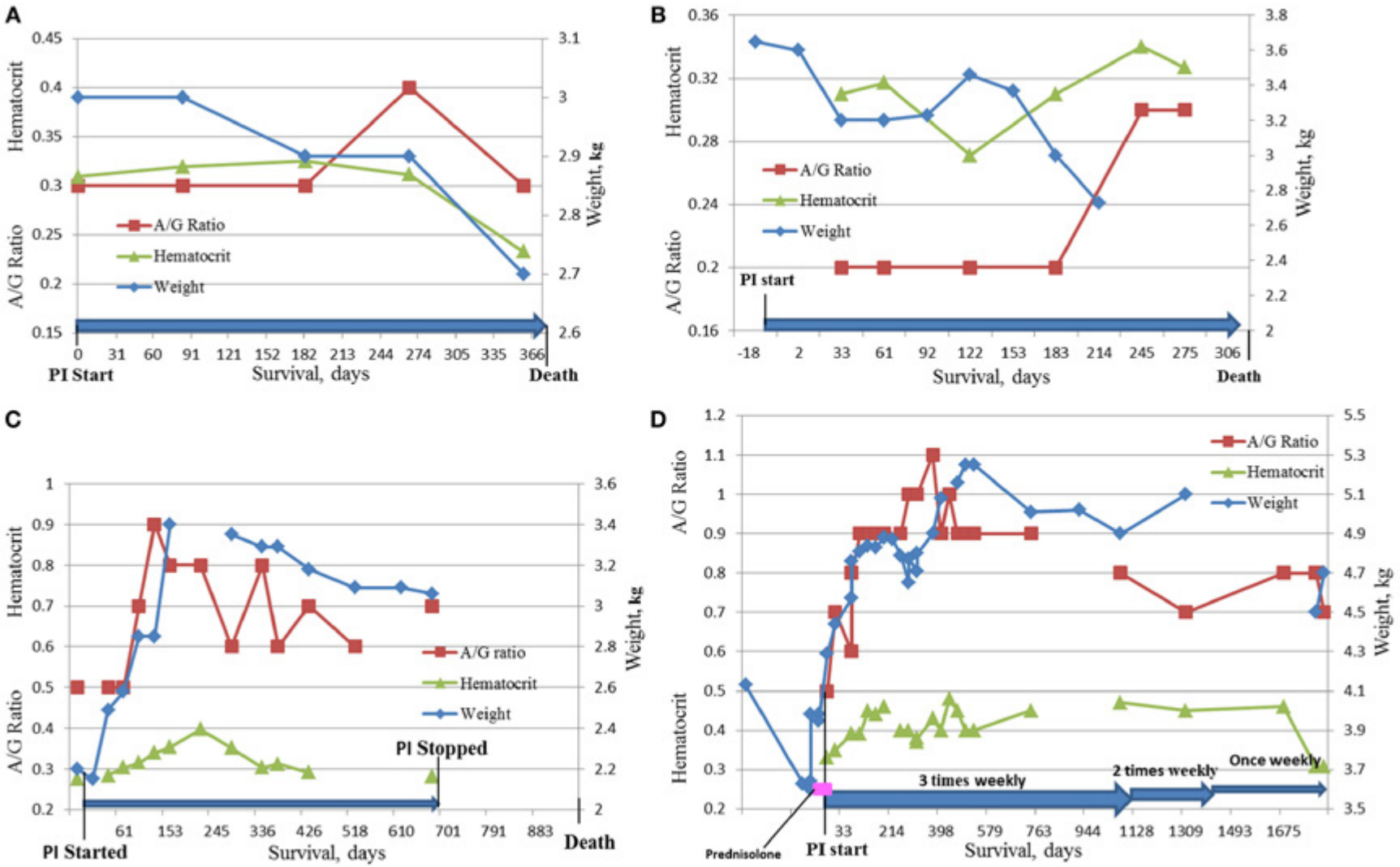

FIGURE 5. *DYNAMICS OF HCT, A/G RATIO, AND WEIGHT IN THE FOUR LONGEST SURVIVORS ON THE STUDY. (**A**) Cat #2, 375 days survival; (**B**) Cat # 52, 334 days survival; (**C**) Cat #78, 965 days survival; (**D**) Cat #105, 1,829 days survival.*

Tabla de contenido:

reduce survival times when given concurrently with PI (**Figure 4B**).

We accepted all cats that met the inclusion criteria and did not intentionally select less severe cases for the study. No assessment of the severity of the disease was done. Our first assumption was that corticosteroids were used in the more severe cases but we could find no justification for that assumption. But the small sample size does not allow ruling out that the shorter survival times of the cats concurrently treated with PI and corticosteroids may indirectly reflect the severity of the disease.

We observed no statistically significant differences between survival of the cats either for the subforms of the disease (ocular, neurologic, gastrointestinal, non-localized, or mixed), or between different age groups (≤6 months, 7–12 month, 13–24 months, and over 25 months) which may be because of a very wide variance in each of the groups. We did not have a sufficient number of cats in each group for the statistical power to validate any conclusions.

The literature offers limited data on survival times of cats with dry form FIP with or without treatments. Cats in the literature with dry form FIP treated with corticosteroids and supportive care had a survival range of 1–200 days (n = 51). One early study[8] examined field records for intestinal, granulomatous manifestation (n = 26), and reported the survival time in this non-effusive FIP form as "up to 9 months" in a cat that was lost to follow-up. More careful retrospective record studies reported the survival time at 7–45 days in cats with histologically confirmed diagnosis(8 effusive, 5 non-effusive, no separate data provided; 9), and 7–60 days (n = 4, 1 of 4 was on IFNα-rHU; 10). Reports of clinical studies in natural infection put survival with dry FIP at 38 days (n = 1; 11), 1–171 days (n = 11; 6), 6–33 days without treatment (n = 4), and 4–42 days in cats previously given an FIP vaccine as a preventative (n = 4; 13).

The literature mentions an individual cat diagnosed with the dry form that survived for 200 days with glucocorticoid and ω-interferon treatment[6] and two cats survived 181 and 477 days with mixed dry/wet form treated with glucocorticoid, human α-interferon, and nelfinavir[7]. A recent mention[18] of a natural survival time of over a year without treatment in cats with dry form FIP referred to cats that were treated with PI[15].

The survival times from this study were compared to published data of cats with dry form FIP. The data suggest a lengthening of the survival times in cats treated with PI. The survival times equaled or exceeded the longest survival times reported in the literature for dry FIP, with 8 of 60 cats in our study exceeding the maximum reported number of 200 days.

The diagnosis of dry FIP in our field study was done at different levels of diagnostic certainty, and we compared survival times in the subgroups—diagnosed, or not, by histologic, cytologic, or immunostaining tests and found no statistically significant differences based on the proof of the diagnosis, although there were significant differences between cats treated with corticosteroids concurrently with PI or not. There was no significant difference in the survival times of cats who did not receive steroids regardless of whether they were diagnosed by any biopsy-based method or diagnosed without invasive methods. The survival time was the same regardless of the diagnostic method used.

A comparison of mean and SD values for the survival times of cats with dry FIP showed that the survival time of cats in our study was significantly longer for the group treated with PI without concurrent corticosteroid treatment (201.4 ± 378.6 days, n = 27) than the published 38.4 ± 48.8 days (n = 11; 7); p = 0.04. The survival time for the group treated with corticosteroids concurrently with PI was 47.45 ± 49.26 days (n = 31), which does not significantly differ (p = 0.59) from the published value[7]. Corticosteroids are the usual treatment

for FIP signs; however, they need to be used with caution if PI is used.

Feline infectious peritonitis is considered 100% fatal which, in our study plan, precluded for ethical reasons an experimental design that used an untreated placebo group. For statistical comparison of data, a placebo group is ideal. This study was modeled on human studies that use single-arm trials of anti-cancer drugs with survival as the end-point. In those studies, no placebo or best accepted therapy is used because of the lack of known beneficial treatment and the universally grave prognosis[19]. Our study was a field trial dependent on primary care veterinarians to identify candidates for the study and treat those accepted in accordance with the protocol. On ethical grounds and considering the reluctance of cat owners to participate in a placebo-controlled study, we elected the presented study design.

Our decision to exclude cats with wet form FIP was based on the rapid progression of the wet form (median of 9 days; 5) which we assumed correctly to be faster than the expected median time needed for acceptance of a cat into the study (which turned out to be 22.97 ± 21.60 days). Additionally, our prior limited studies of PI in effusive FIP did not appear promising while treatment of dry form FIP was encouraging.

The diagnosis of dry FIP was done at three levels of certainty:[1] highly probable, diagnosis supported by history, clinical signs, and laboratory findings without specialized lab tests; this category included neurologic forms in which biopsies are not possible and ocular forms when ocular centesis was used;[2] with histologic or cytologic confirmation of the diagnosis with or without immunostaining; this group also included cats with confirmation done on necropsied tissues; and its subgroup[3] with the confirmation of the presence of the FCoV antigen by IHC. There was no statistically significant difference in the survival between the cats diagnosed at different certainty levels and treated with PI only. We could not compare survival times between the subgroups treated with corticosteroids due to insufficient sample power.

Making an antemortem diagnosis of dry FIP is notoriously hard in the absence of a single, standardized test and relies on the combination of non-specific "disease-indicators" especially if the owners do not allow invasive procedures. Test data in clinical chemistry, hematology, serology, clinical assessment, histology, etc. have different predictive values and are not pathognomonic[2,14,18,20]. In the absence of a single, 100% reliable diagnostic approach, all diagnostic efforts are aimed to increase the "index of suspicion" as it was called by Diaz and Poma[21]. The requirements for a "gold standard" vary between research groups with some accepting histopathology and others stressing IHC[14,20], while the consensus document by Addie et al.[2] avoids the definition of the "definitive diagnosis" altogether. In clinical practice, most diagnoses are made by laboratory findings consistent with FIP and excluding other diseases[3,18].

The primary care veterinarians and veterinary specialists treating these cats were comfortable with the diagnosis of dry form FIP, but we are using the term "presumptive FIP" because the "gold standard" of coronavirus antigen by IHC in lesions consistent with dry form FIP was not achieved with all cats. The cats in this study were diagnosed by their veterinarians based on the assessment of the combination of signalment, clinical signs, and as many tests as necessary to rule out other diseases and support the diagnosis, relying on experience[18], in the mode documented by Rohrbach et al.[22]. The diagnosis, made by the primary care veterinarian in conjunction with specialists as needed, was reviewed by AML using criteria consistent with the evidence-based algorithm[2]. Specialized testing such as histology, cytology, CSF tap, PCR on aqueous humor, IHC, and necropsy was done in 36/60 cats. When the presumptive diagnosis is made, owners are of-

Tabla de contenido:

ten reluctant to get the confirmation through the invasive procedures.

The diagnosis for each cat on the study was established individually, and we also compared our data to the data published for cats with a confirmed diagnosis of FIP[1,7,20,23]. Signalment and housing density were similar to the reported data[23] with the majority of the cats being young, non-pedigreed, and originating from multi-cat households. The age distribution of the FIP patients in our study underscored a well-established age bias for FIP[12,22]. Clinical presentations observed in our sample were similar to the reported for the cats with dry form FIP[20,23].

Diseases that may be clinically similar to FIP were excluded by specific tests in most cats. Feline leukemia virus (FeLV) antigen and FIV antibody were measured in 50/60 cats and one of those was positive for FIV antibodies. The FIP diagnosis of the only FIV-positive cat was given by a neurologist based on the results of MRI and CSF tap. Antibodies for toxoplasma were measured in 18 cats and all were negative. Ten cats were not tested for FeLV and FIV; six had the diagnosis confirmed by specialized tests on the biopsied tissues (four IHC and two histology), and the other four had a number of strong FIP indicators and considered presumptive.

We analyzed the initial biochemical parameters of diagnostic value for FIP. In young cats, with high serum globulin and low A/G ratios, there are few conditions except FIP that are likely. Plasma cell and B-lymphocyte malignancies can produce a monoclonal gammopathy, but these conditions are rare in young cats. Chronic inflammatory or infectious conditions such as chronic abscesses and pyothorax could increase serum globulin levels but these conditions would likely be identified by diagnostic evaluation. Hyperglobulinemia and/or an albumin/globulin (A/G) ratio ≤0.6 were noted in 48 of the 50 (96%) cats; two cats had A/G ratio equal 0.8. At the initial evaluation of the cats in our study, there was hyperglobulinemia in 64.7% of the cats, which is similar to the value reported in the literature in the combined wet-dry group[7]. The albumin/globulin ratio offers the highest positive prediction value for FIP[20]. In our group it was 0.37 ± 0.14, which is consistent with the reported values for FIP in general[20] and lower than the mean and SD reported for the initial presentation by Tsai et al.[7].

Hyperbilirubinemia occurred in 16% of the cats which is consistent with dry form FIP and is lower than reported in the groups with wet FIP[7,23]. Hyperglobulinemia is an important diagnostic sign because it is rarely associated with diseases other than FIP in young kittens, which is the most often affected group.

The initial hematologic parameters of diagnostic value for FIP were analyzed. Leukocytosis and neutrophilia were found in 45.1 and 54.5% of the cats, respectively; both values are consistent with the published[23]. Lymphopenia was present in 33.3% of the study cats consistent with previous studies[23].

Coronavirus antibody titers are somewhat helpful in the diagnosis of FIP[2,4,17]. Negative serum antibody titers make the diagnosis of FIP unlikely, while high and very high titers are supportive of the diagnosis. However, mid-range to high antibody titers can occur with coronavirus infection without FIP. All tested cats accepted to the study had coronavirus antibody titers with most of the cats having high (400–1,280; n = 13), very high (>1,280, n = 36), or extremely high positive titers (>12,800, n = 10).

The only cat on the study without signs of FIP was diagnosed by the veterinarian after a pre-neuter checkup. The follow-up cytology of the mesenteric lymph node aspirate was inconclusive because the cellularity was insufficient to definitively demonstrate pyogranulomatous inflammation, although there was no evidence of neoplasia or other infectious organisms. The cat developed effusion and died after 148 days of the treatment with PI and corticosteroids concurrently.

The four cats who survived over 300 days had clinical signs and diagnostic tests consistent with FIP at initial examination. A detailed history and diagnostic information is given in Table 8 and Figures 5A–D. Although cats 52 and 78 did not have histopathology or IHC, their age (12 and 6 months), exposure in multi-cat, FCoV environments, clinical signs, laboratory findings, and high FCoV antibody titers were sufficient to establish a clinical diagnosis. Cats 2 and 105 had their diagnoses confirmed by histology and cytology and IHC. All four showed clinical improvement and returned to normal behavior. Cats 2 and 52 had only modest improvements in laboratory findings despite clinical improvements (**Figures 5A,B**). The clinical decline in those two cats started 2 weeks before their death and was accompanied by weight loss, anorexia, worsening anemia, reappearance of abdominal masses at the resection site (Cat 2), and pleural effusion in the same cat. Redevelopment of the abdominal mass and progression of the disease is common in the cats where the masses were resected[8].

The two longest survivors, cats 78 and 105, had weight loss at diagnosis but gained weight while on the PI treatment. The weight gain in cat 105 started 1 week before the trial and was attributed to prednisolone (5 mg daily). The prednisolone treatment was tapered and stopped during the first week of the PI treatment and the cat continued gaining weight. The clinical improvements and weight gain in both cats were accompanied by improvements in A/G ratios and hematocrits (**Figures 5B,C**). In both cats, PI treatment was either stopped or its frequency was decreased after over 2 years of survival. Cat 105 died of renal failure. Both cats declined at the end of life and were euthanized in extremis. The lack of pyogranulomatous changes in the biopsied tissues in cat 78 is consistent with the observation that cats experimentally infected with FIPV and who had clinical disease and survived were "free of lesions" post-mortem[14,24]. The necropsy changes of mild hepatic firbrosis and mild chronic lymphoplasmacytic interstitial nephritis are not adequate to account for the demise of the cat. There was no evidence of lesions of FIP. The A/G ratio of 0.5 at the initial diagnosis was returned to normal suggesting resolution of the FIP. No necropsy was performed on cat 105.

We did not use a formalized assessment of the quality of life. We collected information about clinical and behavior changes from progress reports, communications, and veterinarian charts. All progress reports and notes indicated an improved quality of life, returning to normal pre-diagnosis behavior as expressed in comments by both owners and veterinarians, e.g., "Very energetic … doing well," "acts normal," etc. In the medical records, cats were noted as having more energy, being more playful, interacting more with owners, and essentially returning to their pre-FIP behavior. In the 34 cats that lived for 30 + days, clinical improvement, i.e., an improvement in one or more signs such as increased appetite or an abatement of fever, was anecdotally noted after 10–14 days (four to six doses of PI); 10/34 reported weight gain, 5/34 reported weight loss, the weight remained stable for 17 cats, no records were filed for 2 cats. Generally speaking, the cats treated with PI returned to regular routines with occasional "bad days" until a precipitous decline led to death or euthanasia within days.

Our results suggest that PI benefits cats clinically diagnosed with dry FIP by increasing survival times and improving quality of life but a controlled study will be needed to verify the benefit of PI in the treatment of FIP. While not a cure, PI may maintain FIP cats as a chronic condition as opposed to the fast-progressing fatal disease. It may be possible to predict and monitor the survival by the normalization of A/G ratio and hematocrit. Survival times with PI treatment are significantly longer when corticosteroids are not used concurrently.

Author Contributions

AL: conception and design of the work, interpretation of the data, revising the manuscript critically for important intellectual content, final approval of the version to be published. TK: post-study acquisition of the data, analysis and interpretation of data for the work, drafting the work and revising it critically for important intellectual content, final approval of the version to be published. GG: data acquisition, entry and organization, final approval of the version to be published. VB: data acquisition, entry and organization, revising the data and the manuscript critically for important intellectual content, final approval of the version to be published. RH: data analysis and interpretation, revising the manuscript critically for important intellectual content, final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest Statement

AL, GG, VB, and RH do not have a financial interest in Sass & Sass, Inc. TK is an employee and a minor stakeholder in Sass & Sass, Inc. VB and RH were consultants to Sass & Sass. No financial incentives were provided to owners and veterinarians participating on the study.

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https://axoncomunicacion.net/tratamiento-inmunoestimulante-de-poliprenilo-de-gatos-con-peritonitis-infecciosa-felina-presuntiva-no-efectiva-en-un-estudio-de-campo/

Tabla de contenido:

Dietas de **proteína hidrolizada** en el diagnóstico de reacciones adversas al alimento con manifestaciones cutáneas

Javier Manzanares Rodríguez

Veterinario, Comunicación Científica de Royal Canin

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Se denomina reacciones adversas al alimento con manifestaciones cutáneas (cAFR) al conjunto de alteraciones dermatológicas asociadas a la ingestión de un determinado componente presente en algún/os ingrediente/s del alimento. Este componente normalmente es una glucoproteína de 10 a 70 kilodaltons de tamaño, siendo las proteínas más comúnmente incluidas en la composición de la dieta de una población concreta las que más frecuentemente inducen reacción adversa alimentaria. Las más comunes en perros son las de ternera, lácteos, pollo y trigo. En el gato, los alérgenos alimentarios más reportados son las proteínas de ternera, pescado y pollo.

El signo dermatológico más frecuente es el prurito no estacional, pudiendo ser este generalizado o localizado. Las lesiones cutáneas son muy variables y poco específicas, no existiendo un patrón lesional patognomónico aunque, en gatos, la distribución de éstas se ha observado más frecuentemente en las regiones facial y cervical.

“El signo dermatológico más frecuente es el prurito no estacional”

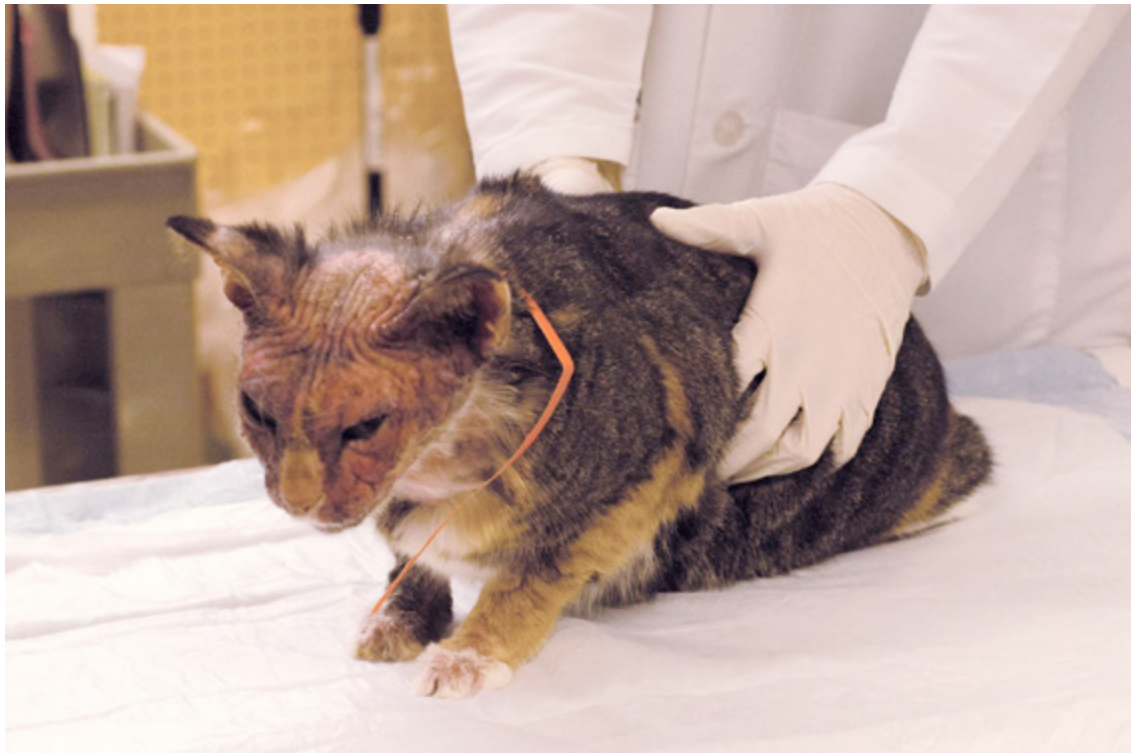

Figura 1. *En los gatos pueden aparecer excoriaciones en la cabeza y cuello como parte del patrón de dermatitis miliar secundario a cAFR. © Karen L. Campbell*

Fisiopatología de las cAFRs

La fisiopatología de la cAFRs inmunomediadas (comúnmente conocidas como alergias alimentarias) en gatos y perros no es completamente conocida, aunque se cree que las reacciones de hipersensibilidad más frecuentemente implicadas son las de tipo I (mediadas por IgE). La reacción alérgica se produce como consecuencia de la liberación de mediadores celulares (histamina) de los mastocitos previamente sensibilizados y que provocan una respuesta inflamatoria causando signos dermatológicos, respiratorios, gastrointestinales o una combinación de estos. Para que esto tenga lugar se requiere la unión de dos secuencias de aminoácidos de la misma proteína a dos IgE situadas en la superficie del mastocito.

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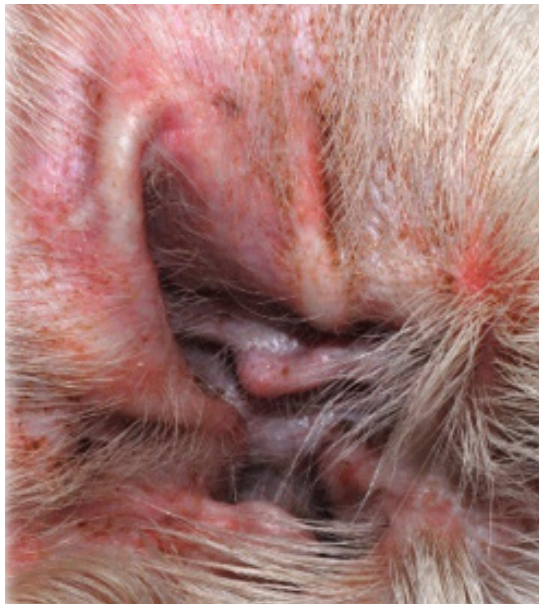

Figura 2. *En los cachorros, las dietas basadas en proteína hidrolizada deben cumplir las necesidades nutricionales de la fase de crecimiento.*

“Para evaluar la eficacia de estas dietas se debe valorar el producto final, y no únicamente las proteínas hidrolizadas utilizadas”

Dietas basadas en proteína hidrolizada

El manejo de las cAFRs se basa en la elección de la dieta más adecuada. Las opciones disponibles son: por un lado, las dietas con proteína seleccionada (proteína novel) que pueden ser caseras o comerciales, y por otro, las dietas basadas en proteína hidrolizada. Las primeras, se basan en la elección de una proteína a la que el animal no haya estado expuesto anteriormente y su recomendación para este fin empieza a estar en desuso debido a que se utilizan multitud de fuentes de proteína en los alimentos comerciales de las distintas marcas, además del potencial riesgo de reactividades cruzadas entre las distintas fuentes de proteína.

La proteína hidrolizada se obtiene tras un proceso denominado hidrólisis enzimática que consiste en la división de la proteína en péptidos más pequeños. Como consecuencia de la disminución del tamaño de las proteínas se reducen las probabilidades de que se produzca la unión con dos IgE y que se libere histamina. Por otro lado, la gran digestibilidad de estos péptidos también reduce el tiempo de permanencia en el intestino, haciendo que la posibilidad de reacciones alérgicas o intolerancias sea mínima.

Las dietas hipoalergénicas basadas en proteína hidrolizada además, deben tener en cuenta otros factores, como es el control y limpieza en todo el proceso de fabricación para evitar una contaminación con antígenos alimentarios indeseados. Por tanto, para evaluar la eficacia de estas dietas se debe valorar el producto final, y no únicamente las proteínas hidrolizadas utilizadas.

Dietas basadas en proteína extensivamente hidrolizada

La probabilidad de una respuesta alérgica disminuye con estas dietas basadas en proteína hidrolizada, consiguiéndose excelentes resultados en un 90-95% de los casos. Sin embargo, una reacción alérgica todavía es posible en un pequeño porcentaje y para estos casos se han desarrollado las dietas basadas en proteína extensivamente hidrolizada. Las diferencias con las dietas de proteína hidrolizada “clásicas” se refieren tanto al grado de hidrólisis de la proteína como a la selección del resto de sus componentes y a las medidas higiénicas y de control llevadas a cabo durante su producción.

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Respecto al grado de hidrólisis de estas dietas, la fuente de proteína se somete a una hidrólisis extrema y su peso molecular es inferior a 1 kD del que el 88% está constituido por aminoácidos libres. En cuanto a la selección de los componentes no proteicos, es recomendable que estas dietas incluyan una única fuente de hidratos de carbono y que esté purificada, es decir, que no contenga ningún resto proteico. Por otro lado, es necesario tomar medidas de control exhaustivas a lo largo de todo el proceso de producción, desde limpieza de toda la línea de producción, hasta análisis de las materias primas y control del producto final mediante PCR para garantizar que no ha habido contaminación en todo el proceso.

Debido a todas las razones anteriormente expuestas, estas dietas basadas en proteína extensivamente hidrolizada tienen un potencial alérgeno muy bajo, convirtiéndolas en las de elección para ser utilizadas como dieta de eliminación en el diagnóstico de las cAFRs.

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Gracias a 50 años de ciencia, a una observación meticulosa y a la colaboración con veterinarios, sabemos que podemos utilizar una nutrición específica desde el diagnóstico de la enfermedad hasta su manejo nutricional a largo plazo.

Por eso, hemos desarrollado una amplia gama de soluciones nutricionales a medida para dar respuesta a cada una de las fases del proceso clínico, incluyendo **HYPOALLERGENIC PUPPY:** la nueva respuesta nutricional específicamente formulada para cachorros a partir de los 6 meses y hasta el final del crecimiento.

DERMATOLOGY

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Tres suplementos alimentarios que ayudan a mantener la función cardíaca

La adición de determinados suplementos aminoacídicos y componentes derivados de aminoácidos a la dieta de las mascotas puede ayudar al tratamiento de determinadas patologías. Además, la deficiencia de algunos de ellos puede ser la causa directa de enfermedades del corazón.

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Taurina

La taurina es uno de los aminoácidos libres más abundantes. Se encuentra en elevadas concentraciones en los tejidos del músculo cardiaco, músculo esquelético, sistema nervioso central y plaquetas. Actúa en numerosos procesos metabólicos, ejerciendo diversas funciones:

- Antioxidación
- Actividad en las células fotorreceptoras de la retina
- Estabilización de las membranas neuronales
- Desarrollo del sistema nervioso
- Reducción de la agregación plaquetaria
- Reproducción
- Actividad miocárdica[1]:
  - Modulación de las concentraciones de calcio en los tejidos y su disponibilidad.
  - Inactivación de los radicales libres y cambio de la osmolaridad celular.
  - Efectos en la osmorregulación del miocardio.
  - Otros mecanismos específicamente relacionados con la función miocárdica incluyen la N-metilación de los fosfolípidos de la membrana celular, efectos directos en las proteínas contráctiles e interacciones con el sistema renina-angiotensina-aldosterona.

¿Qué ocurre en casos de deficiencia de taurina?

En **gatos**, la taurina es un aminoácido esencial y su deficiencia puede causar miocardiopatía dilatada (MCD), degeneración de la retina y anomalías reproductivas. Existen evidencias de que la MCD causada por su deficiencia puede ser reversible con la suplementación de este aminoácido[2,3].

En **perros**, hasta hace unos años, la taurina no se consideraba un aminoácido esencial ni se conocía su papel en el desarrollo de la MCD[4]. Sin

CALIER

embargo, diversos estudios han demostrado que sí lo es en perros alimentados con dietas restrictivas en proteína y que, al igual que los gatos, pueden desarrollar MCD secundaria a la deficiencia de taurina[5].

L-carnitina

La L-carnitina es un derivado aminoacídico que se obtiene de la proteína de la dieta o por síntesis endógena en el hígado, siendo la lisina y la metionina los aminoácidos precursores. La síntesis requiere hierro, vitamina C y vitamina B6 como cofactores. El músculo esquelético y el cardiaco son los lugares donde se almacena hasta el 95-98% de la carnitina del cuerpo.

Entre las funciones de la carnitina, la más importante es la de cofactor de algunas enzimas necesarias para el transporte de ácidos grasos de cadena larga al interior de las mitocondrias, donde se oxidan para la generación de energía para el corazón, que obtiene de esta manera aproximadamente el 60% de su producción de energía total.

¿Qué es la miocardiopatía dilatada (MCD)?

Se trata de una enfermedad del corazón muy habitual, progresiva y, en gran medida, irreversible, que puede conducir a fallo cardiaco congestivo o muerte súbita. Es la segunda enfermedad cardiaca más habitual en perros, con una prevalencia superior al 50% en algunas razas10. La nutrición está actualmente aceptada como un importante adyuvante a la terapia médica en perros y gatos con MCD.

¿Qué ocurre en casos de deficiencia de L-carnitina?

La deficiencia de L-carnitina puede ser un trastorno primario o secundario.

- Las deficiencias primarias pueden aparecer por defectos genéticos en la síntesis, transporte, absorción o degradación. En personas se han asociado con cardiomiopatías.
- Las deficiencias secundarias son más comunes en pacientes que siguen dietas restrictivas

Se ha demostrado en perros que la deficiencia de L-carnitina puede favorecer el desarrollo de MCD en perros. Además, varios estudios[6,7,8,9,10] han puesto de manifiesto que suplementar con carnitina mejora el tiempo de supervivencia de perros con MCD.

Hidrolizado de levadura de cerveza

La levadura de cerveza es un subproducto de la industria cervecera que puede ser un ingrediente beneficioso en la alimentación de las mascotas, ya que aporta el contenido nutricional que necesitan los perros y gatos[11]. Esterilizada y sin poder leudante, es una levadura inactiva compuesta por el organismo unicelular Saccharomyces cerevisiae.

Su administración tiene efectos beneficiosos sobre la salud intestinal y la función inmune de los perros, estimulando las respuestas Th1 y, en consecuencia, la inflamación. Además, mejoran la palatabilidad de las dietas[12]. Esto resulta especialmente útil para los perros con poco apetito a consecuencia de una enfermedad crónica.

Es una fuente proteínica rica en aminoácidos esenciales y vitaminas del grupo B:

- Los aminoácidos ayudan a la mascota a construir y mantener sus músculos, huesos, sangre, órganos, sistema inmunitario y pelaje y uñas. En particular, la arginina es un aminoácido esencial que reacciona con el oxígeno para producir óxido nítrico. El óxido nítrico relaja los músculos lisos de los vasos sanguíneos y reduce la presión arterial[13]. La hipertensión puede contribuir a las cardiopatías y a la insuficiencia cardíaca crónica, por lo que es conveniente controlar la tensión arterial de cualquier perro sospechoso de padecer una cardiopatía.
- Las vitaminas del grupo B contribuyen a la función cerebral, la fuerza muscular, la producción de glóbulos rojos y la digestión de los animales.

Por otro lado, se ha demostrado que estimula la producción de determinados marcadores de defensa antioxidantes, lo que ayuda a mejorar la salud cardiovascular de los animales[14].

- A medida que progresa la insuficiencia cardiaca congestiva, aumenta el daño a las células cardiacas por la formación de radicales libres. Los estudios realizados en perros con insuficiencia cardíaca congestiva han demostrado que estos pacientes presentan un aumento de oxidantes reactivos y una disminución de antioxidantes a medida que progresa la enfermedad[15].
- En perros con fallo cardiaco, la oxigenación y el metabolismo celular no funcionan de forma apropiada, lo que conlleva la producción de elevadas cantidades de radicales libres. Los radicales libres son responsables de los principales daños celulares, lo que se denomina estrés oxidativo[16].

Adicionalmente, la levadura de cerveza contiene sodio, calcio, magnesio y potasio. Muchos de los medicamentos utilizados para tratar las cardiopatías disminuyen los niveles sanguíneos de potasio y magnesio.

o Unos niveles inadecuados de potasio y magnesio pueden favorecer las arritmias cardiacas y debilitar las contracciones del músculo cardiaco[17].

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