UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE

COMPARATIVE STUDY OF THE EFFICACY AND SAFETY BETWEEN AMINOSIDINE (PAROMOMYCIN) AND MEGLUMINE ANTIMONATE FOR THE TREATMENT OF CANINE LEISHMANIOSIS DUE TO *LEISHMANIA INFANTUM*

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

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Γ.Π.Α.: Γεωπονικό Πανεπιστήμιο Αθηνών

Π.Θ.: Πανεπιστήμιο Θεσσαλίας

AG: albumin/globulin

AHCC: active hexose correlated compound

BAER: brainstem auditory evoked potentials

BUN: blood urea nitrogen

CanL: canine leishmaniosis

Cr: creatinine

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

IFAT: indirect immunofluorescence antibody testing

Ig: immunoglobulin

IL: interleukin

INF-γ: interferon-γ

iP: inorganic phosphorus

LST: leishmanin skin test

nPCR: nested polymerase chain reaction

PCR: polymerase chain reaction

PO: *per os*

RNA: ribonucleic acid

RT-PCR: real-time polymerase chain reaction

Slc11a1: solute carrier family 11-member a1

Th1: T-helper 1 lymphocytes

Th2: T-helper 2 lymphocytes

TLR: toll-like receptors

TNF-a: tumor necrosis factor-a

UPC: urine protein/creatinine ratio

WHO: World Health Organization

ΠΡΟΛΟΓΟΣ

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PART ONE-REVIEW OF THE LITERATURE

1. Introduction

Leishmanioses are a group of protozoan diseases caused by the intracellular protozoa of the genus *Leishmania* that infect humans, dogs and many other animal species. In human medicine they are considered neglected but emerging diseases and they have variable clinical manifestations. Human leishmanioses are classified into cutaneous (including mucocutaneous) and visceral forms, with an estimated incidence of 700,000-1,500,000 and of 200,000-500,000 cases per year, respectively, and with a 10% mortality rate for the visceral forms (Alvar et al., 2012).

Canine infection by *Leishmania* spp. is endemic in more than 70 countries and approximately 2.5 million dogs in southwestern Europe are considered to be infected (Pennisi, 2015). Among the more than 30 species of the genus *Leishmania*, at least 10 are pathogenic for dogs [*L. amazonensis*, *L. arabica*, *L. braziliensis*, *L. colombiensis*, *L. donovani*, *L. infantum* (Syn. *L. chagasi*), *L. major*, *L. mexicana*, *L. peruviana*, and *L. tropica*]. However, in the Mediterranean basin, the most important and widespread is *L. infantum* with only sporadic cases attributed to other species like *L. donovani* or *L. tropica* (Saridomichelakis, 2009; Pennisi, 2015).

Numerous treatment options for CanL have been studied over the years. Currently, the treatment of choice includes the oral administration of allopurinol along with either parenteral meglumine antimoniate or oral miltefosine (Solano-Gallego et al., 2009). However, the emergence of parasite strains that are resistant to the drugs commonly used for the treatment of CanL and human leishmanioses is alarming and renders research for alternative treatment options highly important from both a veterinary and a public health point of view (Olliaro, 2010).

2. Life cycle, transmission and epidemiology

Leishmania spp. are diphasic parasites that complete their life cycle in two hosts: an intermediate host (vector) that harbors the flagellated promastigotes and supports their development to the infective metacyclic promastigote stage, and a vertebrate final host where the parasites develop to the amastigote form (Solano-Gallego et al., 2011). Biting female sand-flies of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) are the only proven vectors of the parasites. The vectorial

capacity of other arthropods (e.g. *Rhipicephalus sanguineous*, *Ctenocephalides felis*) has been proposed, but there is no convincing evidence, mainly because it has not been proven that they can support parasite development to the infective stage (Paz et al., 2010; Medeiros-Silva et al., 2015). Only 98 out of the more than 800 sand-fly species are proven intermediate hosts of different *Leishmania* spp. In Europe, the sand-flies that are proven vectors of *L. infantum* include *P. ariasi*, *P. balcanicus*, *P. kandelaki*, *P. langeroni*, *P. neglectus*, *P. perfiliewi*, *P. perniciosous* and *P. tobbi*, whereas, some additional species are suspect vectors, such as *P. mascitii* that has been incriminated as a possible vector in non-endemic areas of Austria, Belgium, France and Germany (Antoniou et al., 2013; Mencke, 2013).

Various mammalian species can be infected by *L. infatum* and the role of cats, lagomorphs and even humans in parasite's epidemiology has been recently revised (Jimenez et al., 2013; Miro et al., 2014; Moreno et al., 2014). However, dogs are considered the main reservoir host (Gonzalez et al., 2015). Unique features that render dogs an ideal reservoir for this parasite include the high prevalence of asymptomatic infections, the long incubation period and the high parasitic density in the superficial dermis where sand fly vectors bite their hosts (Saridomichelakis, 2009; Pennisi, 2015).

The transmission of *L. infantum* among dogs is typically accomplished by the inoculation of the parasite during the meal of infected female sand flies. Non-vectorial transmission, that may occur through blood product transfusions or by the vertical, venereal and direct (bite wounds) route, is of minor epidemiological importance in endemic areas (de Freitas et al., 2006; Tabar et al., 2008; Silva et al., 2009; Naucke and Lorentz, 2012; Ben Slimane et al., 2014; Vida et al., 2016) but may be important and explain some autochthonous cases in areas where a suitable vector is absent (Baneth et al., 2008; Daval et al., 2016).

Canine leishmaniosis is endemic in all southern European countries (Cyprus, southern France, Greece, Italy Malta, Portugal, Spain) but the prevalence of infection, of seropositivity and of CanL varies and there are foci with decreased (hypoendemic) or increased (hyperendemic) prevalence, probably associated with ecological and socioeconomical factors affecting canine population and sand-fly activity (Gramiccia, 2011). For example, seroprevalence rates range from 1.7 % in Cyprus to more than 40% in southern Italy and in a recent study they varied between 2 and 30% in

different districts of Greece (Athanasiou et al., 2012; Maia and Cardoso, 2015). However, considering that the vast majority of asymptomatic but infected dogs are seronegative, the true prevalence of the infection is much higher. This has been demonstrated in numerous studies that combined molecular, serological and/or delayed-type hypersensitivity testing (leishmanin skin test-LST). The results of these studies show that more than 60% of dogs in endemic areas are infected (PCR- and/or LST-positive), while the seroprevalence rates of the same dogs vary between 12 and 26% (Solano-Gallego et al., 2001a; Leontides et al., 2002; Oliva et al., 2006; Lombardo et al., 2012). Moreover, when naïve dogs were introduced into a hyperendemic area for three consecutive transmission periods, without protection against sand fly bites, 84.5% of them became infected (Oliva et al., 2006). Even in hyperendemic areas, clinical disease (CanL) occurs in a small percentage (2-5%) of infected dogs. Many more (10-30%) infected dogs are seropositive but clinically healthy and the majority of infected dogs are seronegative and clinically healthy with positive LST (Saridomichelakis, 2009; Pennisi, 2015). The low prevalence of seropositivity and the even lower prevalence of CanL among infected dogs has important epidemiological implications for veterinary medicine and public health because, at least the seropositive asymptomatic dogs are able to transmit *L. infantum* to the sand fly vectors (Michalsky et al., 2007; Laurenti et al., 2013).

In recent years, a worrisome spread of CanL towards northern areas of Europe has been documented and has been attributed to ecological and socioeconomic factors (Shaw et al., 2009; Maia and Cardoso, 2015). Increases in average temperature have led to a northward spread of sand-fly vectors of *L. infantum*. Increased traveling of non-infected dogs to endemic areas (e.g. during summer vacations) and translocation of infected stray dogs from the Mediterranean region to central Europe poses a risk for the emergence of CanL in previously non-endemic areas (Alcover et al., 2013). Examples of the geographical expansion of CanL include northern Italy, northern Spain and previously non-endemic areas of southern France, where autochthonous cases are increasingly recognized (Maroli et al., 2008; Dereure et al., 2009; Morosetti et al., 2009; Miro et al., 2012; Ballart et al., 2013).

3. Pathogenesis and immunology

Dogs living in endemic areas, especially those spending the night outdoors, are continuously exposed to *L. infantum* throughout the transmission period. Under favorable environmental conditions, during the night, a dog may be bitten by up to 100 sand-flies per hour and on average 1% of the vectors are infected and able to inoculate, in the superficial dermis of the dog, a variable number of metaclyclic promastigotes. The outcome of the exposure to the parasite depends on multiple factors, including the force of the infection (i.e. number of inoculated metaclyclic promastigotes), the parasite's virulence, the immunomodulatory effect of vector's saliva, the genetic background and the ability of the dog to mount a protective immune response, and the presence of co-morbidities (Saridomichelakis, 2009).

Even though, the immunology of CanL is complex and not fully understood, it is clear that resistance or susceptibility to the disease depends largely on the development of a protective cellular (Th1-like) or a non-protective humoral (Th2-like) immune response, respectively (Baneth et al., 2008). Control of *L. infantum* replication requires the generation of a parasite-specific, cell-mediated immune response that is associated with the production of INF-γ, IL-2 and TNF-α; these cytokines activate infected macrophages to produce nitrogen oxide and reactive oxygen species that kill the intracellular parasites. On the contrary, humoral immunity and the ensuing antibody production does not protect the host and may be even detrimental, mainly due to the formation of immune complexes that are implicated in the pathogenesis of glomerulonephritis, uveitis, polyarthritis and vasculitis (Saridomichelakis, 2009; Papadogiannakis and Koutinas, 2015). However, in naturally infected dogs the immune responses are usually mixed (Th1 and Th2) and organ specific and the final outcome depends on a delicate balance of different effector cells and cytokines. This explains the wide spectrum of clinical presentations that starts from the infected seronegative and asymptomatic dogs, continues with the seropositive asymptomatic dogs and ends with the dogs with CanL which also exhibit marked variation in disease severity, ranging from mild and self-limiting symptoms to severe clinical presentations that can led to death (Lombardo et al., 2014; Pennisi, 2015).

Resistance to CanL is partially mediated by the genetic background of the dog. This is exemplified by the case of some canine breeds that are autochthonous in endemic areas and present natural resistance, probably due to natural selection. The

typical example are the Ibizan hounds that have a similar prevalence of infection, a higher prevalence of *Leishmania*-specific cell-mediated immune response and a lower prevalence of CanL compared to the general canine population living in the same area. On the contrary, some other breeds, like boxers, German shepherds and Rottweilers, have a clear predisposition for development of CanL (Solano-Gallego et al., 2000; Miranda et al., 2008). Even though the genetics of resistance and susceptibility to *L. infantum* are complex and breed-specific, they may be related to the genes encoding for major histocompatibility complex. Also, in boxers from Europe, polymorphisms and mutations of the *Slc11a1* gene are strongly associated with susceptibility to CanL. This gene encodes an ion transporter protein involved in the control of parasite replication and macrophage activation (Altet et al., 2002; Quinnell et al., 2003).

The immune responses to *L. infantum* can change from a predominantly cell-mediated to a non-protective humoral immune response during the host's life. The factors contributing to this phenomenon remain poorly understood and may include the cumulative exposure to the parasite and vectors and some co-morbidities. This may explain why the prevalence of seropositivity and of CanL presents 2 peaks: one in young dogs (2-4 years) that may be genetically predisposed and a second in older dogs (>6 years) that may have become susceptible due to concurrent diseases and/or due to the natural age-related decline of the immune system (Miranda et al., 2008; Saridomichelakis, 2009).

4. Clinical signs and clinicopathological abnormalities

Canine leishmaniosis is a multisystemic disease with variable and unpredictable clinical manifestations. Many different tissues and organs can be affected, mainly due to granulomatous inflammation (e.g. lymph nodes, spleen, liver, bones), or due to immune-mediated mechanisms, such as the deposition of immune complexes and perhaps of autoantibodies (e.g. kidneys, joints, uveal tract, blood vessels) or due to a combination of the above (e.g. skin) (Koutinas and Koutinas, 2014). Two clinical grading systems have been proposed, based on the clinical signs and clinicopathological abnormalities in conjunction with the results of serology, in an effort to guide treatment and provide prognostic information (Paltrinieri et al., 2010; Solano-Gallego et al., 2017).

Weight loss, with or without anorexia, leading to poor body condition and even cachexia, is a common presenting complaint of dogs with the disease. Peripheral lymphadenomegaly is considered the most common clinical sign, encountered in the majority of the patients. Splenomegaly is also prevalent although typically it is not severe enough to be detected upon physical examination (Ciaramella et al., 1997; Koutinas et al., 1999; Mylonakis et al., 2005; Giunchetti et al., 2008; Melendez-Lazo et al., 2018).

Skin lesions are diverse and they are present in up to 90% of the cases. Moreover, even the normal-looking skin of dogs with CanL is inflamed, since 50-100% of the biopsies obtained from the macroscopically healthy skin, exhibit microscopic lesions (Ciaramella et al., 1997; Koutinas et al., 1999; Solano-Gallego et al., 2004; Papadogiannakis et al., 2005; Melendez-Lazo et al., 2018). The main cutaneous presentations include exfoliative, ulcerative, nodular, sterile pustular and papular dermatitis, lesions at the site of parasite inoculation and onychogryphosis (Saridomichelakis and Koutinas, 2014). Interestingly, the nodular and papular dermatitis of CanL have been linked to the general immune responses of the dog, with the former encountered in dogs with humoral responses and the latter been associated with strong cell-mediated immunity (Fondevila et al., 1997; Ordeix et al., 2005; Lombardo et al., 2014).

Kidney involvement in CanL, mainly due to glomerulonephritis (usually membranoproliferative or mesangioproliferative) and tubulointerstitial nephritis, is observed in up to 100% of renal biopsies (Costa et al., 2003; Zatelli et al., 2003; Plevraki et al., 2006). The clinical manifestations are diverse and range from asymptomatic proteinuria, to arterial hypertension, nephrotic and uremic syndrome. Advanced kidney disease carries a poor prognosis and is the most common cause of death in CanL (Cortadellas et al., 2006; Cortadellas et al., 2008).

Ocular involvement is detected in up to 25% of CanL patients. The most common manifestations include blepharitis, conjunctivitis, keratoconjunctivitis sicca and uveitis (Ciaramella et al., 1997; Koutinas et al., 1999; Pena et al., 2000; Melendez-Lazo et al., 2018).

Muscle and skeletal manifestations are quite common. Masticatory myositis and appendicular muscle myopathy are usually manifested as muscle atrophy without locomotor disorder (Vamvakidis et al., 2000; Paciello et al., 2009). Arthritis, either

ulcerative or not, can be symmetrical and involve, simultaneously or sequentially multiple joints (Agut et al., 2003; Sbrana et al., 2014). Long bone granulomatous osteomyelitis has been reported and is accompanied by proliferative and/or lytic lesions (Agut et al., 2003).

Leishmaniosis is one of the most common causes of epistaxis in endemic areas where this signs appears in 5-15% of the patients. Epistaxis can be unilateral or bilateral, acute or chronic and severe enough to result in life-threatening hemorrhagic anemia (Mylonakis et al., 2008; Petanides et al., 2008). The pathogenesis of epistaxis is complicated with numerous mechanisms, such as thrombocytopenia, thrombocytopathy, hyperviscosity syndrome and nasal inflammation, contributing to its appearance (Juttner et al., 2001; Petanides et al., 2008).

Granulomatous inflammation of the liver is detected histologically in almost all specimens examined but, from a clinical perspective, hepatitis is subclinical; even though increases in liver enzyme activity are common and hepatomegaly is sporadically detected, clinical signs of liver disease or, even more, of liver failure are extremely rare (Rallis et al., 2005; Melo et al., 2008). Small and, especially, large intestine is affected by granulomatous inflammation that is usually asymptomatic, but in some dogs may result in chronic small intestinal diarrhea and/or colitis (Adamama-Moraitou et al., 2007; Pinto et al., 2011).

Neurological signs attributed to CanL are rare and their causal relationship with the disease usually remains elusive. Seizures, meningitis, paraparesis, tetraparesis have been attributed to *Leishmania*-induced brain, meningeal or spinal cord granuloma formation, diffuse granulomatous inflammation, vasculitis and/or hyperviscosity syndrome (Maia et al., 2015). Similarly, urogenital (e.g. chronic prostatitis) and cardiopulmonary (myocarditis, pneumonia) involvement is rarely reported in CanL (Diniz et al., 2005; Mir et al., 2012).

Non-regenerative anemia is the most common abnormality of complete blood count and has been detected in up to 75% of CanL cases (Ciaramella et al., 1997; Koutinas et al., 1999; Foglia Manzillo et al., 2013). The pathogenesis of anemia is multifactorial because it can occur because of bleeding (e.g. epistaxis), hemolysis (due to anti-erythrocyte auto-antibodies), chronic kidney disease, anemia of chronic disease and/or dyserythropoiesis (bone marrow erythroid suppression) (Silvestrini et al., 2012; Nicolato et al., 2013; De Tommasi et al., 2014). Thrombocytopenia is the

second most common hematological abnormality, is encountered in approximately 30% of the cases and it is usually mild and rarely leads to bleeding (Ciaramella et al., 1997; Terrazano et al., 2006; Cortese et al., 2008; Petanides et al., 2008; Cortese et al., 2009).

Common abnormalities found on serum biochemistry and urinalysis, include hyperglobulinemia (commonly accompanied by increased total protein concentration), hypoalbuminemia, decreased serum AG ratio and proteinuria. Hyperglobulinemia is usually polyclonic and appears due to increased β- and γ-globulin production. Hypoalbuminemia has been attributed to urine losses, decreased liver production and poor nutritional plane. Additional, but less common clinicopathological abnormalities include increased BUN, Cr and iP concentrations due to chronic kidney disease, and increases in liver and muscle enzyme activities (Ciaramella et al., 1997; Koutinas et al., 1999; Corona et al., 2004; Cortadellas et al., 2006; Plevraki et al., 2006; Petanides et al., 2008).

5. Acute phase proteins in canine leishmaniosis

Acute phase proteins are blood proteins that reflect the systemic response of the body to any kind of inflammatory insults. Their high sensitivity in detecting inflammation is accompanied by a low specificity regarding the cause of the inflammation. In recent years their use in the diagnosis, prognosis and treatment monitoring in veterinary medicine is continuously evolving (Eckersall and Bell, 2010).

The concentration of positive acute phase protein in the serum and urine show considerable increase in CanL and also in experimentally infected dogs, where these increases precede the clinical manifestations of the disease (Matrinez-Subiela et al., 2002; Matrinez-Subiela et al., 2011; Matrinez-Subiela et al., 2014; Garcia-Martinez et al., 2015). The most important and well-studied positive acute phase proteins in CanL include C-reactive protein, ferritin and paroxonase-I (Pardo-Marin et al., 2020). Their concentration in the serum was found to be strongly correlated with the two clinical grading systems of CanL (Paltrinieri et al., 2010; Solano-Gallego at al., 2017; Pardo-Marin et al., 2020) and C-reactive protein concentration at diagnosis has been proposed as a prognostic marker due to the good correlation between high levels and mortality (Silvestrini et al., 2013). Recently, a new clinical classification system that

takes into consideration, not only the clinical signs, clinicopathologic abnormalities and serology, but also the concentration of acute stage proteins has been proposed (Ceron et al., 2018).

Moreover, during effective treatment of CanL, acute phase protein abnormalities resolve quickly and they precede the clinical and clinicopathological improvement and the reduction of *Leishmania*-specific antibodies. The latter is not observed in non-responders making acute phase proteins a promising biochemical marker for treatment monitoring (Sasanelli et al., 2007; Matrinez-Subiela et al., 2016; Rubio et al., 2016; Daza Gonzalez et al., 2019).

6. Diagnosis

Various diagnostic tests can be used for a definite diagnosis of CanL in dogs with compatible clinical and clinicopathological alterations, for investigation of the infection by *L. infantum* in otherwise healthy animals that live or have travelled in endemic areas and that will be relocated in non-endemic areas, for blood donors and for treatment monitoring (Noli and Saridomichelakis, 2014). Commonly used diagnostic methods include the qualitative or quantitative (e.g. IFAT, ELISA) detection of *Leishmania*-specific antibodies, the microscopic observation of the parasite on cytology or histopathology and molecular methods of identification or quantitative measurement of parasite DNA (PCR, nPCR, RT-PCR) (Baneth and Aroch, 2008).

The activation of humoral immune responses in CanL leads to increased production of *Leishmania*-specific but non-protective antibodies (Papadogiannakis and Koutinas, 2015). The concentration of *Leishmania*-specific IgG is positively correlated with the severity of clinical signs and clinicopathological abnormalities (Reis et al., 2006; Proverbio et al., 2014). Furthermore, detection of high IgG titer is highly sensitive and specific for the diagnosis of CanL, especially in dogs with compatible clinical signs (Solano-Gallego et al., 2011; Noli and Saridomichelakis, 2014), but low titers can be found occasionally in infected but healthy dogs or in infected dogs without CanL but with various other diseases. Also, a minority of dogs with CanL may be seronegative. For all these reasons, the interpretation of serology must always be done in conjunction with the clinical signs, the clinicopathological abnormalities and the exclusion of major differentials (Solano-Gallego et al., 2001a;

Oliva et al., 2006; Lombardo et al., 2014; Noli and Saridomichelakis, 2014). Even though there is some debate about the most appropriate serological test, IFAT is currently considered the gold standard (Paltrinieri et al., 2010).

Qualitative, commercially available, in-house serological tests for the detection of *Leishmania*-specific antibodies are commonly used in the everyday clinical practice, mainly when a fast result is needed to help confirm or reject the diagnosis of CanL (Maia and Campino, 2008; Bourdeau et al., 2014). Their sensitivity and specificity are variable and should be validated in independent studies. Nevertheless, even if they are accurate, their major disadvantage is that a positive result must be followed by quantitative test (Noli and Saridomichelakis, 2014).

Microscopic observation of *Leishmania* amastigotes in tissue cytology samples is an easy to perform, low cost test, with absolute (100%) diagnostic specificity when done by an experienced examiner and with variable sensitivity in dogs with CanL. Even though there are only few comparative studies on the sensitivity of microscopy among different tissues, lymph nodes, bone marrow, spleen and skin are usually recommended (Paltrinieri et al., 2010; Solano-Gallego et al., 2011; Bourdeau et al., 2014). In any case, the sensitivity depends on the parasitic load, the quality of the preparation, the experience of the examiner and the number of oil immersion fields that are examined (Alvar et al., 2004). Regarding lymph node cytology, bone marrow cytology and their combination, the sensitivity for the diagnosis of CanL has been reported to be 84%, 73% and 93%, respectively, when 100 oil immersion fields were examined and 93%, 88% and 95%, respectively, at 1,000 oil immersion fields. On the contrary, cytology has poor sensitivity in detecting asymptomatic infected dogs due to their low parasitic load (Mylonakis et al., 2005; Saridomichelakis et al., 2005a).

Molecular techniques (PCR, nPCR, RT-PCR), depending on the applied methodology, can be very sensitive for the detection of infection by *Leishmania* spp., even in asymptomatic dogs with a low parasitic burden (Hernandez et al., 2015). Various tissues can be used and tissue selection can affect the sensitivity due to the differential tropism of the parasite. Although there is no general consensus, lymph nodes, bone marrow, conjunctiva and spleen show the highest sensitivity and peripheral blood the lowest (Francino et al., 2006; Lombardo et al., 2012; Almeida et al., 2013). Molecular techniques are powerful tools in epidemiological studies but in

clinical practice they must be used with extreme caution (Oliva et al., 2006; Lombardo et al., 2012). A positive result in a sick dog simply means that the dog is infected but does not prove that the clinical signs are due to CanL and the prevalence of positive results, even among healthy dogs living in endemic areas, can be very high (Solano-Gallego et al., 2009). Current recommendation is to use molecular techniques only in those rare cases where serology and cytology fail to confirm the diagnosis but the index of suspicion for CanL is still high and all other differentials have been ruled-out with reasonable certainty (Noli and Saridomichelakis, 2014).

Leishmanin skin test is the intradermal injection of parasitic antigen followed, 48 to 72 hours later, by the physical examination for a delayed hypersensitivity reaction (Cardoso et al., 1998; Solano-Gallego et al., 2001b). A positive test result is indicative of strong parasite-specific cell-mediated immune response, so it is only occasionally positive in dogs with CanL but can become positive after effective treatment (Fernandez-Bellon et al., 2005; Cardoso et al., 2007). For these reasons, LST is largely used in epidemiological studies and for treatment monitoring and not to diagnose CanL (Oliva et al., 2006; Gomez-Ochoa et al., 2009; Silveira et al., 2012).

7. Treatment

The goals of CanL treatment are to eliminate clinical signs and clinicopathological abnormalities, to reduce parasitic load, to modify the immune response against the parasite in favor of cellular immunity in order to avoid future relapses, and to eliminate or at least to reduce the probability of the dog to transmit *L. inafntum* to sand fly vectors. Additional considerations include safety, route of administration and cost of the medication and the avoidance of first-line drugs for the treatment of human visceral leishmaniosis in the same geographical area (Noli and Auxilia, 2005; Noli and Saridomichelakis, 2014). Parasitological cure is rarely achieved, regardless of the therapeutic regimen, and its importance has been questioned, especially for dogs living in endemic regions where the possibility of reinfection is high (Baneth and Aroch, 2008; Oliva et al., 2010). Pentavalent antimonials (meglumine antimoniate), allopurinol, miltefosine, amphotericin-B, spiramycin, metronidazole, enrofloxacin, marbofloxacin and aminosidine (paromomycin), among others, have been used for the treatment of CanL as monotherapy or in various combinations (Oliva et al., 2010) and guidelines for first-

line treatment options, depending on the clinical staging, have been published (Oliva et al., 2010; Solano-Gallego et al., 2017). In adition, in recent years immunomodulatory interventions, including the periodic administration of domperidone and dietary supplementation with nucleotides and AHCC has been proposed.

7.1. Pentavalent antimonials

Pentavalent antimonials are leishmanicidals. Their activity is mediated by inhibition of enzymes that are necessary for essential metabolic processes of the parasite, by alterations of the sulphur redox balance and by competition with zinc for binding to zinc-finger proteins that are involved in protein-nucleic acid interactions, thus causing DNA fragmentation and apoptosis-like cell death. Meglumine antimoniate, its liposomal form and sodium stibogluconate are the drugs of this group and the former has been studied extensively for the treatment of CanL. The clinical improvement or cure rate after meglumine antimoniate monotherapy varies tremendously from 25% up to 100% of treated dogs. This variation has been attributed to the different treatment protocols that have been used; doses ranged between 50 and 150 mg/kg/day, frequency of administration from every 4 hours to every 48 hours, the route of administration was subcutaneous, intravenous or intramuscular and treatment duration varied from 3 to 25 weeks (Poli et al., 1997; Slappendel and Teske, 1997; Moritz et al., 1998; Oliva et al., 1998; Denerolle and Bourdoiseau, 1999; Riera et al., 1999; Bianciardi et al., 2004; Ikeda-Garcia et al., 2007; Paradies et al., 2012; Luciani et al., 2013). In any case, the currently recommended dose regimen is usually 100 mg/kg, once daily, subcutaneously for 4 weeks.

Parasitological cure is rarely achieved and the relapse rate ranges between 70-100% at 6-12 months after the end of treatment. Common side effects are depression, gastrointestinal symptoms, pain and inflammation at the injection site, increases in liver enzyme activity and acute kidney disease (Poli et al., 1997; Slappendel and Teske, 1997; Oliva et al., 1998; Denerolle and Bourdoiseau, 1999; Riera et al., 1999; Ikeda-Garcia et al., 2007; Paradies et al., 2012). In comparative studies, meglumine antimoniate monotherapy was found to be inferior to its combination with allopurinol, non-inferior to miltefosine or aminosidine and superior to allopurinol, enrofloxacin or

the enrofloxacin-metronidazole combination (Oliva et al., 1998; Denerolle and Bourdoiseau, 1999; Mateo et al., 2009; Miro et al., 2011; Paradies et al., 2012). Even though meglumine antimoniate is a first-line drug for the treatment of CanL (Miro et al., 2008), current recommendations are against its use as monotherapy, mainly because of the high relapse rate (Solano-Gallego et al., 2009; Solano-Gallego et al., 2017) and the increased chances for development of resistance after repeated use. Contrary to the extensive research on antimonial resistance in human leishmanioses (Hendrickx et al., 2018), relatively few data exist for CanL. However, it has been established that resistance to meglumine antimoniate increases up to 41 times after repeated treatment cycles and resistant strains of *L. infantum* have been isolated (Gramiccia et al., 1992; Carrio and Portus, 2002; Maia et al., 2013; Gómez Pérez et al., 2016).

7.2. Allopurinol

Allopurinol is a leishmaniostatic hypoxanthine analogue that inhibits protein synthesis of *Leishmania* amastigotes (Koutinas et al., 2001). Allopurinol monotherapy has been extensively studied in CanL at various dose regimens ranging from 10 to 40 mg/kg/day, that have been administered every 8 to 24 hours for 1-24 months (Noli and Auxilia, 2005). Clinical remission rates range from 18% to 100% and may be dose-related. Also, a reduction of *Leishmania*-specific antibody titer and of positive acute phase protein concentrations has been documented. Parasitological cure is rarely achieved but parasite transmission to sand flies is reduced after a 6 month treatment period (Vercammen et al., 1995; Cavaliero et al., 1999; Denerolle and Bourdoiseau, 1999; Koutinas et al., 2001; Vercammen et al., 2002; Martinez-Subiela et al., 2003; Sasanelli et al., 2007; Martinez-Subiela et al., 2011; Miro et al., 2011; da Silva et al., 2012; Paradies et al., 2012; Martinez-Subiela et al., 2014).

Advantages of allopurinol monotherapy are the low cost and the fact that it can ameliorate proteinuria in patients without chronic kidney disease. Also side effects are minimal: although xanthine crystalluria is common, urolithiasis is rarely reported (Cavaliero et al., 1999; Koutinas et al., 2001; Papadogiannakis et al., 2010; Torres et al., 2011; Paradies et al., 2012). Moreover, allopurinol is not a therapeutic option for human visceral leishmaniosis and it is recommended for the treatment of CanL by WHO (Yasur-Landau et al., 2016). Disadvantages of allopurinol

monotherapy are the moderate efficacy and the high relapse rate, that ranges from 89% to 100%, 2 weeks-11 months after treatment withdrawal (Moritz et al., 1998; Denerolle and Bourdoiseau, 1999; da Silva et al., 2012; Paradies et al., 2012; Noli and Saridomichelakis, 2014). Allopurinol is considered a first-line treatment option for CanL (Miro et al., 2008), but it should never be used as the sole treatment (Solano-Gallego et al., 2017). Unfortunately, this recommendation is not followed and allopurinol monotherapy is the most common treatment proposed by veterinarians in endemic areas of Europe (Solano-Gallego et al., 2011; Bourdeau et al., 2014). The periodic administration of allopurinol has no value for the prevention of CanL (Saridomichelakis et al., 2005b), but the long-term administration has been shown to reduce the possibility of relapse although, at the same time, it induces resistant strains of *L. inafntum* (Ginel et al., 1998; Noli and Auxilia, 2005; Yasur-Landau et al., 2016; Yasur-Landau et al., 2017; Yasur-Landau et al., 2018).

7.3.Various treatments

Miltefosine is an alkyphospholipid, initially developed as an antineoplastic agent and subsequently used for the treatment of human visceral leishmaniasis. Its leishmanicidal activity is based on the disruption of signaling pathways and cell membrane synthesis, leading to parasite death. It also stimulates T-cells and macrophages and the production of reactive nitrogen and oxygen species, thus enhancing parasite clearance (Sindermann and Engel, 2006; Dorlo et al., 2012). As a monotherapy, at a dose of 2 mg/kg PO, once daily for 4 weeks, leads to clinical and clinicopathological improvement in up to 100% of the cases, to reduction of antibody titer and parasitic load, to activation of cell-mediated immunity and to reduced infectivity to sand-flies (Manna et al., 2008a; Manna et al., 2008b; Manna et al., 2009; Mateo et al., 2009; Miro et al., 2009; Woerly et al., 2009; Andrade et al., 2011; Manna et al., 2015; dos Santos Nogueira et al. 2019). Gastrointestinal side effects (vomiting, diarrhea) are seen in up to 31% of the dogs but they are usually mild and self-limited (Manna et al., 2008a; Manna et al., 2008b; Manna et al., 2009; Mateo et al., 2009; Miro et al., 2009; Woerly et al., 2009; Andrade et al., 2011; dos Santos Nogueira et al. 2019). In addition, it may preserve kidney function better than meglumine antimoniate (Bianciardi et al., 2009). Although it is a first line drug for the treatment of CanL it should not be used alone because clinical relapses are expected

6-12 months after treatment discotninuation (Manna et al., 2008a; Woerly et al., 2009; Andrade et al., 2011).

Amphotericin-B disrupts cell membrane synthesis leading to the parasite death (Plotnick, 2000). Intravenous administration has a high clinical remission rate and may lead to parasitological cure. In addition to the side effects (thrombophlebitis, gastrointestinal symptoms, acute kidney injury), this drug is not recommended for CanL because it is the first-line treatment for human visceral leishmaniosis in Europe (Noli and Auxilia 2004).

The use of spiramycin, metronidazole and enrofloxacin, as monotherapy or in different combinations, has been evaluated in three clinical trials. A good clinical response was seen in 50-70% of dogs with CanL, but a high relapse rate (50%) followed their discontinuation (Bianciardi et al., 2004; Pennisi et al., 2005). Marbofloxacin, a second generation fluoroquinolone, was tested *in vitro* and *in vivo*. Leishmanicidal activity, mediated by TNF-α and nitric oxide synthase pathways, results in a significant reduction of parasitic load and clinical improvement in up to 70% of dogs, but the relapse rate of at 6 months following withdrawal is more than 50% (Vouldoukis et al., 2006; Rougier et al., 2008; Farca et al., 2012; Rougier et al., 2012; Pineda et al., 2017).

Domperidone is a dopamine-2 receptor antagonist, with antiemetic and prokinetic action, that promotes release of prolactin from hypophysis, which in turn enhances innate immunity and Th1 immune responses (Gomez-Ochoa et al., 2009; Noli and Saridomichelakis, 2014). When used in mild cases of CanL, a good clinical response accompanied by a reduction of antibody titers was achieved (Gomez-Ochoa et al., 2009). Side effects were uncommon and mild (diarrhea, galactorrhea). Other advantages include the relatively low cost, oral administration and the fact that it cannot promote resistance because it has no direct anti-*Leishmania* activity (Gomez-Ochoa et al., 2009; Sabate et al., 2014).

Recently, dietary nucleotides and AHCC that modulate immune responses, probably through a TLR-mediated mechanism, in favor of cell-mediated immunity, have been tested for the treatment of CanL. They are safe and may represent an effective alternative to allopurinol for long-term management (Segarra et al., 2017; Segarra et al., 2018).

7.4. Drug combinations

The combination of meglumine antimoniate (for 4-6 weeks) with allopurinol (for at least 12 months) is currently recommended as the first-line treatment of CanL stage II and III, according to LeishVet clinical staging (Ikeda-Garcia et al., 2007; Miro et al., 2008; Solano-Gallego et al., 2009; Oliva et al., 2010; Solano-Gallego et al., 2011; Solano-Gallego et al., 2017). Clinical and clinicopathological improvement occurs in >95% of the dogs during the first 3 months and is followed by a significant reduction of antibody titer (Noli and Auxilia, 2005; Miro et al., 2009; Torres et al., 2011; Paradies et al., 2012; Manna et al., 2015). Parasitological cure is uncommon but parasitic load is significantly reduced from the first month and there is evidence for a change in *Leishmania*-specific immune response towards cell-mediated immunity. Also, treated dogs hardly transmit parasites to sand fly vectors, at least under experimental conditions (Miranda et al., 2007; Manna et al., 2008c; Miro et al., 2009; Miro et al., 2011; Manna et al., 2015).

At the level of clinical and clinicopathological improvement this combination is superior than meglumine antimoniate, allopurinol monotherapy and spiramycin-metronidazole combination and equal compared to miltefosine-allopurinol combination. The major advantage is the low relapse rate (0-13%) even after a 6 year follow-up period (Denerolle and Bourdoiseau, 1999; Noli and Auxilia, 2005; Torres et al., 2011; Paradies et al., 2012; Manna et al., 2015) which is mainly attributed to the long-term administration of allopurinol (Noli and Auxilia, 2005). Strict criteria for allopurinol discontinuation have not been established, but it is usually recommended after clinical cure, amelioration of all clinicopathological abnormalities (with the possible exception of proteinuria) and marked (at least 3 to 4-fold) reduction of antibody titer; however, some dogs may never reach these end-points (Noli and Saridomichelakis, 2014; Solano-Gallego et al., 2017).

Miltefosine (for 4 weeks) and allopurinol (for at least 12 months) combination is the alternative first-line treatment protocol for dogs with CanL stage II and III (Solano-Gallego et al., 2011; Solano-Gallego et al., 2017). It is more effective compared to miltefosine monotherapy and there was no difference from the meglumine antimoniate-allopurinol combination regarding clinical and clinicopathological improvement, reduction of parasitic load, side effects and short-term relapse rates (Manna et al., 2008b; Manna et al., 2009; Miro et al., 2009; Farca et

al., 2012; Manna et al., 2015). However, in a long-term study with a 6 year follow-up, relapses were more common for the miltefosine-allopurinol compared to the meglumine antimoniate-allopurinol combination (Manna et al., 2015).

8. Aminosidine

Aminosidine is an aminoglycoside antibiotic effective against Gram-positive and Gram-negative bacteria and protozoa. Inhibition of 16S ribosomal RNA and alteration of cell membrane synthesis with subsequent parasite death is the main mode of action against the different species of *Leishmania* (Maarouf et al., 1997; Lynch et al., 2003; Mehta and Champney, 2003).

The effectiveness and safety of aminosidine in human leishmanioses have been extensively studied. It has been used as an alternative treatment, mainly in areas with a widespread resistance to antimonials and when the high cost or low availability of miltefosine and amphotericin-B restrict their use (Shakya et al., 2011). Topical or systemic administration of aminosidine in cutaneous and visceral leishmanioses, either alone or in combination with other drugs, has shown promising results (Thakur et al., 1992; Melaku et al., 2007; Musa et al., 2010; Musa et al., 2012; Ben Salah et al., 2013; Atia et al., 2015; Jamil et al., 2015). Its main advantages are the low cost and the effectiveness against antimony-resistant strains (Kulshrestha et al., 2011). However, resistance to aminosidine has been also documented, at least *in vitro* (Hadighi et al., 2007; Hendrickx et al., 2014), and its administration can cause nephrotoxicity, deafness and vestibular disease that may be reversible if they are detected early and treatment is immediately discontinued (Amato et al., 2007).

The efficacy and safety of aminosidine for the treatment of CanL has been evaluated in 7 clinical trials that enrolled a total of 147 dogs (Persechino et al., 1994; Perscechino et al., 1995; Poli et al., 1995; Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013). There is also a single case report where the combination of aminosidine with meglumine antimoniate was administered (Santos et al., 2006).

There are differences between these studies regarding the dose regimen (from 7 mg/kg/day up to 80 mg/kg/day), the duration of treatment (2-4 weeks), and the route of administration (intramuscularly or subcutaneously). The evaluation of efficacy in all studies was based on the clinical and clinicopathological improvement and the

time needed to be achieved (Persechino et al., 1994; Perscechino et al., 1995; Poli et al., 1995; Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013). Reduction of parasitic load was assessed in 6 of them by lymph node and/or bone marrow cytology (Persechino et al., 1994; Perscechino et al., 1995; Oliva et al., 1998; Vexenat et al., 1998), by parasite culture from lymph nodes (Poli et al., 1997) or by combining lymph node and bone marrow cytology and PCR (Athanasiou et al., 2013).

Rates of clinical improvement ranged from 54% (Oliva et al., 1998) to 100% (Poli et al., 1997; Vexenat et al., 1998; Athanasiou et al., 2013) and they were accompanied by a significant reduction of antibody levels (Poli et al., 1997; Athanasiou et al., 2013). Reduction of parasitic load was also documented by lymph node and/or bone marrow cytology (Persechino et al., 1994; Perscechino et al., 1995; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013) and PCR (Athanasiou et al., 2013). The prevalence of side effects was dose-dependent and was 0% at 3.5 mg/kg every 12 hours (Oliva et al., 1998) and at 15 mg/kg every 24 hours (Athanasiou et al., 2013), 5-8% at 5 mg/kg every 12 hours (Perscechino et al., 1995; Poli et al, 1995; Poli et al., 1997) and 25-50% at the upper end of the tested doses (Persechino et al., 1994; Vexenat et al., 1998); also, a mortality rate of 50% was recorded when 40 or 80 mg/kg/day were administered (Vexenat et al., 1998). Side effects included anorexia, weight loss, depression, renal impairment, deafness, keratitis and death. The relapse rate after treatment discontinuation was high, ranging from 54 to 100% in studies with a follow up period of at least 3 months (Persechino et al., 1994; Perscechino et al., 1995; Oliva et al., 1998; Vexenat et al., 1998), whereas in a single study no relapses were recorded after 6 months (Athanasiou et al., 2013). Comparative studies between aminosidine and meglumine antimoniate monotherapy showed no differences at the rate of clinical and clinicopathological improvement, reduction of parasitic burden and relapse rate (Poli et al, 1995; Poli et al., 1997; Oliva et al., 1998).

Based on these data, aminosidine is currently considered a second-line drug for the treatment of CanL (Miro et al., 2008; Solano-Gallego et al., 2011). Also, a systematic literature review of the literature published between 1980 and 2004 concluded that there is fair evidence in favor of recommending the administration aminosidine for the treatment of CanL at a dose of 5 mg/kg every 12 hours,

subcutaneously, and strong evidence against its use at higher doses (20-80 mg/kg/day) (Noli and Auxilia, 2005).

The real value of aminosidine for the treatment of CanL is a subject of further investigation. There are only three randomized comparative studies with a first-line drug (meglumine antimoniate) but none of them was blinded (Poli et al., 1995; Poli, et al 1997; Oliva et al., 1998). Also, the methods used for randomization are either not reported (Oliva et al., 1998) or unclear (Poli et al., 1995; Poli et al., 1997). The evaluation of hearing impairment was based on clinical criteria (Poli et al., 1997; Vexenat et al., 1998), which is a method with very low sensitivity and specificity. Furthermore, no studies evaluated the combination of short-term aminosidine administration with long-term allopurinol.

9. Brainstem auditory evoked potentials

This is an electrophysiologic test that objectively examines auditory function and hearing acuity (Scheifele and Clark, 2012) and the only accurate way to determine the prevalence of hearing loss during and after treatment of CanL with aminosidine. In the past, evaluation of hearing loss was based on the behavioral responses to sounds. However, results were highly unreliable because they depend on dog's mental state, rate of adaptation to the sound stimulus and sensitivity to concurrent visual and vibratory stimuli. On the contrary, BAER is a non-invasive, safe examination that produces reliable results and helps to determine the pathophysiology and to locate anatomically the cause of deafness (Munro and Cox, 1997; Strain, 2004; Wilson and Mills, 2005).

Brain stem auditory evoked potentials represent the electrical activity of cranial nerve VIII and of brainstem auditory tracts, in response to externally applied, controlled, acoustic stimuli (Scheifele and Clark, 2012). Recording of this electrical activity results in a waveform that consists of up to seven separate waves that normally occur within 10 msec after stimulus (Poncelet et al., 2006; Wilson et al., 2006). Each positive wave is labelled sequentially with Roman numbers (I-VII). A separate wave IV may be absent because it merges with III or V and only the first five waves are considered clinically important in veterinary medicine (Kawasaki and Inada, 1994; Eger and Lindsay, 1997; Wilson and Mills, 2005).

Currently it is believed that, with the exception of waves I and II, each of the remaining components of BAER receives contribution from more than one anatomic structure. Conversely, each anatomic structure contributes to more than one wave (Scheifele and Clark, 2012). From a neuroanatomic point, BAER components are generated at the distal portion of cranial nerve VIII (wave I), the proximal portion of the same nerve as it enters the brainstem (wave II), the second order neurons in the area of cochlear nuclei (wave III) and at multiple sites, including the inferior colliculus and the medial geniculate body for waves IV and V (Wilson and Mills, 2005).

The evaluation of the BAER includes the assessment of the following parameters:

- Wave morphology: it is assessed based on the presence of four or five vertex positive peaks (waves) and a large trough after the fifth of them. The first peak is generated 1-2 msec after the stimulus and subsequent waves appear at approximately 1 msec intervals. Waves III and IV or IV and V occasional merge, making identification of all five waves impossible (Scheifele and Clark, 2012).
- Wave latencies: they are the time intervals from the presentation of the acoustic stimulus to the peak of each wave and they are of high diagnostic value (Kawasaki and Inada, 1994; Eger and Lindsay, 1997; Kemper et al., 2013).
- Interwave latencies: they represent the time period between two peaks. Of particular clinical importance are the I-III (electrical activity of cranial nerve VIII and proximal brainstem), III-V (electrical activity within the brainstem) and I-V (total electrical activity of cranial nerve VIII and the auditory portion of the brainstem) (Poncelet et al., 2000a).
- Intraural comparisons: they are the comparison between BAER recordings regarding wave latencies, morphology and interwave latencies (Wilson and Mills, 2005).
- Wave V latency-intensity curve: this is the graphical representation of wave V latencies in response to at least 3 different intensities of stimuli (Poncelet et al., 2000a; Harcourt-Brown et al., 2011; Scheifele and Clark, 2012).
- Hearing threshold: is the lowest intensity of acoustic stimulus that can induce an identifiable V wave. It evaluated by presenting stimuli at gradually decreasing intensities (Poncelet et al., 2006; Harcourt-Brown et al., 2011).

- Repeatability: the BAER recordings are considered repeatable and thus reliable, when wave V latencies in two consecutive recordings differ by less than 0.1 msec (Kemper et al., 2013).

Brain stem auditory evoked potentials can be used to investigate diseases of the central nervous system that involve the brainstem, such as tumors, degenerative and infectious diseases, head trauma (Fischer and Obermaier, 1994; Steiss et al., 1994; Vanhaesebrouck et al., 2010) and to examine for deafness. Indeed, BAER is the golden standard examination for the diagnosis of congenital, including inherited skin pigmentation-associated, sensorineural deafness (Poncelet et al., 2000a; Poncelet et al., 2000b; Strain, 2004). Also, it is useful for the etiological diagnosis of acquired deafness because it can differentiate sensorineural (e.g. otitis interna, ototoxin exposure, presbycusis) from conductive deafness (e.g. otitis externa, otitis media, middle ear polyps) (Eger and Lindsay, 1997; Poncelet et al., 2000a; Poncelet et al., 2000b; Ter Haar et al., 2009; Harcourt-Brown et al., 2011; Cole et al., 2018; Paterson, 2018a; Paterson 2018b).

PART TWO-OUR STUDY

1. Aims of the study

Because the efficacy and the safety of aminosidine-allopurinol combination for the treatment of CanL has not been previously evaluated, we conducted a randomized, blinded, positive-controlled (meglumine antimoniate-allopurinol combination) clinical trial, with a 6 month duration, to investigate: a) the prevalence of side effects and specifically of nephrotoxicity and ototoxicity, b) the evolution of the prevalence and intensity of CanL-associated clinical signs and the occurrence of relapses during the trial period, c) the evolution of the prevalence and intensity CanL-associated clinicopathological abnormalities, d) the efficacy for the reduction of parasitic load, and e) the effect on the *Leishmania*-specific cell-mediated and humoral immune response.

The methodology, the results and their interpretation are presented in the following two articles.

2. Article No 1

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Evaluation of nephrotoxicity and ototoxicity of aminosidine (paromomycin)-allopurinol combination in dogs with leishmaniosis due to *Leishmania infantum*: A randomized, blinded, controlled study

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ABSTRACT

Canine leishmaniosis due to *Leishmania infantum* is a widespread zoonotic disease. Although aminosidine can be an effective treatment, current therapeutic recommendations do not advocate its use, mainly due to concerns regarding the potential nephrotoxicity and ototoxicity of this drug. The aim of this randomized, blinded, controlled study was to evaluate the nephrotoxicity and ototoxicity of aminosidine-allopurinol combination and compare it with that of meglumine antimonate-allopurinol combination in non-azotemic dogs with leishmaniosis. Forty dogs with leishmaniosis were randomly assigned to be treated with either aminosidine at 15 mg/kg, subcutaneously, once daily for 28 days (group A) or with meglumine antimonate at 100 mg/kg, subcutaneously, once daily for 28 days (group B). In addition to either drug, dogs in both groups were administered allopurinol at 10 mg/kg *per os* twice daily for 2 months. Kidney function was evaluated through measurement of serum creatinine, urea nitrogen, inorganic phosphorus, and cystatin-c concentrations and complete urinalysis, including protein-to-creatinine ratio, at baseline and after 14, 28, and 60 days from the beginning of the treatment. At the same time points, vestibular and auditory functions were evaluated through neurological examination and brainstem auditory evoked response (BAER) recordings of wave I, wave V, inter-wave I–V latencies, and minimum hearing thresholds. None of the dogs developed clinicopathological evidence of kidney disease during the study. Serum creatinine concentration increased > 0.3 mg/dl over baseline in 2 dogs in group A and in 5 dogs in group B. Parameters of kidney function were not significantly different or were improved compared to baseline and the only difference between the two groups was the lower concentration of serum creatinine in group A. None of the dogs developed peripheral vestibular syndrome or hearing impairment. At the end of the study, parameters of auditory function were not significantly different or were improved compared to baseline and there were no differences between the two groups. The results of this study show that the nephrotoxicity and ototoxicity of aminosidine, when administered to non-azotemic dogs with leishmaniosis at 15 mg/kg subcutaneously once daily for 28 days along with allopurinol, is minimal and does not differ from that of meglumine antimonate.

1. Introduction

Canine leishmaniosis (CanL) due to infection with *Leishmania infantum* (syn. *L. chagasi*) (Maurício et al., 2000) is a widespread zoonosis that is endemic in many regions, including southern Europe and Latin America (Pennisi, 2015). Of the various drugs that have been evaluated for the treatment of CanL, the combination of allopurinol with either meglumine antimonate or miltefosine is currently considered the treatment of choice (Solano-Gallego et al., 2011; Noli and Saridomichelakis, 2014). The combination of meglumine antimonate

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(100 mg/kg, subcutaneously, once daily, for 28 days) with allopurinol (10 mg/kg, *per os*, twice daily, for at least 6–12 months) demonstrates good clinical efficacy, results in a considerable reduction of parasitic load, minimizes the chances of parasite transmission to sand fly vectors and is usually well tolerated (Solano-Gallego et al., 2011; Noli and Saridomichelakis, 2014). However, meglumine antimonate is considered nephrotoxic and, even though this adverse effect is sparsely documented in the literature (Ikeda-Garcia et al., 2007; Bianciardi et al., 2009; Mateo et al., 2009; Torres et al., 2011), it is not recommended for dogs with CanL and stage III or stage IV chronic kidney disease (CKD) (Solano-Gallego et al., 2011).

Aminosidine (paromomycin) is an aminoglycoside with leismanicidal activity (Reguera et al., 2016). When used as the sole agent for the treatment of CanL, it demonstrated good clinical efficacy but was associated with severe, dose-dependent nephrotoxicity and ototoxicity (Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998). Therefore, an evidence-based systematic review, concluded that there was only fair evidence for recommending aminosidine at the dosage regimen of 5 mg/kg, twice daily, for 3–4 weeks and fair evidence against recommending its use at daily doses exceeding 20 mg/kg (Noli and Auxilia, 2005). However, in a recent study, when aminosidine was administered at 15 mg/kg once daily for 21 days to healthy dogs and to non-proteinuric and non-azotemic dogs with leishmaniosis, it resulted in effective blood concentrations without appreciable nephrotoxicity (Athanasiou et al., 2014).

The reported incidence of aminosidine-associated nephrotoxicity and ototoxicity in CanL range from 0 to 37.5% and from 0 to 100%, respectively. Such wide variability may be explained by the differences among published studies in the total daily dose administered (ranging from 5 mg/kg to 80 mg/kg), dosing interval (every 12 h or 24 h), and route of administration (subcutaneous or intramuscular administration) (Persechino et al., 1994, 1995; Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013). As a result, the toxicity of this drug, when used at low dosages that are still considered effective for the treatment of CanL, remains unclear.

Long-term allopurinol administration, in addition to the short-term use of leishmanicidal drugs is considered mandatory to improve therapeutic efficacy and to avoid relapses of CanL (Manna et al., 2015; Reguera et al., 2016). To our knowledge, there are no published studies evaluating the safety of aminosidine-allopurinol combination for the treatment of CanL.

The aim of this randomized, blinded, controlled study was to evaluate the nephrotoxicity and ototoxicity of aminosidine (given at 15 mg/kg, subcutaneously, once daily, for 28 days)-allopurinol combination and compare it with that of meglumine antimonate (given at 100 mg/kg, subcutaneously, once daily, for 28 days)-allopurinol combination in non-azotemic dogs with leishmaniosis.

2. Materials and methods

2.1. Dogs, study design, and treatment

This study was randomized, blinded, and controlled with 2 treatment arms. Forty dogs with clinical signs of leishmaniosis, positive serology (indirect immunofluorescence antibody testing titer ≥1/200) and positive lymph node and/or bone marrow microscopy for *Leishmania* amastigotes were enrolled. Exclusion criteria were the presence of stage III CKD based on the International Renal Interest Society (IRIS) guidelines, concurrent diseases, previous vaccination for CanL, treatment with leishmanicidal (meglumine antimonate, miltefosine, aminosidine, or amphotericin-B) or leishmaniostatic (allopurinol, ketoconazole, metronidazole, or fluoroquinolones) drugs in the previous 12 and 3 months, respectively, or history of administration of nephrotoxic or ototoxic drugs.

Dogs were randomly assigned into groups A or B, with an allocation ratio of 1:1, using a randomization table. Dogs in group A were treated with aminosidine (Gabbrocol®, CEVA) at the target dose of 15 mg/kg, subcutaneously, once daily, for 28 days plus allopurinol (Zylapour®, Farmanic) at the target dose of 10 mg/kg, *per os*, twice daily for 2 months. Dogs of group B were treated with meglumine antimonate (Glucantime®, Aventis) at the target dose of 100 mg/kg, subcutaneously, once daily for 28 days plus allopurinol at the same dosage regimen as dogs in group A. During the first 28 days of the study, dogs were hospitalized, in individual cages, after which time they were returned to their owners for continuing treatment at home. During the hospitalization period dogs were fed a commercial dry canine food with low purine concentration (Urinary Low Purine®, Royal Canin), had free access to water, and received daily physical examination by a non-blinded member of the research team (MKC) who was also responsible for treatment administration. Evaluation of kidney, vestibular and auditory function was conducted at baseline (time 0) and on days 14 (time 1), 28 (time 2), and 60 (time 3) from the beginning of the treatments by the first author (DK) who was blinded to each dog's group. Dogs were enrolled between September 2009 and June 2012 at the Clinic of Medicine, Faculty of Veterinary Science, University of Thessaly, Greece.

2.2. Evaluation of kidney function

Blood samples were obtained by jugular venipuncture after withholding food overnight. Serum creatinine, urea nitrogen, and inorganic phosphorus concentrations were measured immediately after serum separation using an automated chemistry analyzer (Vet Test Chemistry Analyzer, IDEXX). The remaining serum was stored at −20 °C for batch measurement of cystatin-c concentration, using a species-specific commercial ELISA (canine cystatin c ELISA kit, BioVendor - Laboratorni Medicina, Brno, Czech Republic) (Tvarijonaviciute et al., 2013). A reference interval for cystatin-c serum concentration (775–1339 ng/ml) was established as the central 95th percentile of serum concentrations in 30 seronegative dogs, living in a non-endemic area and having serum creatinine concentrations within reference interval.

Morning urine samples, collected by aseptic cystocentesis, were used for measurement of urine specific gravity (USG), dipstick examination, sediment microscopy, aerobic culture, and measurement of urine protein-to-creatinine ratio (UPC). USG was measured with a handheld refractometer (Clinical Refractometer, American Optical) and Multistix 10 SG (Bayer) colorimetric strips were used for dipstick examination. Subsequently, urine samples were centrifuged (1,500 rpm for 5 min) and the sediment was examined microscopically for the presence of red blood cells, white blood cells, epithelial cells, casts, crystals, and bacteria. Aerobic bacterial culture, followed by antimicrobial susceptibility testing, was performed in all dogs at baseline and when bacteriuria was seen upon sediment microscopy at subsequent time-points. In case of a positive result, bacterial culture was repeated 7 days after the end of systemic antibacterial treatment. In the absence of severe hematuria [macroscopic hematuria or > 250 red blood cells/high power field (HPF)], pyuria (> 3 white blood cells/HPF), and positive urine culture (Lees et al., 2005), supernatant obtained after urine centrifugation was stored at −20 °C for batch measurement of UPC. Urine protein concentration was determined by the pyrogallol red method (urinary proteins LR, Cesan) in undiluted samples, but, if the result was > 250 mg/dL, samples were diluted 1:5 with distilled water and reanalyzed to fit into the linear range of the assay. Urine creatinine concentration was measured with a modified Jaffe reaction method (creatinine kinetic, Flowcytogen Laboratories) in samples diluted 1:20 in distilled water. Dogs were classified according to the current IRIS guidelines as non-proteinuric if UPC < 0.2, borderline proteinuric if UPC = 0.2–0.5, and proteinuric if UPC > 0.5 (Vaden and Elliott, 2016).

For each of the dogs, possible treatment-related nephrotoxicity was considered when serum creatinine concentration increased > 0.3 mg/dl from baseline (Hokamp and Nabity, 2016) or when glycosuria and/or casts were present at urinary sediment examination. Development of

azotemia, defined as a serum creatinine concentration > 2.0 mg/dl with concurrent USG < 1035, was a study end-point necessitating aminosidine or meglumine antimonate discontinuation, unmasking randomization for the particular dog and institution of symptomatic treatment, if necessary. At the group level, statistically significant increases of serum creatinine, urea nitrogen, ionized phosphorus, cystatin-c concentrations and/or significant increases of UPC were considered evidence of treatment-related nephrotoxicity.

2.3. Evaluation of vestibular and auditory function

Brainstem auditory evoked responses (BAER) for both ears were recorded in a quiet, but not soundproof, room with a 3-channel electrodiagnostic system (Madsen, Octavus®, GN Otometrics) by the first author. Dogs were sedated by intramuscular administration of dexmedetomidine hydrochloride (Dexdomitor®, Zoetis) at 100 μg/m^2 and butorphanol (Dolorex®, Intervet) at 0.2 mg/kg and, at the end of the procedure, sedation was reversed with atipamezole (Antisedan®, Zoetis) at 150 μg/kg, intramuscularly. Initially, otoscopy was performed bilaterally with a hand-held otoscope (Welch Allyn) to exclude dogs with visually-observeable abnormal ears from further testing. Recordings were obtained with stainless hypodermic needles (Ambu®, Neuroline), which were inserted at the vertex (positive non-inverting electrode), in front of the tragus of both ears (negative inverting electrodes) and at the base of the neck (ground electrode) and stimuli were delivered with insert earphones (Ear 3A, Nicolet Biomedical Instruments). The signal was filtered between 160 and 3,000 Hz, averaged, and displayed on a computer screen. BAER were elicited by 0.1 ms click stimuli with a repetition rate of 24 Hz. An average of 1,000 stimuli were delivered and the responses from the first 10 ms were averaged until a stable waveform had been produced. A band-limited white noise with an intensity of 20 dB normal hearing level (dBnHL) less than the click stimulus was delivered to the non-tested ear.

Tracings were obtained for decreasing intensities from 70 dBnHL to the minimum hearing threshold (MHT) or until 0 dBnHL, using 10 dB increments. MHT was defined as the midway point between the intensities for which a repeatable wave V was recorded and the one that did not. For recordings with a repeatable wave V at the lowest stimulus intensity used (0 dBnHL), MHT was arbitrary assigned to a value of 0 dBnHL (Kemper et al., 2013). At least two recordings were performed at 70 dBnHL and at the MHT vicinity to ensure repeatability, defined as the presence of an identifiable wave V within 0.1 ms in two consecutive tracings at a given stimulus intensity (Scheifele and Clark, 2012). Wave V latency was recorded at all stimulus intensities where this wave was identifiable, wave I and inter-wave I–V latencies were recorded only at 70 dBnHL and for all these variables the mean of repeated measurements was calculated. Auditory function of each ear was classified as grade 0 (normal hearing) if MHT ≤25 dBnHL, grade 1 (mild hearing loss) if MHT = 26–40 dBnHL, grade 2 (moderate hearing loss) if MHT = 41–60 dBnHL, grade 3 (severe hearing loss) if MHT = 61–80 dBnHL, and grade 4 (profound hearing loss) if MHT ≥ 81 dBnHL (Mason et al., 2013). Ears with abnormal auditory function at baseline, defined as absence of repeatable waveforms at 70 and 90 dBnHL were excluded from further analysis (Scheifele and Clark, 2012).

For each individual dog, development of peripheral vestibular syndrome (i.e., head tilt, jerk nystagmus, absence of oculocephalic reflex, and/or vestibular ataxia in the absence of other cranial nerve and postural deficits) and hearing impairment (i.e., any increase of auditory dysfunction at any level) were study end-points necessitating unmasking randomization for the particular dog, aminosidine discontinuation and institution of symptomatic treatment, if deemed necessary. For each group, statistically significant increases of wave I, V, and/or inter-wave I–V latencies and of MHT and were considered evidence of treatment-related ototoxicity.

2.4. Statistical analysis

Continuous data were assessed for normality with the Shapiro-Wilk test and were subsequently presented by their mean and standard deviation (SD) or by their median and range, if distribution was found to be normal or non-normal, respectively. Frequencies and percentages were used to describe categorical data.

The two groups were compared at time-point 0 regarding sex (Pearson's χ^2 test) and age (Mann-Whitney-U test) of the dogs and the actual dose of allopurinol per kg body weight (unpaired *t*-test); serum creatinine, urea nitrogen, ionized phosphorus, and cystatin-c concentrations and UPC (Mann-Whitney-U test); percentage of dogs with serum cystatin-c concentration above the upper limit of the reference interval (Pearson's χ^2 test); percentage of borderline proteinuric and/or proteinuric dogs (Pearson's χ^2 test or Fisher's exact test); wave I, wave V, and inter-wave I–V latencies (unpaired *t*-test); and MHT (Mann-Whitney-U test). The dropout rate was compared between the two groups using a Fisher's exact test.

The effect of each treatment on kidney function, as assessed by serum creatinine, urea nitrogen, ionized phosphorus, and cystatin-c concentrations, percentage of dogs with serum cystatin-c concentration above the upper limit of the reference interval, UPC and percentage of dogs with borderline proteinuria and/or proteinuria and auditory function, as assessed by wave I, wave V, inter-wave I–V latencies, and MHT were examined by comparing time-point 0 and time-point 3 values, of each group separately, with paired Student's t-tests or Wilcoxon signed rank (continuous data) and McNemar's tests (categorical data). Kidney and auditory functions were also compared between the two treatment groups at the end of the study (time-point 3) using an unpaired *t*-test or a Mann-Whitney-U test (continuous data), and a Pearson's χ^2 or a Fisher's exact test (categorical data). Also, Kaplan-Meier survival plots and a log-rank test were used to compare the interval from time-point 0 until serum creatinine concentration increased > 0.3 mg/dl over baseline between the two groups. To account for dogs that dropped-out before the end of the study, intention-to-treat analysis, using the last known observation-carrying forward rule, was performed, including all dogs with at least one post-time-point 0 evaluation.

Significance was set at 0.05 and all statistical analyses were performed using Stata 13.1 (Stata Statistical Software, College Station, TX, USA). Study power calculation revealed that 19 dogs per group were needed to have an 80% chance to show a significant difference between the two groups in the incidence of nephrotoxicity and/or ototoxicity, assuming a 10% incidence in group B and at least 4-fold higher incidence in group A.

2.5. Ethics

Handling of the dogs was in accordance with the European Communities Council Directive 86/609/EEC and state laws. The experimental protocol had been approved by State authorities (license No 3699/20-10-08) and a signed informed consent was obtained from all dog owners.

3. Results

3.1. Dogs, treatment and follow-up

Group A included 12 (60%) intact male and 8 (40%) intact female dogs that belonged to 7 different breeds (Greek hound: 4 dogs; Doberman pincher, English pointer, English setter, German shepherd, German wirehaired pointer, and Segugio Italiano: 1 dog, each) or were mixed-breed (n = 10; 50%) and their age ranged from 1.5 to 12 years (median: 3.7 years). Group B included 13 (65%) intact male and 7 (35%) intact female dogs that belonged to 8 different pure breeds (Greek hound and German shorthaired pointer: 4 dogs, each; English

setter and Rottweiler: 2 dogs, each; Caucasian shepherd, Doberman pincher, English pointer, and German wirehaired pointer: 1 dog, each) or were mixed-breed (n = 4; 20%) and their age ranged from 1 to 12 years (median: 5 years). Sex distribution ($p = 0.74$) and age ($p = 0.44$) of the dogs were not significantly different between the two groups.

In group A, the mean doses of aminosidine and allopurinol administered were 14.8 mg/kg (SD: 0.56) and 10.2 mg/kg (SD: 1.7), respectively. In group B, the mean doses of meglumine antimonate and allopurinol administered were 99.4 mg/kg (SD: 1.1) and 10.2 mg/kg (SD: 1.1), respectively. There was no significant difference in the mean dosage of allopurinol between the two groups ($p = 0.983$).

A total of 33 dogs (15 from group A and 18 from group B) completed the 60-day study period. Two group A dogs were lost to follow-up at time-point 3, two died from reasons unrelated to treatment (i.e., car accident) between time-points 2 and 3, and one dog died during hospitalization, one week after treatment commencement. Although permission for necropsy to determine the cause of death was not obtained from the owner, severe kidney disease was ruled out based on serum creatinine concentration measured just before death (0.8 mg/dl). One dog from group B was lost to follow-up at time-point 3 and another dog died of acute pancreatitis in the first week of the study. Diagnosis was based on increased pancreatic lipase immunoreactivity concentration (> 1000 μg/L, reference interval: 0–200 μg/L) and necropsy findings. None of the dogs were withdrawn due to azotemia, peripheral vestibular syndrome, or hearing impairment. Dropout rates did not differ significantly between groups ($p = 0.407$). Therefore, intention-to-treat analysis, using the last known observation-carrying forward rule, for the evaluation of kidney, auditory, and vestibular functions included the 19 dogs from group A and 19 dogs from group B that were alive at time-point 1. Additionally, auditory testing results from five ears (from four different dogs) were excluded due to severe otitis externa-media (two ears of one dog in group A) or unilateral deafness (one ear in one dog from group A and two ears in two dogs from group B), which had included 71 ears (35 ears from the 19 dogs of group A and 36 ears from the 19 dogs of group B).

3.2. Evaluation of kidney function

At baseline, UPC was not determined in 7 dogs (3 from group A and 4 from group B) because of positive urine cultures.

Serum creatinine, urea nitrogen, ionized phosphorus, and cystatin-c concentrations and UPC were not significantly different between the two groups. Also, the percentage of dogs with serum cystatin-c concentrations above the upper limit of the reference interval and the percentage of dogs with borderline proteinuria (0.2 < UPC < 0.5) or proteinuria (UPC > 0.5) did not differ significantly between the two groups (supplementary data, Table S1).

None of the dogs developed azotemia during the study. However, based on serum creatinine concentration increases > 0.3 mg/dl over baseline, there was evidence of possible treatment-related nephrotoxicity in 2 dogs of group A (one each at time-points 2 and 3) and in 5 dogs of group B (two dogs each at time-points 1 and 2 and one dog at time-point 3). Glycosuria and urinary casts were not observed in any dog and any time-point. The incidence of possible nephrotoxicity did not differ significantly between the two groups ($p = 0.405$).

At the group level, there was no evidence of treatment-related nephrotoxicity with either treatment. Serum cystatin-c concentration (group B), the percentage of dogs with serum cystatin-c concentration above the upper limit of the reference interval (group B), and UPC (both groups) were lower at time-point 3 compared to baseline measurements (Table 1, Fig. 1). Also, serum creatinine concentration at time-point 3 was significantly lower ($p = 0.046$) in group A compared to group B, but there were no differences between the two groups regarding serum urea nitrogen, ionized phosphorus, and cystatin-c concentrations, percentage of dogs with serum cystatin-c concentration above the upper limit of the reference interval, UPC, or percentage of dogs with borderline proteinuria and/or proteinuria (supplementary data, Table S2). Finally, the time interval until serum creatinine concentration increased > 0.3 mg/dl over baseline did not differ significantly between the two groups ($p = 0.217$).

Table 1
Comparison of renal function parameters of dogs with leishmaniosis before (time-point 0; baseline) and 60 days after treatment (time-point 3) with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B).

Parameter (unit)	Group	Baseline	Time-point 3	*p value*
Creatinine (mg/dl)	A	0.7 (0.4–1.3)[d]	0.8 (0.4–1.2)	0.269
	B	0.8 (0.5–1.5)	0.9 (0.6–1.7)	0.106
Urea nitrogen (mg/dl)	A	10 (6–26)	12 (4–37)	0.458
	B	11 (6–20)	12 (7–45)	0.537
Inorganic phosphorus (mg/dl)	A	4.4 (1.8–6.8)	4.4 (3.2–5.4)	0.924
	B	4.7 (3.1–6.6)	4.0 (1.4–6.7)	0.074
Cystatin-c (ng/ml)	A	1,408 (796–4,564)	1,250 (706–2,129)	0.064
	B	1,661 (876–2,713)	1,432 (283–2,050)	< 0.001
Increased cystatin-c[f]	A	11/19 (57.9%)[e]	9/19 (47.4%)	0.625
	B	16/19 (84.2%)	10/19 (52.6%)	0.031
UPC	A	0.36 (0.09–2.9)	0.28 (0.08–1.4)	0.039
	B	0.68 (0.02–6.1)	0.23 (0.02–2.6)	0.009
UPC ≥ 0.2[a]	A	12/17 (70.6%)	15/19 (78.9%)	0.5
	B	10/16 (62.5%)	10/19 (52.6%)	1
UPC = 0.2–0.5[b]	A	5/17 (29.4%)	12/19 (63.2%)	0.031
	B	2/16 (12.5%)	7/19 (36.8%)	0.063
UPC > 0.5[c]	A	7/17 (41.2%)	3/19 (15.8%)	0.125
	B	8/16 (50%)	3/19 (15.8%)	0.063

Abbreviations: UPC: urine protein-to-creatinine ratio.
[a] Dogs with borderline proteinuria and proteinuria.
[b] Dogs with borderline proteinuria.
[c] Dogs with proteinuria.
[d] Median (range).
[e] Number of dogs (percentage).
[f] Dogs with cystatin-c above 1,339 ng/ml (upper limit of the reference interval calculated for this study from the central 95th percentile in 30 clinically healthy dogs).

3.3. Evaluation of vestibular and auditory function

Wave I, wave V, inter-wave I–V latencies, and MHT did not differ between the two groups (supplementary data, Table S3) at baseline. In group A, auditory function was classified as grade 0 in 31/35 (88.6%) and as grade 1 in 4/35 (11.4%) ears, whereas for group B auditory function was classified as grade 0 in 34/36 (94.4%) and as grade 1 in 2/36 (5.6%) ears.

Peripheral vestibular syndrome and hearing impairment (i.e., any increase of auditory function grade) were not observed in group A or group B dogs at any time point of the study.

At the group level, there was no evidence of treatment-related ototoxicity. On the contrary, a significant decrease of right ear MHT at time-point 3 compared to baseline was witnessed in both groups (Table 2, Fig. 2). Also, there were no differences between the two groups considering wave I, wave V, inter-wave I–V latencies, and MHT for both ears (supplementary data, Table S4).

4. Discussion

In addition to the usual laboratory parameters (i.e., serum creatinine, urea nitrogen, and ionized phosphorus concentrations and UPC), the effect of aminosidine-allopurinol and meglumine antimonate-allopurinol treatment of CanL on kidney function were also evaluated by the incidence of serum creatinine concentration increases > 0.3 mg/dl over baseline, serum cystatin-c concentration, and incidence of serum cystatin-c concentration above the upper limit of the reference interval.

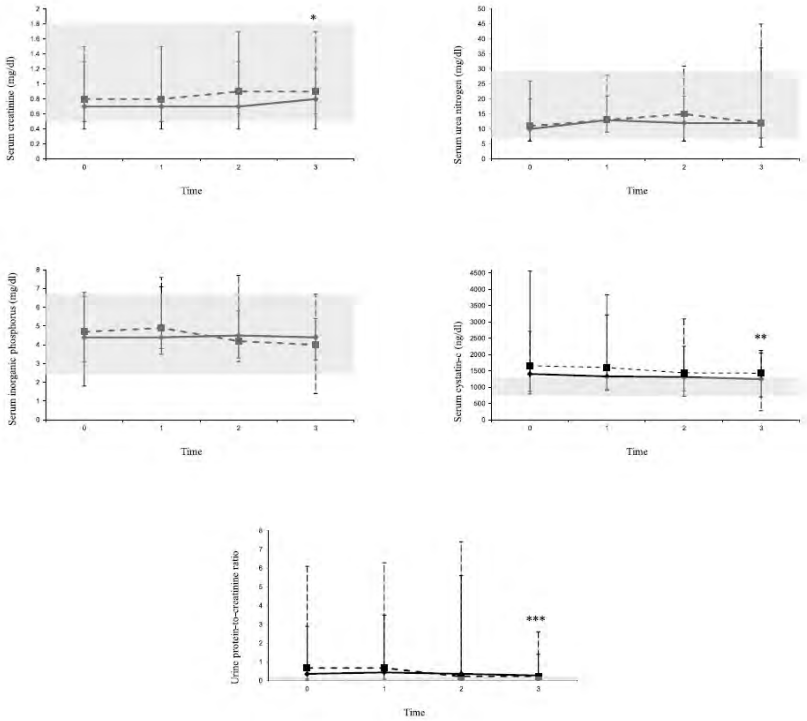

Fig. 1. Median and range of serum creatinine, urea nitrogen, inorganic phosphorus, and cystatin-c concentrations and of urine protein-to-creatinine ratio of dogs with leishmaniosis before (time-point 0; baseline) and after 14 (time-point 1), 28 (time-point 2), and 60 (time-point 3) days of treatment with aminosidine plus allopurinol (group A; solid line) or with meglumine antimonate plus allopurinol (group B; interrupted line). Shaded areas represent reference intervals. *Significant difference between group A and group B. **Significant difference compared to baseline for group B. ***Significant difference compared to baseline for group A and group B.

Small increases in serum creatinine concentration may suggest a decreased glomerular filtration rate (GFR), even if serum creatinine concentration remains within the reference interval (Hokamp and Nabity, 2016). Taking into consideration that inter-individual variability of serum creatinine concentration in an individual animal over weeks to months is minimal, at least in healthy dogs, increases > 0.3 mg/dl are considered clinically relevant (Pagitz et al., 2007). Such increases have been associated with increased mortality in a critical care setting and with an average of 27% reduction of GFR in x-linked hereditary nephropathy (Lee et al., 2011; Thoen and Kerl, 2011; Nabity et al., 2015). Serum cystatin-c was used as an additional surrogate marker of GFR. Although the effect of various biological factors and concurrent diseases on serum cystatin-c concentration has not been fully elucidated (Ghys et al., 2014), it shows a stronger correlation with GFR compared to serum creatinine concentration (Wehner et al., 2008; Miyagawa et al., 2009) and it differs significantly between dogs with CKD, healthy dogs, and dogs with diseases that do not involve the kidneys (Jensen et al., 2001; Almy et al., 2002; Antognoni et al., 2005; Wehner et al., 2008; Miyagawa et al., 2009).

Clinical signs of kidney disease and/or increases of serum creatinine > 2 mg/dl were not observed in any of the dogs in group A. Serum creatinine concentration was increased (an increase was considered a difference of > 0.3 mg/dl over baseline) in only 10.5% of the dogs (2/19) in the same group. In previous studies, up to 50% of dogs with leishmaniosis died, most likely due to kidney disease, after aminosidine administration at daily doses of 40–80 mg/kg (Vexenat et al., 1998) and increased serum creatinine concentrations were detected in 37.5% of dogs with leishmaniosis treated at 21 mg/kg once daily and in 8.3% of the dogs treated at 5 mg/kg twice daily (Persechino et al., 1994; Poli et al., 1995). Furthermore, aminosidine treatment of CanL at the dose employed in the present study (i.e., 15 mg/kg once daily) or at 3.5 mg/kg given twice daily, resulted in no deterioration or in only mild and transient deterioration of renal function in previous studies (Oliva et al., 1998; Athanasiou et al., 2014). Collectively, the results of these studies may imply that the nephrotoxicity of aminosidine, when used for the treatment of CanL, depends on both the dose and frequency of administration.

A high aminoglycoside dose will result in high maximum plasma concentration, an important risk factor for kidney damage (Destache, 2014). This may explain the lower incidence of nephrotoxicity in dogs

Table 2
Comparison of auditory function parameters of dogs with leishmaniosis before (time 0; baseline) and 60 days after treatment (time-point 3) with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B).

Parameter (unit)	Group	Time 0	Time 3	*p value*
R; wave I latency (msec)	A	1.94 ± 0.31[a]	1.86 ± 0.15	0.722
	B	1.89 ± 0.11	1.9 ± 0.14	0.155
L; wave I latency (msec)	A	1.92 ± 0.18	1.8 ± 0.13	0.737
	B	1.89 ± 0.26	1.83 ± 0.09	0.862
R; wave V latency (msec)	A	4.23 ± 0.42	4.24 ± 0.16	0.832
	B	4.24 ± 0.26	4.25 ± 0.27	0.774
L; wave V latency (msec)	A	4.19 ± 0.27	4.19 ± 0.26	0.91
	B	4.27 ± 0.39	4.29 ± 0.24	0.888
R; inter-wave I–V latency (msec)	A	2.28 ± 0.29	2.37 ± 0.18	0.266
	B	2.38 ± 0.24	2.32 ± 0.27	0.235
L; inter-wave I–V latency (msec)	A	2.26 ± 0.29	2.27 ± 0.36	0.985
	B	2.36 ± 0.21	2.45 ± 0.26	0.641
R; MHT (dBnHL)	A	5 (0–35)[b]	5 (0–25)	0.175
	B	5 (0–45)	5 (0–15)	0.068
L; MHT (dBnHL)	A	5 (0–45)	2.5 (0–25)	0.028
	B	5 (0–40)	0 (0–15)	0.007

Abbreviations: L: left ear; MHT: minimum hearing threshold; R: right ear.
[a] Mean ± standard deviation.
[b] Median (range).

with leishmaniosis when aminosidine was administered at doses ranging from 3.5 to 20 mg/kg (0–9.9%) compared to doses ranging from 21 to 80 mg/kg (33.3–50%) (Persechino et al., 1994; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013, 2014). Furthermore, once daily versus more frequent administration is thought to decrease aminoglycoside accumulation into renal tubular cells and to increase the drug-free period. In humans, extended dosing intervals have been associated with a substantial reduction of the nephrotoxicity rate from 17% to 0–5% (Wargo and Edwards, 2014). Therefore, the minimal nephrotoxicity of aminosidine found in the present study may be attributed to both the conservative dose (i.e., 15 mg/kg) and the once daily administration of the drug, as has been suggested in a previous pharmacokinetic study (Athanasiou et al., 2014).

In addition, to increased safety, once daily administration of aminoglycosides has therapeutic advantages, at least in patients with bacterial infections, due to the concentration-dependent bactericidal activity, the post-antibiotic effect of these drugs, and the minimization of adaptive bacterial resistance (Pagkalis et al., 2011; Wargo and Edwards, 2014). Whether or not this applies for the treatment of CanL is unknown. However, deterioration of renal function during treatment of CanL depends on both the potential nephrotoxicity of the drug and its efficacy in halting the progression of CanL-associated CKD (Plevraki et al., 2006). The lack of significant differences between time-point 3 and baseline for both groups (with the exception of the improvement of c-cystatin serum concentration in group B) and the lack of significant differences between the two groups at time-point 3 (with the exception of the lower serum creatinine concentration in group A) denotes a comparable nephrotoxicity/efficacy balance for aminosidine-allopurinol and meglumine antimonate-allopurinol.

Proteinuria of glomerular, tubular, or mixed origin is considered an early marker of kidney injury that precedes azotemia (De Loor et al., 2013; Cianciolo et al., 2016). Almost all dogs with leishmaniosis have histologic evidence of kidney disease (mainly glomerulonephritis) and proteinuria is an early and common laboratory finding in these patients (Zatelli et al., 2003; Noli and Saridomichelakis, 2014). Reduced severity of proteinuria can be seen during treatment of CanL and is considered a positive prognostic indicator (Koutinas et al., 2001; Pierantozzi et al., 2013; Proverbio et al., 2016). Previous open-label studies on the treatment of CanL with the same dosage regimen of aminosidine (15 mg/kg, once daily) did not include dogs with proteinuria due to the potential nephrotoxicity of this drug (Athanasiou et al., 2013, 2014). However, the majority of dogs in the present study were borderline proteinuric or proteinuric at baseline (supplementary data, Table S1) and a significant reduction of the UPC was witnessed, in both groups, at time-point 3. This finding may indicate a positive effect of aminosidine plus allopurinol and of meglumine antimonate plus allopurinol treatment on kidney function of dogs with leishmaniosis and shows that aminosidine can be safely administered to proteinuric non-azotemic CanL patients.

Aminoglycoside ototoxicity may affect the vestibular system and/or the cochlea, depending on the specific molecule. For example, gentamicin is preferentially toxic to the vestibular system, while amikacin targets mainly the cochlea (Oishi et al., 2012). No signs of vestibular dysfunction were seen in the present or in previous studies of CanL treatment with aminosidine and thus it can be concluded that this drug does not preferentially affect the vestibular system, regardless of the dosage regimen (Persechino et al., 1994; Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998; Athanasiou et al., 2013).

In the present study, there was no evidence of toxicity to the cochlea for either treatment group. In previous studies where aminosidine was used for the treatment of CanL, deafness, reversible or not, was reported in 6/6 dogs treated at 80 mg/kg once daily, in 4/12 dogs treated at 40 mg/kg once daily, in 4/8 dogs treated at 21 mg/kg once daily, but also in one dog treated at 5 mg/kg twice daily (Persechino et al., 1994; Poli et al., 1997; Vexenat et al., 1998). Deafness at high aminosidine dosage regimens reflects the dose-dependent ototoxicity of aminoglycosides (Rizzi and Hirose, 2007), whereas the most probable explanation for that dog that was treated at 5 mg/kg twice daily is the presence of concurrent azotemia, therefore the impaired renal function resulted in delayed clearance and accumulation of aminosidine in the plasma (Guthrie, 2008).

Aminosidine treatment of human visceral leishmaniasis results in a reversible high-frequency hearing loss in approximately 5% of the patients whereas severe hearing impairment is rare (Sundar et al., 2007; Hailu et al., 2010; Musa et al., 2012). Unfortunately, frequency-specific hearing thresholds could not be tested in this study (tone burst stimuli were not available) and thus frequency-specific hearing loss in dogs of group A cannot be excluded. However, high-frequency hearing loss in human patients with visceral leishmaniasis is not considered a reason to withdraw aminosidine treatment (Sundar et al., 2007; Hailu et al., 2010; Musa et al., 2012).

5. Conclusion

Administration of aminosidine, at 15 mg/kg, subcutaneously, once daily, for 28 days, plus allopurinol, to non-azotemic dogs with leishmaniosis, with or without proteinuria, resulted in minimal nephrotoxicity and in no evidence of ototoxicity. Furthermore, the impact on renal, vestibular, and cochlear function did not differ between aminosidine-allopurinol and meglumine antimonate-allopurinol. Therefore, aminosidine-allopurinol combination, at the above dosage regimen, may be considered a safe treatment option for non-azotemic dogs with CanL.

Conflicts of interest

The authors have no conflicts of interest to declare.

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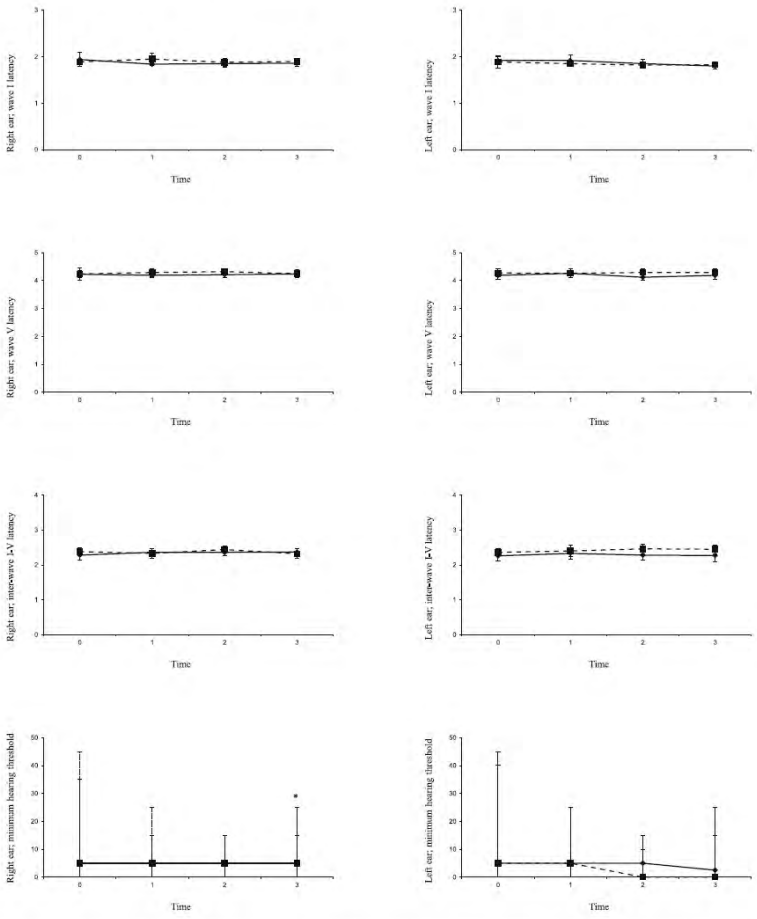

Fig. 2. Mean and standard deviation of wave I, wave V, and inter-wave I–V latencies and median and range minimum hearing threshold for the right and left ear of dogs with leishmaniosis before (timepoint 0; baseline) and after 14 (timepoint 1), 28 (timepoint 2), and 60 (timepoint 3) days of treatment with aminosidine plus allopurinol (group A; solid line) or with meglumine antimonate plus allopurinol (group B; interrupted line). *Significant difference compared to baseline for group A and group B.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2019.107768.

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Table S1. Comparison of renal function parameters between dogs with leishmaniosis treated with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B) at the beginning of the study

Parameter (unit)	**Group A**	**Group B**	***p value***
Creatinine (mg/dl)	0.7 (0.4-1.3)[d]	0.8 (0.5-1.5)	0.234
Urea nitrogen (mg/dl)	10 (6-26)	11 (6-20)	0.624
Inorganic phosphorus (mg/dl)	4.4 (1.8-6.8)	4.7 (3.1-6.6)	0.644
Cystatin-c (ng/ml)	1,408 (796-4,564)	1,661 (876-2,713)	0.057
Increased cystatin-c	11/20 (55%)[e]	16/20 (80%)	0,091
UPC	0.36 (0.09-2.9)	0.68 (0.02-6.1)	0.533
UPC ≥ 0.2[a]	12/17 (70.6%)	10/16 (62.5%)	0.525
UPC = 0.2-0.5[b]	5/17 (29.4%)	2/16 (12.5%)	0.398
UPC >0.5[c]	7/17 (41.2%)	8/16 (50%)	0.61

[a]Dogs with borderline proteinuria and proteinuria; [b]Dogs with borderline proteinuria; [c]Dogs with proteinuria; [d]Median (range); [e]Number of dogs (percentage)

Abbreviations: UPC: urine protein-to-creatinine ratio

Table S2. Comparison of renal function parameters of dogs with leishmaniosis 60 days after treatment with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B)

Parameter (unit)	**Group A**	**Group B**	***p value***
Creatinine (mg/dl)	0.8 (0.4-1.2)[d]	0.9 (0.6-1.7)	0.046
Urea nitrogen (mg/dl)	12 (4-37)	12 (7-45)	0.863
Inorganic phosphorus (mg/dl)	4.4 (3.2-5.4)	4 (1.4-6.7)	0.138
Cystatin-c (ng/ml)	1,250 (706-2,129)	1,432 (283-2,050)	0.795
Increased cystatin-c	9/19 (47.4%)[e]	10/19 (52.6%)	0.74
UPC	0.28 (0.08-1.4)	0.23 (0.02-2.6)	0.37
UPC ≥ 0.2[a]	15/19 (78.9%)	10/19 (52.6%)	0.087
UPC = 0.2-0.5[b]	12/19 (63.2%)	7/19 (36.8%)	0.105
UPC >0.5[c]	3/19 (15.8%)	3/19 (15.8%)	1

[a]Dogs with borderline proteinuria and proteinuria; [b]Dogs with borderline proteinuria; [c]Dogs with proteinuria; [d]Median (range); [e]Number of dogs (percentage)

Abbreviations: UPC: urine protein-to-creatinine ratio

Table S3. Comparison of auditory function parameters between dogs with leishmaniosis treated with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B) at the beginning of the study

Parameter (unit)	**Group A**	**Group B**	***p value***
R; wave I latency (msec)	1.94 ± 0.31[a]	1.89 ± 0.11	0.634
L; wave I latency (msec)	1.92 ± 0.18	1.89 ± 0.26	0.301
R; wave V latency (msec)	4.23 ± 0.42	4.24 ± 0.26	0.708
L; wave V latency (msec)	4.19 ± 0.27	4.27 ± 0.39	0.806
R; inter-wave I-V latency (msec)	2.28 ± 0.29	2.38 ± 0.24	0.916
L; inter-wave I-V latency (msec)	2.26 ± 0.29	2.36 ± 0.21	0.302
R; MHT (dBnHL)	5 (0-35)[b]	5 (0-45)	0.892
L; MHT (dBnHL)	5 (0-45)	5 (0-40)	0.471

[a]Mean ± standard deviation; [b]Median (range)

Abbreviations: L: left ear; MHT: minimum hearing threshold; R: right ear

Table S4. Comparison of auditory function parameters of dogs with leishmaniosis 60 days after treatment with aminosidine plus allopurinol (group A) or with meglumine antimonate plus allopurinol (group B)

Parameter (unit)	**Group A**	**Group B**	***p value***
R; wave I latency (msec)	1.86 ± 0.15[a]	1.9 ± 0.14	0.205
L; wave I latency (msec)	1.8 ± 0.13	1.83 ± 0.09	0.806
R; wave V latency (msec)	4.24 ± 0.16	4.25 ± 0.27	0.483
L; wave V latency (msec)	4.19 ± 0.26	4.29 ± 0.24	0.987
R; inter-wave I-V latency (msec)	2.37 ± 0.18	2.32 ± 0.27	0.179
L; inter-wave I-V latency (msec)	2.27 ± 0.36	2.45 ± 0.26	0.181
R; MHT (dBnHL)	5 (0-25)[b]	5 (0-15)	0.61
L; MHT (dBnHL)	2.5 (0-25)	0 (0-15)	0.616

[a]Mean ± standard deviation; [b]Median (range)

Abbreviations: L: left ear; MHT: minimum hearing threshold; R: right ear

2. Article No 2

D. Kasabalis, M.K. Chatzis, K. Apostolidis, T. Petanides L.V. Athanasiou, P.G. Xenoulis, A. Mataragka, J. Ikonomopoulos, L.S. Leontides, M.N. Saridomichelakis. **A randomized, blinded, controlled clinical trial comparing the efficacy of aminosidine (paromomycin)-allopurinol combination with the efficacy of meglumine antimoniate-allopurinol combination for the treatment of canine leishmaniosis due to *Leishmania infantum***. Under review

A randomized, blinded, controlled clinical trial comparing the efficacy of aminosidine (paromomycin)-allopurinol combination with the efficacy of meglumine antimoniate-allopurinol combination for the treatment of canine leishmaniosis due to *Leishmania infantum*

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ABSTRACT

The aim of this 6-month, randomized, blinded, controlled clinical trial was to compare the efficacy and safety of aminosidine-allopurinol combination with that of meglumine antimoniate-allopurinol combination for the treatment of leishmaniosis in dogs without stage III or IV chronic kidney disease. Forty client-owned dogs were randomly assigned to group A [n=20; aminosidine (15 mg/kg, subcutaneously, once daily, for 28 days) and allopurinol (10 mg/kg, *per os*, twice daily, for 6 months)] or group B [(n=20; meglumine antimoniate (100 mg/kg SC, once daily, for 28 days) and allopurinol (10 mg/kg, *per os*, twice daily, for 6 months)]. Clinical and clinicopathological evaluations, parasitic load measurement (lymph node and bone marrow microscopy, bone marrow real-time PCR), specific serology and leishmanin skin test (LST) were performed at baseline (time 1) and after 14 (time 2), 28 (time 3), 60 (time 4) and 180 (time 5) days. Both treatments were safe and resulted in significant clinical and clinicopathological improvement, reduction of parasitic load and of indirect immunofluorescence antibody test (IFAT) titer and induction of positive LST. There was no significant difference between groups with regards to the primary outcome measures of the trial that included the proportion of dogs that presented severe treatment-related side effects, were cured and were parasitologically negative at time 5. However, some (proportion of dogs that presented no clinical signs, no hyperglobulinemia and negative serology at time 5) secondary outcome measures showed significant differences in favor of the meglumine antimoniate-allopurinol treatment arm. Treatment-related death occurred in one dog in each group, while injection site reactions appeared at a similar frequency in both groups. Due to the differences in some secondary outcome measures in association with the low power of this trial, it cannot be definitively concluded that the two treatments are equally effective. Therefore, the aminisodine-allopurinol combination cannot be proposed as a first-line treatment of CanL but rather as a second-line treatment that may be particularly useful to avoid repeated administration of meglumine antimoniate and in countries where the latter is not available or registered.

Keywords: allopurinol, aminosidine, dog, leishmaniosis, meglumine antimonate, paromomycin

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic disease caused by the protozoan *Leishmania infantum* (*syn*: *L. chagasi*). The prevalence of infection in the Mediterranean basin is very high and the disease can be fatal if left untreated (Pennisi, 2015). Treatment recommendations for dogs with typical clinical signs and laboratory abnormalities of CanL and without advanced chronic kidney disease (CKD), include the administration of allopurinol plus either meglumine antimoniate or miltefosine, since these combinations are reasonably safe and usually lead to clinical remission, amelioration of clinicopathological abnormalities and reduction of parasitic load (Solano-Gallego et al., 2011; Noli and Saridomichelakis, 2014).

Aminosidine (paromomycin) is an aminoglycoside leishmanicidal agent that inhibits parasitic 30S ribosomal subunit (Reguera et al., 2016). The efficacy of aminosidine as monotherapy of CanL has been previously assessed in open and controlled clinical trials. Despite some notable differences in the design of these trials, a good short-term clinical efficacy was generally demonstrated but relapses after treatment discontinuation and dose-dependent side effects were exceedingly common (Persechino et al., 1994; Persechino et al., 1995; Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998). Accordingly, a systematic review of studies published until 2004, concluded that there was fair evidence against recommending aminosidine at doses higher than 20 mg/kg and fair evidence for recommending it at the dosage regimen of 5 mg/kg, twice daily (Noli and Auxilia, 2005). However, more recent studies showed that subcutaneous (SC) administration of aminosidine at an optimized dose regimen (15 mg/kg, once daily) for 21 days, results in effective peak plasma concentrations (Athanasiou et al., 2014) and leads, at least on a short-term basis, to clinical and clinicopathological improvement and reduction of parasitic load, without side effects (Athanasiou et al., 2013).

Long-term allopurinol administration in addition to the short-term use of leishmanicidal drugs, such as meglumine antimoniate and miltefosine, is considered mandatory (Reguera et al., 2016), not only to improve therapeutic efficacy but also to avoid relapses of CanL (Ginel et al., 1998; Denerolle and Bourdoiseau, 1999; Torres et al., 2011; Manna et al., 2015). To our knowledge, there are no published studies evaluating the efficacy of aminosidine-allopurinol combination for the treatment of CanL.

We have previously shown that the optimized dose regimen of aminosidine-allopurinol combination is neither ototoxic nor nephrotoxic in dogs with CanL (Kasabalis et al., 2019). The aim of this randomized, blinded, controlled clinical trial was to compare the efficacy and safety of aminosidine-allopurinol combination with that of meglumine antimoniate-allopurinol combination, in terms of improvement of CanL-associated clinical signs, avoidance of relapses, amelioration of clinicopathological abnormalities, reduction of parasitic load and evolution of *Leishmania*-specific humoral and cell-mediated immune responses, over the course of 6 months.

2. Materials and methods

The methods and results of this trial are reported according to the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement, available at http://www.consort-statement.org/.

2.1. Dogs, trial design and treatment

This was a prospective, randomized, blinded, controlled clinical trial with two parallel treatment arms, conducted at the Clinic of Medicine, Faculty of Veterinary Science, University of Thessaly, Karditsa, Greece, between September 2009 and June 2012.

Client owned dogs were eligible for inclusion into the trial if they presented at least one clinical sign of CanL, positive serology (indirect immunofluorescence antibody test-IFAT titer ≥1/200) and positive lymph node and/or bone marrow microscopy for *Leishmania* amastigotes. Exclusion criteria were stage III or IV CKD, the presence of any concurrent disease, pregnancy, lactation, previous vaccination against CanL, administration of meglumine antimoniate, miltefosine, aminosidine or amphotericin B during the last 12 months, administration of allopurinol, ketoconazole, metronidazole, fluoroquinolones or pentamidine during the previous 3 months, aggressive behavior prohibiting safe hospitalization of the dog and the owner's unwillingness to participate in the study (Kasabalis et al., 2019).

With the aid of a randomization table, dogs were allocated, at 1:1 ratio, into two groups. Group A dogs were treated with aminosidine (Gabbrocol®, CEVA) at a target dose of 15 mg/kg, SC, once daily, for 28 days, whereas group B dogs were

treated with meglumine antimoniate (Glucantime®, Aventis) at a target dose of 100 mg/kg SC, once daily, for 28 days. Allopurinol (Zylapour®, Farmanic) was administered to dogs in both groups at a target dose of 10 mg/kg, *per os*, twice daily, for 6 months. During the first 4 weeks of the trial, all dogs were hospitalized to ensure appropriate treatment administration and monitoring, were fed a commercial dry food with low purine concentration (Urinary Low Purine®, Royal Canin), had free access to water and received a daily physical examination by a non-blinded member of the research team (MKC), who was also responsible for randomization, treatment administration and recording side effects. After the first 4 weeks, dogs were returned to their owners who were instructed to continue allopurinol administration and to bring the dogs to the Clinic for the scheduled re-examinations (Kasabalis et al., 2019). The randomization sequence was unmasked after all clinical evaluations and laboratory examinations had been completed, with the exception of severe, potentially drug-related, clinical signs (see section 2.6.). Also, before entering into the trial and monthly thereafter, an insect repellent spot-on containing permethrin and imidacloprid (Advantix®, Bayer) was applied to all dogs.

2.2. Clinical evaluation

The presence, extent and severity of 18 clinical signs of CanL were scored at baseline (time 1) and after 14 (time 2), 28 (time 3), 60 (time 4) and 180 (time 5) days, using a predefined grading scale (supplementary material-Table S1). Scoring was done by the first author (DK), who was blinded to the group each dog belonged to. Clinical relapse was defined as the appearance of new or the re-appearance of previously regressed clinical signs of CanL.

2.3. Clinicopathological evaluation

Blood samples (approximately 10 ml) were obtained by jugular venipuncture, after overnight fasting, at times 1, 2, 3, 4 and 5 and used for complete blood count [packed cell volume (PCV), white blood cell count and platelet count (VetAutoread™ Hematology Analyzer, IDEXX) and manual differential count], serum biochemistry [total proteins, albumin, globulins, albumin/globulin (AG) ratio, urea nitrogen, creatinine, glucose, cholesterol, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), lipase, amylase, creatine kinase (CK), calcium, inorganic

phosphorus (VetTest Chemistry Analyzer, IDEXX), potassium and sodium (VetStat Electrolyte Blood Gas Analyzer, IDEXX)] and C-reactive protein [(CRP), Phase™ EIA Canine CRP Assay, Tridelta Development Limited; commercially available solid-phase sandwich immunoassay that has been validated for dogs)].

2.4. Evaluation of parasitic load

Evaluation of parasitic load was performed at times 1, 3, 4 and 5 and included lymph node and bone marrow microscopy and bone marrow real-time PCR.

Lymph node and bone marrow microscopic examination was performed, as previously described (Saridomichelakis et al., 2005), by the first author (DK) who was blinded to dog's group and time of sample collection. Following examination of 10-1,000 oil immersion fields, parasitic load was scored from 0 to 6, using a logarithmic scale (Chulay and Bryceson, 1983).

Bone marrow aspirates, stored in sterile EDTA tubes, were processed for DNA isolation and real-time PCR. The former was performed with the High Pure PCR Template Kit®, according to the instructions provided by the manufacturer (Roche). DNA products were tested with a real-time PCR incorporating primers designed to generate a 145 base pair amplicon from the conserved region of *L. infantum* kinetoplast minicircle DNA, as previously described (Lachaud et al., 2002). Reactions were prepared using the KAPA SYBR FAST qPCR kit protocol (Kapa Biosystems) and consisted of 1x KAPA SYBR FAST qPCR Master Mix® Universal, 5 μg of unacetylated bovine serum albumin, 2μl of template DNA, 0.2 μM of each primer (forward: 5′-CTTTTCTGGTCCCGCGGGTAGG-3′; reverse: 5′-CCACCTGGCCTATTTTACACCA-3) and PCR-grade water to a final volume of 20 μl. The temperature profile consisted of an initial denaturation step at 95ºC for 5 min, followed by 40 cycles of 3 sec at 95ºC, 20 sec at 58ºC and 1 sec at 72ºC; disassociation curve was constructed in the range of 65ºC to 95ºC. Quantification was done by means of a graded scale provided by Real Time PCR Detection Kit (Gene Sig Primer Design). Results were expressed as number of parasites/ml.

The quality of DNA product was assessed for purity and integrity with submerged gel electrophoresis, followed by image analysis (Bio-Rad ChemiDoc XRS+ Molecular Imager; Bio-Rad Laboratories Inc.) and measurement of optical density at 260/280 nm (NanoDrop 8000 Spectrophotometer; Thermo Fisher Scientific

Inc.). The presence of inhibitors in the DNA solutions was assessed with a PCR assay targeting a housekeeping gene (*β-actin*), as previously described (Dowling and Bienzle, 2005). If the latter did not produce the expected amplification product, the assessment was repeated with DNA diluted in PCR-grade water (1:4 v/v) or with DNA isolated *de novo* from the respective stock-sample. During DNA isolation and real-time PCR, positive and negative controls (bone marrow aspirates that had been confirmed as PCR-positive and PCR-negative, respectively) were processed, together with tested samples, at a number approximating 10% of tested samples. The *Ct* values, of the positive controls tested per batch were incorporated in a Levey-Jennings analysis, to monitor their quality throughout the investigation. Approximately 50% of PCR-positive samples were randomly selected and submitted to sequence analysis, to confirm the specificity of the amplification process. Sequence analysis was performed on both strands using the BigDye® Terminator Cycle Sequencing Kit and PRISM® 377 DNA Sequencer (Applied Biosystems). Results were compared to deposited sequences in the GenBank database using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information. DNA isolation and real-time PCR was conducted under ISO17025 (ISO17025:2005) accreditation (E.S.Y.D., No 1042).

2.5. Evaluation of Leishmania-specific humoral and cell-mediated immune responses

Serum samples obtained at times 1, 3, 4 and 5 were batch tested for *Leishmania*-specific IgG by IFAT. Commercially available slides and conjugate (Fluoleish®, Bvt, Biovetotest Diagnostic Veterinaire, France) were used and the results were interpreted by a member of the research team (LVA) blinded to dog's group and time of sample collection. Serum was tested at twofold dilutions in phosphate buffered saline (PBS), starting from 1:50, to the end point titer.

Leishmanin skin test (LST) was performed at times 1, 3, 4 and 5, as previously described (Solano-Gallego et al., 2000). Briefly, 100 μl of soluble *Leishmania* antigen in PBS, obtained from 1 x 10^8/ml *L. infantum* (strain MHOM/GR/78/L4A) promastigotes, was injected intradermally in the non-lesional skin of left groin. As a negative control, the same volume of PBS was also injected at a 2 cm distance. The injection site was examined after 48 and 72 hours and the two perpendicular diameters of any induration were measured and averaged. An average induration diameter >5 mm was considered positive.

2.6. Trial end points

Trial end points were: a) development of severe, potentially drug-related, clinical signs, b) CanL-related or treatment-related death or euthanasia, c) administration of unauthorized drugs and d) reaching day 180 (time 5). In the event of severe clinical signs and/or major deterioration of clinicopathological parameters (e.g. CKD stage III or IV; clinical signs of liver disease along with ALP and/or ALT activity >3x the upper limit of reference range; clinical signs of acute pancreatitis and/or lipase and/or amylase activity >5x the upper limit of reference range; white blood cell count >20.000/μl accompanied by systemic signs such as fever, depression and anorexia; hearing impairment or peripheral vestibular syndrome) treatment was withdrawn, randomization was unmasked and appropriate supportive measures were instituted.

2.7. Primary and secondary outcome measures

The primary outcome measures of the trial were the proportion of dogs that: a) were withdrawn due to severe, potentially treatment-related, clinical signs, or died, or were euthanized, b) were cured of CanL (i.e. presented no clinical signs plus no anemia, hypoalbuminemia, hyperglobulinemia, or decreased AG ratio at time 5), and c) were parasitologically negative (no amastigotes found after microscopic examination of 1,000 oil immersion fields of lymph node and bone marrow aspiration smears plus negative bone marrow real-time PCR) at time 5.

The secondary outcome measures of the trial were: a) the proportion of dogs that presented no clinical signs at time 5, b) the proportion of dogs that did not present anemia, hypoalbuminemia, hyperglobulinemia, decreased AG ratio or increased CRP concentration at time 5, c) the proportion of dogs with negative IFAT (titer <1/200) at time 5, and d) the proportion of dogs that converted from negative to positive LST between times 1 and 5.

2.8. Statistical analysis

Due to lack of published information on the treatment of CanL with the aminosidine-allopurinol combination, it was impossible to make valid hypotheses on the expected results for the primary outcome measures in group A dogs and to

perform power analysis for the determination of sample size. For this reason, and taking into consideration the financial limitations and the relatively long (6 months) duration of the trial, it was decided to enroll a total of 40 dogs with CanL. With this sample size, the trial had 80% power to detect a difference of 40% between the two groups, at 5% level of significance.

The distribution of continuous data was tested for normality using the Kolmogorov-Smirnov test and they are presented as mean and standard deviation (SD) if normally distributed or as median and range (non-normal distribution), whereas proportions are used for categorical data.

For the evaluation of the effects of each treatment and the comparison between the two treatment arms, intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination (treatment efficacy) or all enrolled dogs (treatment safety).

The two groups were compared by Pearson's chi-squared or Fisher's exact test (sex, proportion of breeds predisposed to CanL, proportion of dogs reaching a trial end point, proportion of dogs available for examination at time 5, prevalence of each clinical sign at times 1 and 5, proportion of dogs that presented no clinical sign at time 5, prevalence of each clinicopathological abnormality at times 1 and 5, proportion of dogs that were cured of CanL at time 5, proportion of dogs with positive lymph node and with positive bone marrow microscopy at times 1 and 5, proportion of dogs with positive bone marrow real-time PCR at times 1 and 5, proportion of dogs with positive IFAT at time 5, proportion of dogs with positive LST at times 1 and 5, proportion of dogs that converted from negative to positive LST) and by two-sample *t*-test or Mann-Whitney *U* test (age, body weight at times 1 and 5, actual dose of allopurinol, extent and severity score of each clinical sign at times 1 and 5, number of clinical signs per dog at times 1 and 5, numerical values of each clinicopathological parameter at times 1 and 5, number of clinicopathological abnormalities per dog at times 1 and 5, lymph node and bone marrow parasitic load based on microscopy at times 1 and 5, bone marrow parasitic load based on real-time PCR at times 1 and 5, IFAT titer at times 1 and 5).

Kaplan-Meier survival curves were developed to compare the cumulative probability of change in the prevalence of each clinical sign, clinicopathological abnormality, positive lymph node and bone marrow microscopy, positive bone

marrow real-time PCR, positive IFAT and positive LST over time between groups. The survival curves were compared with a log-rank test. Binary logistic generalized estimating equations were used to compare the probability of change in the prevalence of each clinicopathological abnormality, positive lymph node and bone marrow microscopy, positive bone marrow real-time PCR, positive IFAT and positive LST across all time points. The subject (dog) was considered a random factor, while group and time as fixed factors. Also, Friedman two-way ANOVA was used to compare the evolution of the total number of clinical signs, parasitic load based on lymph node and bone marrow microscopy, parasitic load based on bone marrow real-time PCR and IFAT titer between the two groups throughout the trial.

Linear mixed effect models were constructed to compare the effect of treatment on the rate of reduction of the number of clinical signs per dog across all time points. The subject (dog) was treated as random factor while group, time, and group*time interaction as fixed factors.

McNemar's test (prevalence of each clinical sign, prevalence of each clinicopathological abnormality, proportion of dogs with positive lymph node and with positive bone marrow microscopy, proportion of dogs with positive bone marrow real-time PCR, proportion of dogs with positive IFAT, proportion of dogs with positive LST) and paired samples *t*-test or Wilcoxon signed rank test (body weight, extent and severity score of each clinical sign, number of clinical signs per dog, numerical values of each clinicopathological parameter, number of clinicopathological abnormalities per dog, lymph node and bone marrow parasitic load based on microscopy, bone marrow parasitic load based on real-time PCR, IFAT titer) were used, separately for each group, for pairwise comparisons between time 1 and time 5.

Significance was set at 5% and the analyses were done in Stata 13 and SPSS 20 for Windows.

2.9. Ethics

The experimental protocol had been approved by State authorities (license No 3699/20-10-08) and a signed informed consent was obtained from dog owners. Handling of dogs was in accordance with the European Communities Council Directive 86/606/EEC and the International Guiding Principles for Biomedical

Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences (https://grants.nih.gov/grants/olaw/guiding_principles_2012.pdf). Also, owners were free to withdraw their dogs from the trial, at any time point and for any reason.

3. Results

3.1. Dogs, treatment and follow-up

Of the 48 dogs with CanL that were assessed for eligibility, 40 were included in the trial and eight were not due to one of the exclusion criteria (Figure 1). The 40 eligible dogs were randomized to group A (n=20) and group B (n=20).

Group A included 12 (60%) intact-male and 8 (40%) intact-female dogs. Half of them (10/20-50%) were pure-breed [Greek hound (4 dogs), Doberman pinscher, English pointer, English setter, German shepherd, German wirehaired pointer and Segugio Italiano (1 dog, each)] and half (10/20-50%) were mixed-breed, their age ranged from 1.5 to 12 years (median: 3.7 years) and their mean body weight was 17.7 ± 8.9 Kg. Group B included 13 (65%) intact-male and 7 (35%) intact-female dogs that belonged to 8 different pure breeds [Greek hound and German shorthaired pointer (4 dogs, each), English setter and Rottweiler (2 dogs, each), Caucasian shepherd, Doberman pinscher, English pointer and German wirehaired pointer (1 dog, each)] or were mixed-breed (4/20-20%), their age ranged from 1 to 12 years (median: 5 years) and their mean body weight was 23.4 ± 9.6 Kg (Kasabalis et al., 2019). There were no significant differences between groups regarding the sex, the age and the body weight of dogs (supplementary material-Table S2) and the proportion of breeds that are predisposed to CanL (p=1) according to (Miranda et al., 2008).

The actual doses of aminosidine (group A) and meglumine antimoniate (group B) were 14.8 (± 0.56) mg/kg and 99.4 (± 1.1) mg/kg, respectively. The actual dose of allopurinol [10.2 (± 1.7) mg/kg and 10.2 (± 1.1) mg/kg in groups A and B, respectively] did not differ significantly between groups (p=0.904) (Kasabalis et al., 2019).

From the 40 dogs that were randomized, 21 (52.5%) were lost to follow-up and 19 (47.5%) reached a trial end point (Figure 1); the latter included 8/20 (40%) group A and 11/20 (55%) group B dogs (non-significant difference; p=0.342). Lost to

follow-up group A dogs (n=12) were not available for time 4 (3/12) and time 5 (12/12) re-examinations, and, lost to follow-up group B dogs (n=9) were not available for time 4 (1/9) and time 5 (9/9) re-examinations.

Treatment-related death occurred, during the first week of the trial, in one dog from each group. No other dogs were withdrawn due to severe, potentially treatment-related, clinical signs, or were euthanized. The proportion of dogs that were withdrawn from the trial, died, or were euthanized (primary outcome measure) did not differ between the two groups (p=1)

Unauthorized drugs (prednisolone at immunosuppressive dose) were administered, after time 3, to one group A dog that developed pemphigus foliaceus. The proportion of dogs that were available for the final examination (time 5) was 6/20 (30%) and 10/20 (50%) for groups A and B, respectively (non-significant difference; p=0.197).

3.2. Clinical evaluation

The most common clinical signs at enrolment included lymph node enlargement (38/40-95%), masticatory muscle atrophy (33/40-82.5%), splenomegaly (22/40-55%), poor body condition (21/40-52.5%), appendicular muscle atrophy (21/40-52.5%), arthritis/polyarthritis (18/40-45%), exfoliative dermatitis (18/40-45%), blepharitis (17/40-42.5%), conjunctivitis (17/40-42.5%), nasal hyperkeratosis (17/40-42.5%) and onychogryphosis (15/40-37.5%). The prevalence of onychogryphosis (p=0.003), the extent and severity score of masticatory muscle atrophy (p=0.027) and the extent and severity score of onychogryphosis (p=0.004) were significantly higher in group A compared to group B (supplementary material-Tables S3 and S4). The number of clinical signs per dog did not differ between group A and group B (supplementary material-Table S2).

No clinical relapses, defined as appearance of new or re-appearance of previously regressed clinical signs of CanL (see section 2.2), were witnessed in group A or group B dogs throughout the trial. In fact, the frequency, the extent and severity score and the number of clinical signs per dog, gradually declined during the trial in both groups. The time for the reduction in the prevalence of each clinical sign did not differ between groups with the exception of nasal hyperkeratosis that regressed faster

in group B (p=0.015). The rate of reduction of the number of clinical signs per dog did not differ between groups (p=0.827).

The prevalence of all clinical signs of group A dogs was numerically lower at the end of the trial (time 5) compared to time 1 and this difference was significant for 8/16 clinical signs (Table 1). In addition, the extent and severity score of 12/16 clinical signs (Table 2) and the number of clinical signs per dog (p<0.001) were significantly lower, whereas their body weight was significantly higher (p=0.025) at the end of the trial compared to time 1. Similar results were witnessed in group B dogs with the prevalence of 9/16 (Table 1), the severity of 9/16 (Table 2) and the number of clinical signs per dog (p<0.001) being significantly lower and the body weight being significantly higher (p=0.008) at time 5 compared to time 1.

At the end of the trial there was no difference in body weight between the two groups (p=0.123) but masticatory muscle atrophy (p=0.007) and onychogryphosis (p=0.02) were more common (supplementary material-Table S5), the extent and severity score of masticatory muscle atrophy was higher (p=0.023; supplementary material-Table S6) and the number of clinical signs per dog was higher (p=0.018) in group A compared to group B. Also, the proportion of dogs that presented no clinical signs (secondary outcome measure) was significantly (p=0.044) lower in group A (4/19-21.1%) compared to group B (10/19-52.6%).

3.3. Clinicopathological evaluation

The most common clinicopathological abnormalities at enrolment included decreased PCV (31/40-77.5%), decreased albumin/globulin ratio (23/40-57.5%), increased globulins (22/40-55%) and decreased lymphocyte count (15/40-37.5%). The prevalence of all clinicopathological abnormalities was similar in both groups (supplementary material-Table S7) but the activity of alanine aminotransferase was higher, the activity of amylase was lower and the concentration of potassium was lower in group A compared to group B dogs (supplementary material-Table S8). The number of clinicopathological abnormalities per dog did not differ between group A and group B (supplementary material-Table S2)

In both groups the prevalence, severity and number of clinicopathological abnormalities per dog gradually declined during the trial. The time for the reduction in the prevalence of each clinicopathological abnormality did not differ between groups

with the exception of hyperglobulinemia that regressed faster in group B (p=0.009). The probability of change in the prevalence of each clinicopathological abnormality across all time points did not differ between groups, again with the exception of hyperglobulinemia that was significantly more likely to decline in group B (p=0.02).

In group A dogs, the prevalence of decreased PCV, increased globulin concentration and decreased AG ratio was significantly lower at the end of the trial (time 5) compared to time 1 (Table 3). In addition, PCV, AG ratio, lipase and amylase activities were significantly higher, whereas total protein, globulin, total bilirubin, CRP concentrations (Table 4) and number of clinicopathological abnormalities per dog (p=0.018) were significantly lower at the end of the trial compared to time 1. In group B dogs, the prevalence of increased globulin concentration, decreased AG ratio and increased CRP concentration was significantly lower at time 5 compared to time 1 (Table 3). In addition, PCV, albumin concentration, AG ratio, creatinine and cholesterol concentrations and lipase activity were significantly higher, whereas, total protein, globulin and total bilirubin concentrations, CK activity, inorganic phosphorus and CRP concentrations (Table 4) and number of clinicopathological abnormalities per dog (p<0.001) were significantly lower and at time 5 compared to time 1.

At the end of the trial, the prevalence of hyperglobulinemia (p=0.008) but not the prevalence of anemia (p=0.189), hypoalbuminemia (p=1), decreased AG ratio (p=0.09) or increased CRP concentration (p=0.074) (secondary outcome measures) or of any other clinicopathological abnormality, was higher in group A compared to group B (supplementary material-Table S9). In addition, PCV (p=0.029), lymphocyte count (p=0.044), AG ratio (p=0.028), creatinine (p=0.001) and cholesterol (p=0.046) concentrations were significantly lower, whereas, globulin concentration (p=0.031) was significantly higher in group A compared to group B dogs (supplementary material-Table S10). Also, the number of clinicopathological abnormalities per dog was higher (p=0.001) in group A (4 ± 2.36) compared to group B (1.79 ± 1.18).

The prevalence of dogs that were cured of CanL (i.e. presented no clinical signs plus no anemia, hypoalbuminemia, hyperglobulinemia, or decreased AG ratio) at time 5 (primary outcome measure) was not different (p=0.074) between group A (3/19-15.8%) and group B (8/19-42.1%), but approached significance.

3.4. Evaluation of parasitic load

At enrolment, lymph node microscopy was positive for *Leishmania* amastigotes in all 40 dogs and the parasitic load did not differ between group A and group B (supplementary material-Table S2). The proportion of dogs with positive lymph node microscopy decreased significantly during the trial in both treatment arms ($p<0.001$), starting at time 4 in group A and at time 3 in group B and there was no difference between the groups in the rate of reduction over time ($p=0.592$). Also, the parasitic load decreased significantly during the trial in both treatment arms ($p<0.001$), starting at time 3. The proportion of dogs with positive lymph node microscopy as well as the parasitic load was significantly lower ($p<0.001$ for all comparisons) at time 5 compared to time 1 in both groups (Table 5). At the end of the trial, the proportion of dogs with positive lymph node microscopy ($p=1$) and the parasitic load ($p=0.863$) did not differ between group A and group B.

At enrolment, bone marrow microscopy was positive for *Leishmania* amastigotes in 18/20 (90%) group A and in all 20 (100%) group B dogs ($p=0.487$) but the parasitic load was significantly higher ($p=0.046$) in group A (supplementary material-Table S2). The proportion of dogs with positive bone marrow microscopy decreased significantly during the trial in both treatment arms ($p<0.001$), starting at time 3 ($p=0.69$) and there was no difference in the rate of reduction over time ($p=0.428$). Also, the parasitic load decreased significantly during the trial in both treatment arms ($p<0.001$), starting at time 3. The proportion of dogs with positive bone marrow microscopy as well as the parasitic load was significantly lower ($p<0.001$ for all comparisons) at time 5 compared to time 1 in both groups (Table 5). At the end of the trial, the proportion of dogs with positive bone marrow microscopy ($p=1$) and the parasitic load ($p=0.931$) did not differ between group A and group B.

At enrolment, bone marrow real-time PCR was positive in 18/20 (90%) group A and in all 20 (100%) group B dogs ($p=0.487$) and the parasitic load did not differ between the groups (supplementary material-Table S2). The proportion of dogs with positive bone marrow real-time PCR did not change during the trial but the parasitic load decreased significantly in both treatment arms ($p<0.001$), starting at time 3. The proportion of dogs with positive bone marrow real-time PCR was significantly lower at time 5 compared to time 1 in group B ($p=0.002$) but not in group A ($p=0.25$) whereas the parasitic load was lower at time 5 compared to time 1 in both group A ($p=0.002$) and group B ($p<0.001$) (Table 5). At the end of the trial, the proportion of

dogs with positive bone marrow real-time PCR (p=0.097) and the parasitic load (p=0.931) did not differ between the groups.

At the end of the study, the proportion of dogs that were parasitologically negative (no amastigotes found after microscopic examination of 1,000 oil immersion fields of lymph node and bone marrow aspiration smears plus negative bone marrow real-time PCR) did not differ (p=0.485) between the groups, being 5/19 (26.3%) for group A and 7/19 (36.8%) for group B dogs (primary outcome measure).

3.5. Evaluation of Leishmania-specific humoral and cell-mediated immune responses

At enrolment, IFAT was positive in all 40 dogs (inclusion criterion) and the titer did not differ between the groups (supplementary material-Table S2). Titers decreased significantly during the trial in both treatment arms (p<0.001), starting at time 3 in group A and at time 4 in group B and there was no difference between the groups in the rate of reduction over time (p=0.47). At time 5, the proportion of dogs with positive IFAT was significantly lower compared to time 1 only in group B, whereas IFAT titers were significantly lower compared to time 1 in both groups (Table 5). At the end of the trial, the proportion of dogs with positive IFAT (secondary outcome measure) was significantly lower (p=0.02) and the IFAT titers were significantly higher in group A (p=0.008) compared to group B.

At enrolment, LST was positive in 2/20 (10%) group A and in 6/20 (30%) group B dogs (supplementary material-Table S2). The proportion of dogs with positive LST increased in both treatment arms without between-group differences in the probability for positive LST (p=0.306) or the time for a positive LST to occur (p=0.578). The proportion of dogs with positive LST was significantly higher at time 5 compared to time 1 in both groups (Table 5). At the end of the trial, the proportion of dogs with positive LST and the proportion of dogs that converted from negative to positive LST between times 1 and 5 (secondary outcome measure) did not differed between the groups (p=0.097 and 0.511, respectively).

3.6. Side effects

One dog in each group died during the first week of treatment. Clinicopathological evaluation of the group A dog, just before death, was unremarkable and owners declined necropsy. Even though a definite cause of death

could not be established, it was considered to be treatment-related. The group B dog died of acute pancreatitis that was diagnosed based on an increased pancreatic lipase immunoreactivity concentration (Spec cPL >1000 μg/l, reference range: 0-200 mg/l) and compatible post-mortem macroscopic and histopathologic findings of the pancreas. The death was considered treatment-related because pancreatic lipase immunoreactivity at the time 0 serum sample was <30 μg/l and pancreatitis has been considered to be a rare side effect of meglumine antimonate treatment.

Eleven dogs (55%) in each group (p=1) developed injection site reactions (inflammation, thickening, pain on palpation) during the first month of treatment. All injection site reactions regressed by time 5. No other treatment-related side effects were documented.

4. Discussion

In a previous publication, we have shown that aminosidine-allopurinol combination is neither ototoxic nor nephrotoxic when administered to dogs with CanL without stage III or IV CKD (Kasabalis et al., 2019). In this 6-month, randomized, blinded, controlled clinical trial, using the same dogs, there was no significant difference between aminosidine-allopurinol or meglumine antimoniate-allopurinol combinations regarding the proportion of dogs that presented severe treatment-related side effects and the proportion of dogs that were cured and were parasitologically negative at the end of the trial. However, there were significant differences between groups regarding the proportion of dogs that had no clinical signs, no hyperglobulinemia and negative IFAT at time 5, in favor of the meglumine antimoniate-allopurinol treatment arm. These results should be evaluated with caution taking into consideration the relatively small sample size and the high number of dogs that were lost to follow-up, especially in the aminosidine-allopurinol group.

The clinical sings, clinicopathological abnormalities and their prevalence at time 0 were similar to those reported in previous studies (Ciaramella et al., 1997; Koutinas et al., 1999; Perego et al., 2014) and all dogs were classified as stage II or III, according to LeishVet (Solano-Gallego et al., 2017). Therefore, we consider this population of 40 dogs as representative of dogs with moderate to severe CanL that should normally be treated by either meglumine antimoniate-allopurinol or

miltefosine-allopurinol combination, according with current guidelines (Solano-Gallego et al., 2017).

The clinical efficacy of the aminosidine-allopurinol combination was clearly shown in this study as there were no clinical relapses, the total number of clinical signs was significantly reduced and the body weight of group A dogs was significantly increased by the end of the trial. In previous studies, where aminosidine had been used as monotherapy, clinical relapses were witnessed in 73% of the dogs at the 6 month follow-up (Oliva et al., 1998). Our findings support the current recommendation to use allopurinol to maintain the clinical remission achieved by potent leishmanicidal drugs, such as meglumine antimoniate, miltefosine and aminosidine (Miró et al., 2009; Paradies et al., 2012; Noli and Saridomichelakis, 2014). At time 5, the prevalence of 8/16 clinical signs was significantly lower compared to time 0 (Table 1), whereas the reduction in the prevalence was not significant for the remaining 8/16 clinical signs. However, at enrolment (Table 1) the former were each present in 6 to 18 group A dogs (median: 10) whereas the latter in only 1 to 11 dogs (median: 5.5), an observation implying that the lack of significant differences may have been due to insufficient power of the study. Similarly, the 12 clinical signs with a significantly lower extent and severity score at time 5 compared to time 0 (Table 2) were each initially present in 5 to 18 dogs (median: 9) whereas the 4 clinical signs without a significant reduction in their extent and severity scores were each present in only 1 to 8 dogs (median: 3.5) (Tables 1 and 2). A possible way to overcome the lack of statistical power could have been to use a cumulative scoring system based on the sum of the extent and severity scores of selected clinical signs and to compare the total clinical score between times 1 and 5. However, to the best of our knowledge, none of the total clinical scoring systems has been validated in terms of validity (content, construct, criterion), reliability (inter-observer and intra-observer reliability, internal consistency) and responsiveness (sensitivity to change) (Olivry et al., 2014) and for this reason we decided to avoid this option.

As expected based on previous studies (Denerolle and Bourdoiseau, 1999; Pennisi et al., 2005; Miranda et al., 2007; Manna et al., 2008a; Pennisi et al., 2008; Miró et al., 2009; Todolí et al., 2010; Miró et al., 2011; Torres et al., 2011; Di Muccio et al., 2012; Paradies et al., 2012; Rossi et al., 2014; Cortese et al., 2015; Manna et al., 2015; Segarra et al., 2017; Cantos-Barreda et al., 2018a; Cantos-Barreda et al.,

2018b), a clear improvement without clinical relapses, was also present in group B dogs. Some of the differences in favor of group B in the improvement of clinical signs during the trial may be attributed to their increased prevalence and/or extent and severity scores in group A at enrolment (i.e. the prevalence of onychogryphosis and the extent and severity score of masticatory muscle atrophy were significantly higher in group A compared to group B at both times 1 and 5). In contrast, the faster reduction in the prevalence of nasal hyperkeratosis, the decreased prevalence of masticatory muscle atrophy at time 5, the lower number of clinical signs per dog at time 5, and, especially the higher proportion of dogs that presented no clinical signs at time 5 (secondary outcome measure) imply that the clinical efficacy of meglumine antimoniate-allopurinol may be higher compared to the aminosidine-allopurinol combination. Alternatively, the significant difference in the proportion of dogs presenting no clinical signs at time 5 may be related to the larger number of group A dogs that were lost to follow-up in combination with the intention-to-treat analysis of the data. Indeed, if a per-protocol analysis had been adopted the proportion of dogs without clinical signs at time 5 would have been 4/6 and 8/10 for groups A and B, respectively, and the difference between groups would not have been significant (data not shown).

The changes in the prevalence, severity and number of clinicopathological abnormalities per group A dog mirrored the clinical improvement seen in the aminosidine-allopurinol treatment arm. Considering the five most relevant clinicopathological abnormalities during treatment of CanL (decreased PCV, decreased albumin, increased globulins, decreased AG ratio and increased CRP), a significant reduction in their prevalence at time 5 compared to time 1 was observed for 3/5 (decreased PCV, increased globulins, decreased AG ratio) and a significant reduction of their severity was observed for 4/5 (decreased PCV, increased globulins, decreased AG ratio, increased CRP) (Tables 3 and 4). The lack of a significant decrease in the prevalence of hypoalbuminemia can be explained by the low number (n=3) of group A dogs that had hypoalbuminemia at time 1. In contrast, the increased CRP concentration in 8/19 (42.1%) of group A dogs at the end of the trial implies that CanL-induced inflammation was still present, assuming that there were no other causes of subclinical inflammation that were missed on physical examination.

The changes in the clinicopathological abnormalities in the meglumine antimoniate-allopurinol treatment arm were in line with the results of previous studies showing significant increases in PCV and albumin concentration and significant decreases in total protein and CRP concentrations at 6 months after the beginning of the treatment (Solano-Gallego et al., 2016; Segarra et al., 2017; Cantos-Barreda et al., 2018b). Hyperglobulinemia was the only clinicopathological abnormality having an increased probability to regress, regressed faster and at the end of the study was less common and less severe in group B compared to group A dogs. Given the contribution of the increased gamma-globulins to hyperglobulinemia, this difference is in line with the increased prevalence and the increased titer of *Leishmania*-specific IgG in aminosidine-allopurinol treatment arm at the end of the study and implies that this treatment may be less effective than the meglumine antimoniate-allopurinol combination for the control of the exaggerated humoral immune responses of dogs with CanL. Unfortunately, protein electrophoresis and immunoelectrophoresis that are necessary to substantiate this hypothesis were not performed. Alternatively, the differences between groups with regards to hyperglobulinemia may be simply the result of the increased percentage of group A dogs that were lost to follow-up in combination with the intention-to-treat analysis of the data. As an example, none of the 6 group A dogs (and none of the 10 group B dogs) that were available for re-examination at time 5 had hyperglobulinemia (data not shown).

At the end of the trial, 15.8% (3/19) group A and 42.1% (8/19) group B dogs were considered to be cured (i.e. presenting no clinical signs plus no anemia, hypoalbuminemia, hyperglobulinemia, or decreased AG ratio). This difference approached but did not reach statistical significance ($p=0.074$). This may represent a type II statistical error due to the low power of the trial.

During treatment of CanL, the reduction of the parasitic load is the cumulative effect of the direct anti-*Leishmania* action of the drugs and the activation of parasite-specific cell-mediated immune response. In this trial, parasitic load was assessed semi-quantitatively (lymph node and bone marrow microscopy) and quantitatively (bone marrow PCR). Using all these methods, aminosidine-allopurinol treated dogs had a significant qualitative (positive or negative) and quantitative reduction of their parasitic load starting at 30 days (time 3) and continuing until 6 months (time 5). Consistent with previous studies, meglumine antimonate-allopurinol treated dogs also

had significant reduction in their parasitic load (Manna et al., 2008a; Miró et al., 2009; Manna et al., 2015; Segarra et al., 2017). Despite some minor differences between the two groups (increased parasitic load on bone marrow microscopy of group A at time 1, no significant increase of the proportion of group A dogs with negative bone marrow PCR at time 5 compared to time 1), both treatment were equally effective in reducing the parasitic load and, at the end of the trial, 26.3% of group A and 36.8% of group B dogs were parasitologically negative by all three examinations.

Serology is widely used to monitor treatment of CanL and reversion to a negative antibody titer or a significant (3- to 4-fold) decrease of the initial titer has been proposed as one of the criteria for treatment discontinuation (Solano-Gallego et al., 2011; Roura et al., 2013). In this trial, the median IFAT titer at time 1 was 1/800 for both groups and, therefore, a 3- to 4-fold decrease of the titer during treatment would translate to a negative titer at time 5. In line with previous studies (Denerolle and Bourdoiseau, 1999; Miró et al., 2009; Todolí et al., 2010; Di Muccio et al., 2012; Montserrat-Sangrà et al., 2016; Solano-Gallego et al., 2016; Segarra et al., 2017), a reduction of antibody titer and a high proportion of seroconversion to negative (11/19-57.9%) was found in the meglumine antimoniate-allopurinol treatment arm. In contrast, only few (4/19-21%) group A dogs became seronegative despite the significant overall reduction of antibody titers in this group. Furthermore, at time 5 the proportion of dogs with positive IFAT and the IFAT titers were significantly higher in group A compared to group B. This finding suggests that aminosidine-allopurinol treatment is less effective than meglumine antimoniate-allopurinol for controlling the exaggerated *Leishmania*-specific humoral response during treatment of CanL. These results should be interpreted with caution and in the light of the intention-to-treat analysis and the high, non-treatment related, drop-out rate of group A dogs; indeed 4/6 (66.7%) group A dogs that were available for the time 5 re-examination were seronegative and the same was true for 8/10 (80%) of group B dogs (data not shown).

In this trial, LST was used as a surrogate marker of *Leishmania*-specific cell-mediated immune response (Solano-Gallego et al., 2005). As expected (Lima et al., 2017), LST was positive in a minority of dogs at time 1. Treatment with either aminosidine-allopurinol or meglumine-antimoniate allopurinol combination resulted

in a significant increase of the proportion of dogs with positive LST, which is in line with a previous study where all 11 dogs with CanL that were treated with meglumine-antimoniate allopurinol combination for 1 year converted from negative LST to positive (Miranda et al., 2007). Our results demonstrate that both treatments were able to activate parasite-specific cell-mediated immunity and this activation has been associated with clinical remission, improvement of clinicopathological abnormalities and reduction of parasitic load (Miranda et al., 2007; Manna et al., 2008b; Gómez-Ochoa et al., 2009; Papadogiannakis et al., 2010), as seen in the present trial.

One dog from each group died during the first week of treatment. In the aminosidine-allopurinol treated dog, a final diagnosis could not be reached but renal impairment was ruled-out based on serum biochemistry and urinalysis. The group B dog developed acute pancreatitis, which is a rare and probably idiosyncratic side-effect of meglumine antimoniate in dogs (Aste et al., 2005; Luciani et al., 2013; Xenoulis et al., 2014).

The most common side effect in this trial was the development of injection site reactions with a similar prevalence between groups. Inflammation at the sites of SC administration of meglumine antimoniate is common, with an incidence up to 100% (Noli and Auxilia, 2005; Ikeda-Garcia et al., 2007) but this side-effect has not been previously reported for aminosidine. However, in humans, the intramuscular administration of this drug has been associated with local pain and inflammation, which is a common but minor side-effect that does not necessitate treatment withdrawal (Sundar et al., 2007).

The emergence of *L. infantum* strains with reduced susceptibility and/or resistance to meglumine antimoniate and to the other first-line drugs for the treatment of CanL (miltefosine, allopurinol) is alarming (Gramiccia et al., 1992; Maia et al., 2013; Tsirigotakis et al., 2016; Yasur-Landau et al., 2016; Yasur-Landau et al., 2017). Multiple treatment courses with meglumine antimoniate have been associated with the development of resistant parasites (Gramiccia et al., 1992) and thus they should be avoided. Based on the results of this trial, aminosidine can be a useful alternative to meglumine antimoniate in dogs that necessitate repeated administration of leishmanicidal drugs to control the clinical relapses of CanL. However, caution is advised when aminosidine is used in dogs with CanL suspected to be resistant to meglumine antimoniate, because *in vitro* cross-resistance between these two drugs has

been described in dogs (Gómez Pérez et al., 2016) and humans (Das et al., 2013; Ponte-Sucre et al., 2017).

The main limitations of this trial are the small sample size (20 dogs per treatment arm) and the large number of dogs that were lost to follow-up. Due to the small sample size, this trial had only 80% power to detect a large (40%) difference between the two groups; therefore it cannot be excluded that some significant differences have been missed (type II statistical error). On the other hand, the larger number of group A that were not available for time 5 re-examination, in association with the intention-to-treat analysis of the results may have been responsible for some significant differences in some secondary outcome measures in favor of the group B (type I statistical error).

5. Conclusion

Treatment of dogs with CanL without stage III or IV CKD with a combination of aminosidine (15 mg/kg, SC, once daily, for 28 days) and allopurinol (10 mg/kg, *per os*, twice daily, for 6 months) was safe and resulted in significant clinical and clinicopathological improvement, reduction of parasitic load and of IFAT titer and induction of positive LST. There were no significant differences between aminosidine-allopurinol and meglumine antimoniate-allopurinol groups for the proportion of dogs that presented severe treatment-related side effects, were cured or were parasitologically negative at the end of the trial. However, the proportion of dogs that presented no clinical signs, no hyperglobulinemia and negative IFAT at the end of the trial were significantly higher in the meglumine antimoniate-allopurinol group. It may be concluded that the aminosidine-allopurinol combination should be regarded as second-line treatment that may be particularly useful to avoid repeated administration of meglumine antimoniate and in countries where the latter is not available or registered, but not as a first-line treatment of equal efficacy to the meglumine antimoniate-allopurinol combination. Further, larger, ideally multicenter (to account for regional differences in drug susceptibility of *L. infantum*), RCTs are needed to compare these two drug combinations and studies of longer duration are necessary to compare their long-term efficacy and the relapse rates after treatment discontinuation.

Conflict of interest

The authors have no conflicts of interest to declare.

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Figure 1. Flow diagram detailing trial population recruitment, treatment and follow-up

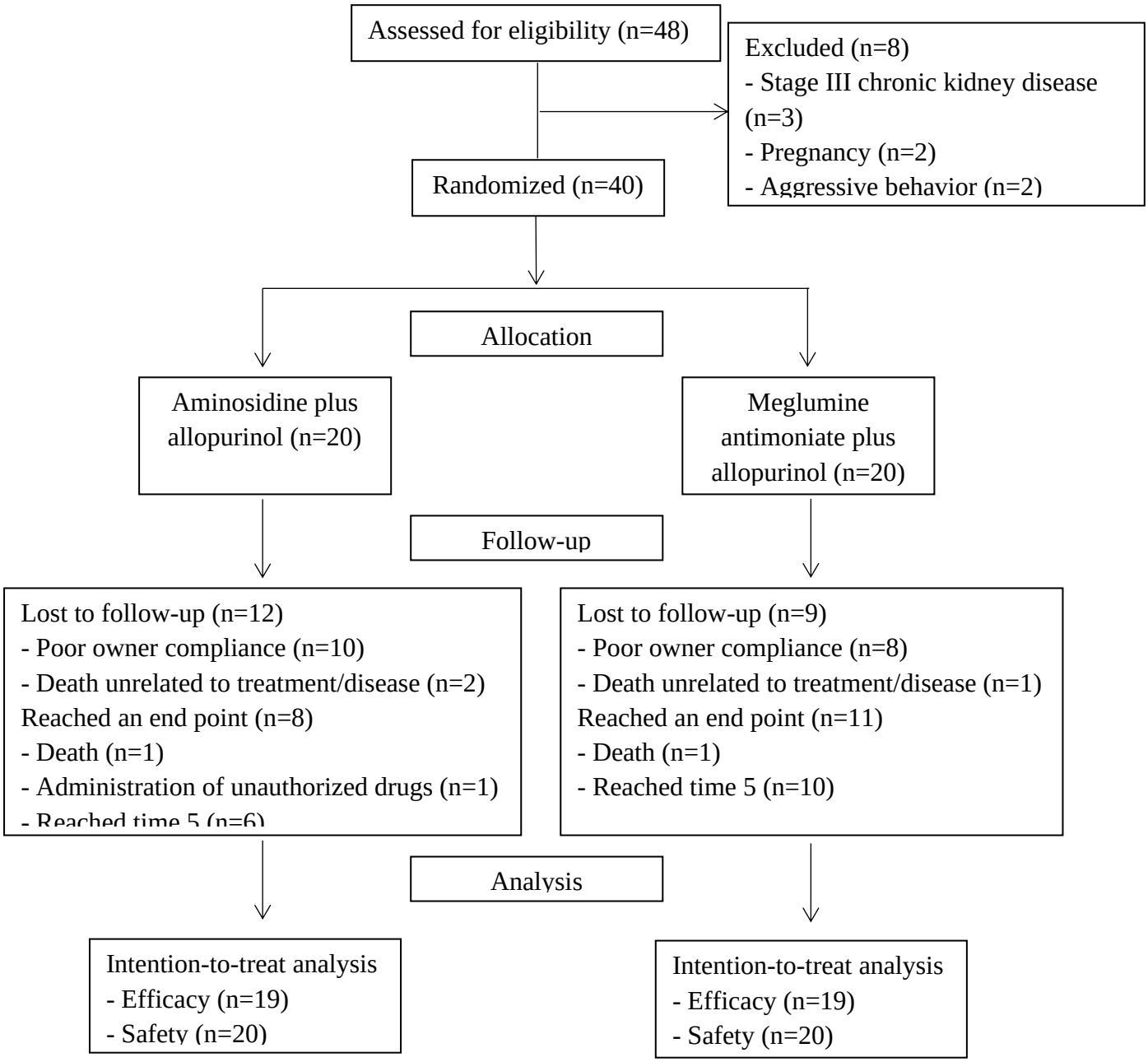

Table 1. Comparison of the prevalence of each clinical sign, between the beginning (time 1) and the end (time 5) of the trial, in dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and in dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B). Intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination

Clinical sign	**Group A (n=19)***			**Group B (n=19)***		
	Time 1	**Time 5**	**p value**	**Time 1**	**Time 5**	**p value**
Poor body condition	11 (57.9%)	3 (15.8%)	**0.008**	9 (47.4%)	1 (5.3%)	**0.008**
Lymph node enlargement	17 (89.5%)	10 (52.6%)	**0.039**	19 (100%)	6 (31.6%)	**<0.001**
Hepatomegaly	6 (31.6%)	0 (0%)	**0.031**	3 (15.8%)	0 (0%)	0.25
Splenomegaly	9 (47.6%)	0 (0%)	**0.004**	12 (63.2%)	0 (0%)	**<0.001**
Blepharitis	9 (47.6%)	1 (5.3%)	**0.008**	7 (36.8%)	1 (5.3%)	**0.031**
Conjunctivitis	6 (31.6%)	1 (5.3%)	0.125	10 (52.6%)	2 (10.5%)	**0.008**
Keratitis	4 (21.1%)	1 (5.3%)	0.25	3 (15.8%)	0 (0%)	0.25
Uveitis	1 (5.3%)	0 (0%)	1	0 (0%)	0 (0%)	
Masticatory muscle atrophy	18 (94.7%)	11 (57.9%)	**0.016**	13 (68.4%)	3 (15.8%)	**0.002**
Appendicular muscle atrophy	11 (57.9%)	3 (15.8%)	**0.008**	8 (42.1%)	2 (10.5%)	0.07
Arthritis/Polyarthritis	7 (36.8%)	2 (10.5%)	0.063	10 (52.6%)	1 (5.3%)	**0.004**
Exfoliative dermatitis	8 (42.1%)	3 (15.8%)	0.125	9 (47.4%)	1 (5.3%)	**0.008**
Skin ulcers	3 (15.8%)	2 (10.5%)	1	3 (15.8%)	0 (0%)	0.25
Nasal hyperkeratosis	9 (47.4%)	2 (10.5%)	**0.016**	7 (36.8%)	0 (0%)	**0.016**
Footpad hyperkeratosis	5 (26.3%)	0 (0%)	0.063	2 (10.5%)	0 (0%)	0.5

Onychogryphosis	11 (57.9%)	6 (31.6%)	0.063	3 (15.8%)	0 (0%)	0.25

*One dog from each group died before the first re-examination and was excluded from the intention-to-treat analysis of treatment efficacy

Table 2. Comparison of the extent and severity score of each clinical sign, between the beginning (time 1) and the end (time 5) of the trial, in dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and in dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B). Intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination

Clinical sign	**Group A (n=19)***			**Group B (n=19)***		
	Time 1	**Time 5**	**p value**	**Time 1**	**Time 5**	**p value**
Poor body condition	3.5 ± 1.39**	2.7 ± 0.87	**0.004**	3.2 ± 1.19	2.4 ± 0.84	**0.001**
Lymph node enlargement	7.2 ± 3.82	2 ± 2.88	**<0.001**	8 (3-12)	0 (0-4)	**<0.001**
Hepatomegaly	0 (0-3)**	0 (0-0)	**0.023**	0 (0-1)	0 (0-0)	0.083
Splenomegaly	0 (0-3)	0 (0-0)	**0.007**	1 (0-3)	0 (0-0)	**0.002**
Blepharitis	0 (0-6)	0 (0-2)	**0.007**	0 (0-6)	0 (0-2)	**0.016**
Conjunctivitis	0 (0-6)	0 (0-2)	**0.041**	2 (0-6)	0 (0-2)	**0.004**
Keratitis	0 (0-4)	0 (0-1)	0.059	0 (0-6)	0 (0-0)	0.109
Uveitis	0 (0-1)	0 (0-0)	0.317	-	-	-
Masticatory muscle atrophy	3.7 ± 1.91	2 ± 2.21	**0.003**	2 (0-6)	0 (0-6)	**0.01**
Appendicular muscle atrophy	4 (0-9)	0 (0-6)	**0.003**	0 (0-9)	0 (0-9)	0.095
Arthritis/Polyarthritis	0 (0-9)	0 (0-4)	**0.018**	1 (0-9)	0 (0-1)	**0.005**
Exfoliative dermatitis	0 (0-9)	0 (0-9)	0.119	0 (0-9)	0 (0-1)	**0.007**
Skin ulcers	0 (0-3)	0 (0-2)	0.414	0 (0-3)	0 (0-0)	0.109
Nasal hyperkeratosis	0 (0-3)	0 (0-3)	**0.015**	0 (0-2)	0 (0-0)	**0.014**
Footpad hyperkeratosis	0 (0-8)	0 (0-0)	**0.038**	0 (0-4)	0 (0-0)	0.18

Onychogryphosis	8 (0-48)	0 (0-48)	**0.01**	0 (0-48)	0 (0-0)	0.102

*One dog from each group died before the first re-examination and was excluded from the intention-to-treat analysis of treatment efficacy

**Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

Table 3. Comparison of the prevalence of each clinicopathological abnormality, between the beginning (time 1) and the end (time 5) of the trial, in dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and in dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B). Intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination

Clinicopathological abnormality*	**Group A (n=19)****			**Group B (n=19)****		
	Time 1	**Time 5**	**p value**	**Time 1**	**Time 5**	**p value**
Decreased packed cell volume	16 (84.2%)	10 (52.6%)	**0.031**	13 (68.4%)	6 (31.6%)	0.065
Decreased white blood cell count	3 (15.8%)	1 (5.3%)	0.5	2 (10.5%)	1 (5.3%)	1
Increased white blood cell count	0 (0%)	2 (10.5%)	0.5	0 (0%)	0 (0%)	-
Decreased neutrophil count	2 (10.5%)	1 (5.3%)	1	2 (10.5%)	0 (0%)	0.5
Increased neutrophil count	2 (10.5%)	2 (10.5%)	1	1 (5.3%)	1 (5.3%)	1
Increased band neutrophil count	0 (0%)	1 (5.3%)	1	1 (5.3%)	0 (0%)	1
Decreased lymphocyte count	9 (47.4%)	4 (21.1%)	0.063	5 (26.3%)	1 (5.3%)	0.219
Increased monocyte count	6 (31.6%)	9 (46.4%)	0.581	1 (10.5%)	4 (21.1%)	0.688
Increased eosinophil count	5 (26.3%)	6 (31.6%)	1	2 (10.5%)	4 (21.1%)	0.688
Decreased platelet count	6 (31.6%)	3 (15.8%)	0.453	4 (21.1%)	2 (10.5%)	0.625
Increased total proteins	8 (42.1%)	2 (10.5%)	0.07	4 (21.1%)	0 (0%)	0.125
Decreased albumins	3 (15.8%)	2 (10.5%)	1	5 (26.3%)	2 (10.5%)	0.375
Increased globulins	13 (68.4%)	7 (36.8%)	**0.031**	9 (47.4%)	0 (0%)	**0.004**
Decreased albumin/globulin ratio	12 (63.2%)	6 (31.6%)	**0.031**	10 (52.6%)	1 (5.3%)	**0.004**
Increased urea nitrogen	0 (0%)	1 (5.3%)	1	0 (0%)	1 (5.3%)	1

Decreased cholesterol	2 (10.5%)	0 (0%)	0.5	1 (5.3%)	0 (0%)	1
Increased alkaline phosphatase	2 (10.5%)	3 (15.8%)	1	2 (10.5%)	0 (0%)	0.5
Increased alanine aminotransferase	1 (5.3%)	1 (5.3%)	1	0 (0%)	0 (0%)	-
Increased lipase	0 (0%)	4 (21.1%)	0.125	1 (5.3%)	5 (26.3%)	0.219
Increased amylase	0 (0%)	0 (0%)	-	1 (5.3%)	0 (0%)	1
Increased creatine kinase	4 (21.1%)	2 (10.5%)	0.688	6 (31.6%)	2 (10.5%)	0.219
Decreased calcium	1 (5.3%)	0 (0%)	1	0 (0%)	0 (0%)	-
Decreased potassium	1 (5.3%)	0 (0%)	1	0 (0%)	0 (0%)	-
Increased sodium	1 (5.3%)	1 (5.3%)	1	0 (0%)	0 (0%)	-
Increased C-reactive protein	13 (68.4%)	8 (42.1%)	0.125	16 (84.2%)	3 (15.8%)	**0.001**

*Clinicopathological parameters that were within reference range in all dogs at both time points (creatinine, glucose, total bilirubin, inorganic phosphorus) have been omitted

**One dog from each group died before the first re-examination and was excluded from the intention-to-treat analysis of treatment efficacy

Table 4. Comparison of the numerical values of each clinicopathological parameter, between the beginning (time 1) and the end (time 5) of the trial, in dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and in dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B). Intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination

Parameter (unit)	**Group A (n=19)***			**Group B (n=19)***		
	Time 1	**Time 5**	**p value**	**Time 1**	**Time 5**	**p value**
Packed cell volume (%)	30 ± 6.5**	36.8 ± 6.7	**<0.001**	32.9 ± 6.9	41.2 ± 5.2	**<0.001**
White blood cell count (10^3/μl)	9.9 ± 3.9	10.1 ± 3.9	0.846	9 ± 3.4	10.8 ± 3.3	0.437
Neutrophil count (10^3/μl)	7.7 ± 3.4	8.2 ± 4.2	0.58	7.5 ± 2.6	7.4 ± 3	0.876
Band neutrophil count (/μl)	145.2 ± 159.3	189.8 ± 282.1	0.536	176.2 ± 303	158.4 ± 168.6	0.835
Lymphocyte count (/μl)	1,207 ± 869	1,624 ± 695	0.083	1,595 ± 989	2,251 ± 1,108	0.059
Monocyte count (/μl)	400.1 ± 299.7	471.1 ± 269.1	0.456	340.4 ± 187.7	352.7 ± 290.1	0.853
Eosinophil count (/μl)	424.1 ± 459.9	480.1 ± 309.2	0.603	301.3 ± 387.2	468.7 ± 253.2	0.156
Platelet count (10^3/μl)	243.2 ± 112.1	282.6 ± 100.8	0.176	268.2 ± 117.9	320.8 ± 122.4	0.155
Total proteins (g/dl)	8 ± 1.5	7 ± 1.2	**0.006**	7.5 ± 1.2	6.6 ± 0.4	**0.003**
Albumins (g/dl)	2.4 ± 0.4	2.6 ± 0.4	0.083	2.5 ± 0.4	2.9 ± 0.5	**0.001**
Globulins (g/dl)	5.6 ± 1.6	4.4 ± 1.1	**0.001**	5.1 ± 1.2	3.7 ± 0.5	**<0.001**
Albumin/globulin ratio	0.5 ± 0.2	0.6 ± 0.2	**<0.001**	0.5 ± 0.2	0.8 ± 0.2	**<0.001**
Urea nitrogen (mg/dl)	12 ± 5.3	13.4 ± 7.4	0.504	12.1 ± 4.2	15.2 ± 8.8	0.195
Creatinine (mg/dl)	0.7 ± 0.2	0.8 ± 0.2	0.619	0.9 ± 0.3	1.1 ± 0.3	**0.012**
Glucose (mg/dl)	96.4 ± 12.5	92.2 ± 13.9	0.216	100.7 ± 15.5	90.8 ± 25.9	0.125
Cholesterol (mg/dl)	164.7 ± 43.7	185.7 ± 50.7	0.149	182.2 ± 53.7	229.1 ± 76.1	**0.001**
Total bilirubin (mg/dl)	0.1 (0.1-0.8)**	0.1 (0.1-0.3)	**0.02**	0.1 (0.1-0.8)	0.1 (0.1-0.5)	**0.043**

Alkaline phosphatase (U/l)	119.5 ± 84.9	106.1 ± 81	0.505	95.9 ± 60.4	88.3 ± 44.4	0.556
Alanine aminotransferase (U/l)	29 (10-568)	29 (10-133)	0.663	22.4 ± 15.5	27.9 ± 14.3	0.069
Lipase (U/l)	512.2 ± 320.6	1,428 ± 1,373	**0.007**	656 ± 439	1,543 ± 1,125	**0.006**
Amylase (U/l)	554.6 ± 141.8	653.8 ± 169	**0.004**	714.4 ± 253.4	693.6 ± 163.6	0.762
Creatine kinase (U/l)	157.3 ± 127.6	113.1 ± 83.2	0.228	205.7 ± 135.5	85.3 ± 55	**<0.001**
Calcium (mg/dl)	9.7 ± 1	9.8 ± 0.8	0.876	9.9 ± 0.8	10.2 ± 0.7	0.352
Inorganic phosphorus (mg/dl)	4.4 ± 1.3	4.4 ± 0.6	0.814	4.6 ± 0.9	4.1 ± 1	**0.045**
Potassium (mmol/l)	4.7 ± 0.5	4.8 ± 0.3	0.483	5.1 ± 0.5	5 ± 0.4	0.54
Sodium (mmol/l)	155.7 ± 4	155.8 ± 3	0.909	155.3 ± 2.9	156.1 ± 2.2	0.35
C-reactive protein (mg/l)	30.1 ± 25	12.3 ± 16.9	**0.009**	28.6 ± 19.8	4.6 ± 7.1	**<0.001**

*One dog from each group died before the first re-examination and was excluded from the intention-to-treat analysis of treatment efficacy

**Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

Table 5. Comparison of the proportion of dogs with positive microscopy, real-time PCR, IFAT and leishmanin skin test and of the parasitic load and the IFAT titer between the beginning (time 1) and the end (time 5) of the trial, in dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and in dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B). Intention-to-treat analysis, by carrying forward the last known value, was performed and included the dogs with at least one re-examination

Parameter	**Group A (n=19)***			**Group B (n=19)***		
	Time 1	**Time 5**	**p value**	**Time 1**	**Time 5**	**p value**
			Lymph node microscopy			
Positive	19 (100%)	2 (10.5%)	**<0.001**	19 (100%)	3 (15.8%)	**<0.001**
Parasitic load	3 (1-5)**	0 (0-4)	**<0.001**	2 (1-6)	0 (0-2)	**<0.001**
			Bone marrow microscopy			
Positive	17 (89.5%)	3 (15.8%)	**<0.001**	19 (100%)	3 (15.8%)	**<0.001**
Parasitic load	2.8 ± 1.46**	0 ± 1.57	**<0.001**	2.1 ± 1.15	0.2 ± 0.37	**<0.001**
			Bone marrow PCR			
Positive	17 (89.5%)	14 (73.7%)	0.25	19 (100%)	9 (47.4%)	**0.002**
Parasitic load (10^3/ml)	6.6 (0-323)	0.05 (0-63.7)	**0.002**	1.7 (24-63.7)	0 (0-0.3)	**<0.001**
			IFAT			
Positive	19 (100%)	15 (78.9%)	0.125	19 (100%)	8 (42.1%)	**0.001**
Titer	1/800 (1/200-1/12800)	1/400 (1/50-1/6400)	**0.001**	1/800 (1/200-1/6400)	1/100 (0-1/1600)	**<0.001**
			Leishmanin skin test			

Positive	2 (10.5%)	9 (47.4)	**0.016**	6 (31.6%)	14 (73.7%)	**0.021**

*One dog from each group died before the first re-examination and was excluded from the intention-to-treat analysis of treatment efficacy

**Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

Table S1. Scale used to score the extent and severity of 17 clinical signs of canine leishmaniosis

Clinical sign	**Scoring scale**
Body condition	1: obese
	2: overweight
	3: ideal
	4: underweight
	5: thin
Lymph node enlargement	0: normal size
	1: mild enlargement
	2: moderate enlargement
	3: severe enlargement
	Each prescapular and popliteal lymph node was scored separately and individual scores where added (maximum score: 12)
Hepatomegaly	0: absent
	1: mild
	2: moderate
	3: severe
Splenomegaly	0: absent
	1: mild
	2: moderate
	3: severe
Blepharitis	0: absent
	1: mild

	2: moderate
	3: severe
	Each eye was scored separately and individual scores where added (maximum score: 6)
Conjunctivitis	0: absent
	1: mild
	2: moderate
	3: severe
	Each eye was scored separately and individual scores where added (maximum score: 6)
Keratitis	0: absent
	1: mild
	2: moderate
	3: severe
	Each eye was scored separately and individual scores where added (maximum score: 6)
Uveitis	0: absent
	1: mild
	2: moderate
	3: severe
	Each eye was scored separately and individual scores where added (maximum score: 6)
Masticatory muscle atrophy	0: absent
	1: mild

	2: moderate 3: severe Right-side and left-side masticatory muscles were scored separately and individual scores where added (maximum score: 6)
Appendicular muscle atrophy	Extent was scored as: 0: absent 1: single muscle or muscles of a single extremity 2: muscles of one side of the body or of the posterior or of the anterior part of the body 3: generalized Severity was scored as: 0: absent 1: mild 2: moderate 3: severe Extent and severity scores where multiplied (maximum score: 9)
Arthritis/Polyarthritis	Joint inflammation was always confirmed by synovial fluid analysis following arthrocentesis Extent was scored as: 0: absent 1: single joint or joints of a single extremity 2: joints of one side of the body or of the posterior or

	of the anterior part of the body
	3: generalized
	Severity was scored as:
	0: absent
	1: mild
	2: moderate
	3: severe
	Extent and severity scores where multiplied
	(maximum score: 9)
Epistaxis	0: absent
	1: unilateral
	2: bilateral
Exfoliative dermatitis	Extent was scored as:
	0: absent
	1: <10% of total body surface
	2: 10-50% total body surface
	3: >50% total body surface
	Severity was scored as:
	0: absent
	1: predominance of psoriasiform (small) scales
	2: both psoriasiform and pytiriasiform (large) scales
	3: predominance of pytiriasiform scales severe
	Extent and severity scores where multiplied
	(maximum score: 9)

Pustular dermatitis	0: absent
	1: <10% of total body surface
	2: 10-50% total body surface
	3: >50% total body surface
Skin ulcers	0: absent
	1: one ulcer
	2: 2-3 ulcers
	3: > 3 ulcers
Nasal hyperkeratosis	0: absent
	1: mild
	2: moderate
	3: severe
Footpad hyperkeratosis	0: absent
	1: mild
	2: moderate
	3: severe
	Each leg was scored separately and individual scores where added (maximum score: 12)
Onychogryphosis	0: absent
	1: mild
	2: moderate
	3: severe
	Each nail was scored separately and individual scores where added (maximum score: 48)

Table S2. Comparison of selected baseline characteristics (signalment, number of clinical signs and clinicopathological abnormalities per dog, parasitic load based on lymph node and bone marrow microscopy, parasitic load based on bone marrow real-time PCR, IFAT titer and proportion of dogs with positive leishmanin skin test) between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

		Group A	**Group B**	**p value**
Sex	Male	12/20 (60%)	13/20 (65%)	0.744
	Female	8/20 (40%)	7/20 (35%)	
Age (years)		3.7 (1.5-12)*	5 (1-12)	0.098
Body weight (Kg)		17.7 ± 8.9*	23.4 ± 9.6	0.058
Number of clinical signs		8.5 ± 3.85	7.05 ± 3.36	0.212
Number of clinicopathologic abnormalities		5.9 ± 3.13	4.65 ± 2.3	0.158
Positive lymph node microscopy		20/20 (100%)	20/20 (100%)	1
Lymph node microscopy parasitic load		3 (1-5)	2 (1-6)	0.219
Bone marrow microscopy parasitic load		2.9 ± 1.45	2.1 ± 1.15	**0.046**
Bone marrow real-time PCR parasitic load (/ml)		2,957 (0-323,010)	1,658 (24-63,701)	0.271
IFAT titer		1/800 (1/200-1/12800)	1/600 (1/200-1/6400)	0.249
Positive leishmanin skin test		2/20 (10%)	6/20 (30%)	0.235

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Table S3. Comparison of the prevalence of each clinical sign at the beginning of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinical sign	**Group A**	**Group B**	**p value**
Poor body condition	11/20 (55%)	10/20 (50%)	0.752
Lymph node enlargement	18/20 (90%)	20/20 (100%)	0.487
Hepatomegaly	6/20 (30%)	3/20 (15%)	0.451
Splenomegaly	10/20 (50%)	12/20 (60%)	0.525
Blepharitis	10/20 (50%)	7/20 (35%)	0.337
Conjunctivitis	7/20 (35%)	10/20 (50%)	0.337
Keratitis	4/20 (20%)	3/20 (15%)	1
Uveitis	1/20 (5%)	0/20 (0%)	1
Masticatory muscle atrophy	19/20 (95%)	14/20 (70%)	0.091
Appendicular muscle atrophy	12/20 (60%)	9/20 (45%)	0.342
Arthritis/Polyarthritis	8/20 (40%)	10/20 (50%)	0.525
Exfoliative dermatitis	9/20 (45%)	9/20 (45%)	1
Skin ulcers	3/20 (15%)	3/20 (15%)	1
Nasal hyperkeratosis	10/20 (50%)	7/20 (35%)	0.337
Footpad hyperkeratosis	5/20 (25%)	3/20 (15%)	0.695
Onychogryphosis	12/20 (60%)	3/20 (15%)	**0.003**

Table S4. Comparison of the extent and severity score of each clinical sign at the beginning of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinical sign	**Group A**	**Group B**	**p value**
Body condition	3.5 ± 1.4*	3.4 ± 1.2	0.811
Lymph node enlargement	7 ± 3.84	7.3 ± 2.81	0.78
Hepatomegaly	0 (0-3)*	0 (0-1)	0.22
Splenomegaly	0.5 (0-3)	1 (0-3)	0.42
Blepharitis	1 (0-6)	0 (0-6)	0.361
Conjunctivitis	0 (0-6)	1 (0-6)	0.349
Keratitis	0 (0-4)	0 (0-6)	0.791
Uveitis	0 (0-1)	0 (0)	0.317
Masticatory muscle atrophy	3.8 ± 1.94	2.4 ± 1.9	**0.027**
Appendicular muscle atrophy	5 (0-9)	0 (0-9)	0.09
Arthritis/Polyarthritis	0 (0-9)	0.5 (0-9)	0.532
Exfoliative dermatitis	0 (0-9)	0 (0-9)	0.467
Skin ulcers	0 (0-3)	0 (0-3)	1
Nasal hyperkeratosis	0.5 (0-3)	0 (0-2)	0.202
Footpad hyperkeratosis	0 (0-8)	0 (0-4)	0.323
Onychogryphosis	12 (0-48)	0 (0-48)	**0.004**

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Table S5. Comparison of the prevalence of each clinical sign at the end of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinical sign	**Group A**	**Group B**	**p value**
Poor body condition	3/19 (15.8%)	1/19 (5.3%)	0.604
Lymph node enlargement	10/19 (52.6%)	6/19 (31.6%)	0.189
Hepatomegaly	0/19 (0%)	0/19 (0%)	-
Splenomegaly	0/19 (0%)	0/19 (0%)	-
Blepharitis	1/19 (5.3%)	1/19 (5.3%)	1
Conjunctivitis	1/19 (5.3%)	2/19 (10.5%)	1
Keratitis	1/19 (5.3%)	0/19 (0%)	1
Uveitis	0/19 (0%)	0/19 (0%)	-
Masticatory muscle atrophy	11/19 (57.9%)	3/19 (15.8%)	**0.007**
Appendicular muscle atrophy	3/19 (15.8%)	2/19 (10.5%)	1
Arthritis/Polyarthritis	2/19 (10.5%)	1/19 (5.3%)	1
Exfoliative dermatitis	3/19 (15.8%)	1/19 (5.3%)	0.604
Skin ulcers	2/19 (10.5%)	0/19 (0%)	0.486
Nasal hyperkeratosis	2/19 (10.5%)	0/19 (0%)	0.486
Footpad hyperkeratosis	0/19 (0%)	0/19 (0%)	-
Onychogryphosis	6/19 (31.6%)	0/19 (0%)	**0.02**

Table S6. Comparison of the extent and severity score of each clinical sign at the end of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinical sign	**Group A**	**Group B**	**p value**
Poor body condition	2.7 ± 0.87	2.4 ± 0.84	0.284
Lymph node enlargement	2 ± 2.88	0 (0-4)	0.223
Hepatomegaly	0 (0-0)	0 (0-0)	1
Splenomegaly	0 (0-0)	0 (0-0)	1
Blepharitis	0 (0-2)	0 (0-2)	1
Conjunctivitis	0 (0-2)	0 (0-2)	0.795
Keratitis	0 (0-1)	0 (0-0)	0.795
Uveitis	0 (0-0)	-	-
Masticatory muscle atrophy	2 ± 2.21	0 (0-6)	**0.023**
Appendicular muscle atrophy	0 (0-6)	0 (0-9)	0.795
Arthritis/Polyarthritis	0 (0-4)	0 (0-1)	0.773
Exfoliative dermatitis	0 (0-9)	0 (0-1)	0.563
Skin ulcers	0 (0-2)	0 (0-0)	0.583
Nasal hyperkeratosis	0 (0-3)	0 (0-0)	0.583
Footpad hyperkeratosis	0 (0-0)	0 (0-0)	1
Onychogryphosis	0 (0-48)	0 (0-0)	0.096

*Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

Table S7. Comparison of the prevalence of each clinicopathological abnormality at the beginning of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinicopathological abnormality*	**Group A**	**Group B**	**p value**
Decreased packed cell volume	17/20 (85%)	14/20 (70%)	0.451
Decreased white blood cell count	4/20 (20%)	2/20 (10%)	0.661
Decreased neutrophil count	2/20 (10%)	2/20 (10%)	1
Increased neutrophil count	2/20 (10%)	1/20 (5%)	1
Increased band neutrophil count	0/20 (0%)	1/20 (5%)	1
Decreased lymphocyte count	10/20 (50%)	5/20 (25%)	0.102
Increased monocyte count	6/20 (30%)	2/20 (10%)	0.235
Increased eosinophil count	5/20 (25%)	2/20 (10%)	0.407
Decreased platelet count	7/20 (35%)	4/20 (20%)	0.288
Increased total proteins	8/20 (40%)	4/20 (20%)	0.168
Decreased total proteins	0/20 (0%)	1/20 (5%)	1
Decreased albumins	3/20 (15%)	6/20 (30%)	0.451
Increased globulins	13/20 (65%)	9/20 (45%)	0.204
Decreased albumin/globulin ratio	12/20 (60%)	11/20 (55%)	0.749
Increased urea nitrogen	0/20 (0%)	1/20 (5%)	1
Decreased cholesterol	2/20 (10%)	1/20 (5%)	1
Increased alkaline phosphatase	3/20 (15%)	2/20 (10%)	1
Increased alanine aminotransferase	2/20 (10%)	0/20 (0%)	0.487

Increased lipase	0/20 (0%)	1/20 (5%)	1
Increased amylase	0/20 (0%)	1/20 (5%)	1
Increased creatine kinase	5/20 (25%)	6/20 (30%)	0.723
Decreased calcium	1/20 (5%)	1/20 (5%)	1
Decreased potassium	1/20 (5%)	0/20 (0%)	1
Increased sodium	1/20 (5%)	0/20 (0%)	1
Increased C-reactive protein	14/20 (70%)	16/20 (80%)	0.465

*Clinicopathological parameters that were within reference range in all dogs (creatinine, glucose, total bilirubin, inorganic phosphorus) have been omitted

Table S8. Comparison of the numerical values of each hematological and serum biochemistry parameter at the beginning of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Parameter (unit)	Group A	Group B	p value
Packed cell volume (%)	29.8 ± 6.36*	32.2 ± 7.49	0.291
White blood cell count (/μl)	9,719.5 ± 3,964.03	9,816.5 ± 3,406.97	0.934
Neutrophil count (/μl)	7.534 ± 3,355.31	7,414.8 ± 2,621.41	0.901
Band neutrophil count (/μl)	107.5 (0-462)*	83 (0-1,270)	0.667
Lymphocyte count (/μl)	1,155.5 ± 877.18	1,584.8 ± 964.12	0.149
Monocyte count (/μl)	385.7 ± 298.72	347.5 ± 185.45	0.631
Eosinophil count (/μl)	408.5 ± 453.01	293.1 ± 378.6	0.388
Platelet count (/μl)	232,500 ± 119,169.27	268,300 ± 114,715.25	0.339
Total proteins (g/dl)	8 ± 1.53	7.3 ± 1.62	0.162
Albumins (g/dl)	2.5 ± 0.37	2.3 ± 0.64	0.51
Globulins (g/dl)	5.5 ± 1.57	4.9 ± 1.34	0.195
Albumin/globulin ratio	0.5 ± 0.16	0.5 ± 0.19	0.869
Urea nitrogen (mg/dl)	12.4 ± 5.59	13.5 ± 7.63	0.604
Creatinine (mg/dl)	0.7 (0.4-1.3)	0.8 (0.5-1.5)	0.107
Glucose (mg/dl)	97 ± 12.41	99.8 ± 15.65	0.542
Cholesterol (mg/dl)	170.3 ± 49.25	181.5 ± 52.35	0.49
Total bilirubin (mg/dl)	0.15 (0.1-0.8)	0.1 (0.1-0.8)	0.802
Alkaline phosphatase (U/l)	131.1 ± 97.65	92.5 ± 60.76	0.141

Alanine aminotransferase (U/l)	29 (10-568)	18.5 (10-64)	**0.043**
Lipase (U/l)	495.5 ± 320.8	629.9 ± 442.99	0.279
Amylase (U/l)	556.5 ± 138.27	703.9 ± 251.16	**0.027**
Creatine kinase (U/l)	128.5 (42-540)	168.5 (79-602)	0.123
Calcium (mg/dl)	9.8 ± 1.05	9.8 ± 0.94	0.987
Inorganic phosphorus (mg/dl)	4.5 ± 1.32	4.6 ± 0.89	0.738
Potassium (mmol/l)	4.8 ± 0.47	5.1 ± 0.49	**0.026**
Sodium (mmol/l)	155.6 ± 3.89	155.4 ± 2.82	0.853
C-reactive protein (mg/l)	31.7 ± 25.45	27.4 ± 20.01	0.558

*Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

Table S9. Comparison of the prevalence of each clinicopathological abnormality at the end of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Clinicopathological abnormality*	**Group A**	**Group B**	**p value**
Decreased packed cell volume	10/19 (52.6%)	6/19 (31.6%)	0.189
Decreased white blood cell count	1/19 (5.3%)	1/19 (5.3%)	1
Increased white blood cell count	2/19 (10.5%)	0/19 (0%)	0.486
Decreased neutrophil count	1/19 (5.3%)	0/19 (0%)	1
Increased neutrophil count	2/19 (10.5%)	1/19 (5.3%)	1
Increased band neutrophil count	1/19 (5.3%)	0/19 (0%)	1
Decreased lymphocyte count	4/19 (21.1%)	1/19 (5.3%)	0.34
Increased monocyte count	9/19 (46.4%)	4/19 (21.1%)	0.087
Increased eosinophil count	6/19 (31.6%)	4/19 (21.1%)	0.461
Decreased platelet count	3/19 (15.8%)	2/19 (10.5%)	1
Increased total proteins	2/19 (10.5%)	0/19 (0%)	0.486
Decreased albumins	2/19 (10.5%)	2/19 (10.5%)	1
Increased globulins	7/19 (36.8%)	0/19 (0%)	**0.008**
Decreased albumin/globulin ratio	6/19 (31.6%)	1/19 (5.3%)	0.09
Increased urea nitrogen	1/19 (5.3%)	1/19 (5.3%)	1
Increased alkaline phosphatase	3/19 (15.8%)	0/19 (0%)	0.23
Increased alanine aminotransferase	1/19 (5.3%)	0/19 (0%)	1
Increased lipase	4/19 (21.1%)	5/19 (26.3%)	1

Increased creatine kinase	2/19 (10.5%)	2/19 (10.5%)	1
Increased sodium	1/19 (5.3%)	0/19 (0%)	1
Increased C-reactive protein	8/19 (42.1%)	3/19 (15.8%)	0.074

*Clinicopathological parameters that were within reference range in all dogs (creatinine, glucose, cholesterol, total bilirubin, amylase, calcium, inorganic phosphorus, potassium) have been omitted

Table S10. Comparison of the numerical values of each hematological and serum biochemistry parameter at the end of the trial between dogs with leishmaniosis treated with aminosidine and allopurinol (group A) and dogs with leishmaniosis treated with meglumine antimoniate and allopurinol (group B)

Parameter (unit)	**Group A**	**Group B**	**p value**
Packed cell volume (%)	36.8 ± 6.7**	41.2 ± 5.2	**0.029**
White blood cell count (10^3/μl)	10.1 ± 3.9	10.8 ± 3.3	0.565
Neutrophil count (10^3/μl)	8.2 ± 4.2	7.4 ± 3	0.532
Band neutrophil count (/μl)	114 (1-1020)**	116 (0-568)	0.817
Lymphocyte count (/μl)	1,624 ± 695	2,251 ± 1,108	**0.044**
Monocyte count (/μl)	471.1 ± 269.1	352.7 ± 290.1	0.201
Eosinophil count (/μl)	480.1 ± 309.2	468.7 ± 253.2	0.902
Platelet count (10^3/μl)	282.6 ± 100.8	320.8 ± 122.4	0.301
Total proteins (g/dl)	7 ± 1.2	6.6 ± 0.4	0.202
Albumins (g/dl)	2.6 ± 0.4	2.9 ± 0.5	0.089
Globulins (g/dl)	4.4 ± 1.1	3.7 ± 0.5	**0.031**
Albumin/globulin ratio	0.6 ± 0.2	0.8 ± 0.2	**0.028**
Urea nitrogen (mg/dl)	13.4 ± 7.4	15.2 ± 8.8	0.489
Creatinine (mg/dl)	0.8 ± 0.2	1.1 ± 0.3	**0.001**
Glucose (mg/dl)	92.2 ± 13.9	90.8 ± 25.9	0.84
Cholesterol (mg/dl)	185.7 ± 50.7	229.1 ± 76.1	**0.046**
Total bilirubin (mg/dl)	0.1 (0.1-0.3)	0.1 (0.1-0.5)	0.603
Alkaline phosphatase (U/l)	106.1 ± 81	88.3 ± 44.4	0.407

Alanine aminotransferase (U/l)	29 (10-133)	24 (13-62)	0.644
Lipase (U/l)	1,428 ± 1,373	1,543 ± 1,125	0.78
Amylase (U/l)	653.8 ± 169	693.6 ± 163.6	0.466
Creatine kinase (U/l)	90 (43-410)	72 (31-241)	0.109
Calcium (mg/dl)	9.8 ± 0.8	10.2 ± 0.7	0.153
Inorganic phosphorus (mg/dl)	4.4 ± 0.6	4.1 ± 1	0.296
Potassium (mmol/l)	4.8 ± 0.3	5 ± 0.4	0.132
Sodium (mmol/l)	155.8 ± 3	156.1 ± 2.2	0.77
C-reactive protein (mg/l)	5.8 (0-63)	2.2 (0-29.4)	0.191

*Normally distributed continuous data are presented as means and standard deviations, whereas non-normally distributed continuous data are presented as medians and ranges

CONCLUSIONS

1) The total number and the severity of clinical signs of CanL were significantly reduced after treatment with aminosidine-allopurinol combination and no clinical relapses were recorded during the 6-month study period.

2) At the end of the trial, the number of clinical signs was higher and the proportion of dogs without clinical signs was lower in aminosidine-allopurinol compared to meglumine antimoniate-allopurinol treated dogs.

3) There were no differences between groups in the probability and the time for resolution of clinicopathological abnormalities with the exception of hyperglobulinemia that was more likely to resolve in meglumine antimoniate-allopurinol treated dogs. At the end of the study, the prevalence and severity of hyperglobulinemia and the total number of clinicopathological abnormalities were significantly higher in the aminosidine-allopurinol group.

4) The rate of clinical cure, defined as absence of clinical signs plus no anemia, hypoalbuminemia, hyperglobulinemia, or decreased AG ratio, did not differ between groups at the end of the trial.

5) Treatment with aminosidine-allopurinol resulted in a significant reduction of the parasitic load that was especially prominent during the first month and did not differ from the meglumine antimoniate-allopurinol group. At the end of the trial, there was no difference between groups regarding the number of dogs that were parasitologicaly cured based on lymph node and bone marrow cytology and bone marrow RT-PCR.

6) A significant reduction in *Leishmania*-specific antibody levels was documented during the study period for both groups. Even though the time needed for this reduction to occur was not different, at the end of the trial the prevalence of seropositivity and the antibody titers were significantly higher in the aminosidine-allopurinol treated dog.

7) Based on LST, the enhancement of parasite-specific cell-mediated immune responses was significant and similar for both treatment arms.

8) Injection site reactions were the most common side effect with similar prevalence in both groups, but they were mild and resolved without specific treatment.

9) One dog from each treatment arm died due to treatment-related side effects. The cause of death of the meglumine antimoniate-allopurinol treated dog was acute pancreatitis, whereas, the cause of death remained unknown in the aminosidine-allopurinol treated dog.

10) Aminosidine at a dose of 15 mg/kg once daily, subcutaneously for 28 days, along with allopurinol, did not result in significant impairment of kidney function in dogs with CanL without azotemia and with or without proteinuria. The same was true for meglumine antimoniate-allopurinol treatment.

11) The significant reduction of UPC during treatment in both groups may reflect a positive protective effect on renal function.

12) Small but potentially clinically relevant increases of creatinine concentrations (>0.3 mg/dl) were recorded with similar frequency in both treatment arms.

13) None of the dogs treated with aminosidine-allopurinol combination presented increases in hearing thresholds, wave I, wave V and interwave I-V latencies. Subsequently, aminosidine was not accompanied by hearing loss in the study population.

14) No events of peripheral vestibular syndrome were recorded in dogs treated with aminosidine-allopurinol during the trial.

In summary, the results of this study show that aminosidine (15 mg/kg, once daily, subcutaneously for 28 days) and allopurinol combination is safe and effective for the treatment of CanL. However, some secondary efficacy parameters, such as the number of clinical signs, the proportion of dogs without clinical signs, the evolution of hyperglobulinemia, the number of clinicopathological abnormalities, the prevalence of seropositivity and the antibody titers were inferior compared to merglumine antimoniate-allopurinol combination. Subsequently, aminisodine-allopurinol cannot be recommended as a first-line but can be used as a second-lie treatment of CanL.

PROPOSALS FOR FUTURE STUDIES

The results of this study show that aminosidine-allopurinol combination is safe and effective and can be used as a second-line treatment of CanL. Future studies should:

1) Compare the therapeutic efficacy between aminosidine-allopurinol and meglumine antimoniate-allopurinol combinations in a larger number of dogs, followed for a longer time period and living in various countries, in order to take into account the patterns of *L. infantum* drug resistance in different geographical areas.

2) Examine the efficacy of aminosidine-allopurinol combination in dogs with CanL that did not responded to the treatment with meglumine antimoniate-allopurinol.

3) Examine the efficacy of aminosidine-allopurinol combination in dogs with CanL that relapsed after one or more treatment cycles with meglumine antimoniate-allopurinol or with miltefosine-allopurinol.

SUMMARY

A COMPARATIVE STUDY OF THE EFFICACY AND SAFETY BETWEEN AMINOSIDINE (PAROMOMYCIN) AND MEGLUMINE ANTIMONIATE IN CANINE LEISHMANIOSIS DUE TO (*LEISHMANIA INFANTUM*)

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Doctoral thesis 2020

Canine leishmaniosis is a zoonotic disease caused by the protozoan *L. infantum* (syn: *L.chagasi*) with a high prevalence of infection in many countries, including Greece. Current treatment recommendations for dogs without advanced kidney disease include the administration of meglumine antimoniate or miltefosine for 4 weeks along with allopurinol for at least 12 months. These combination treatments are relatively safe and lead to significant clinical and clinicopathological improvement, reduction of parasitic load and of *Lesihmania*-specific antibodies, and are accompanied by low relapse rates.

Protozoan resistance to first-line drugs is a well-known problem in human medicine. In CanL, similar reports of drug resistance, although sparse, appear more and more commonly. Drug pressure (e.g. repeated administration of meglumine antimoniate and long-term allopurinol administration) are documented causes for the development of resistance. Due to the frequency of CanL in endemic areas and the zoonotic potential, investigations on alternative treatment options are urgently needed.

Aminosidine (paromomycin) is an aminoglycoside antibiotic with leihmaniocidal properties that has been studied and used mainly in human leishmanioses. The efficacy and safety of aminosidine as monotherapy of CanL has been previously assessed in a small number of clinical trials. Despite some notable differences among the results of these studies, a good short-term clinical and clinicopathological response has been documented but it is accompanied by dose-related severe side effects and by a high relapse rate after treatment discontinuation. Recently, aminosidine administered at an optimized dose regimen (15 mg/kg, once daily, subcutaneously), resulted in effective plasma concentrations, good short-term clinical and clinicopathological improvement and reduction of parasitic load.

To the best of our knowledge there are no published studies evaluating the efficacy of aminosidine-allopurinol combination for the treatment of CanL. However, similarly to the first-line drugs (meglumine antimoniate and miltefosine) the combination of aminosidine with allopurinol is probably mandatory to improve efficacy and, mainly, to avoid relapses.

The aim of this randomized, blinded, controlled study was to compare the efficacy and safety of aminosidine-allopurinol combination with that of meglumine antimoniate-allopurinol, in terms of improvement of clinical signs and clinicopathological abnormalities, relapse rate, parasitic load reduction, evolution of *Leishmania*-specific humoral and cell mediated immune responses and side effects.

Forty client-owned dogs with at least one clinical sign of CanL, without evidence of stage III or IV chronic kidney disease were randomly assigned to group A [n=20; aminosidine (15 mg/kg, subcutaneously, once daily for 28 days) and allopurinol (10 mg/kg, *per os*, twice daily for 6 months] or group B [n=20; meglumine antimoniate (100 mg/kg subcutaneously, once daily for 28 days) and allopurinol (same dose as in above)]. At enrollment (time 1) the dogs were physically examined and biological material were collected (blood, urine, lymph node and bone marrow aspirates). Re-examinations were scheduled after 15 (time 2), 30 (time 3), 60 (time 4) and 180 days (time 5). The examinations included: a) physical examination and scoring of the extent and severity all CanL-associated clinical signs (times 1-5); b) complete blood count including a manual differential count (times 1-5); c) complete serum biochemistry including C-reactive protein and c-cystatin (times 1-5); d) complete urinalysis including UPC (times 1-5) and urine culture (time 1); e) lymph node and bone marrow microscopy for measurement of parasitic density (times 1 and 3-5); f) IFAT (times 1 and 3-5); g) real-time PCR in bone marrow samples for measurement of parasitic density (times 1 and 3-5); h) LST (times 1 and 3-5); i) BAER testing (times 1-4). All examinations were conducted blindly regarding the group of each dog. During the first month (injectable treatment) all dogs were hospitalized and daily physical examinations were conducted by a non-blinded investigator who was also responsible for randomization and treatment administration.

Primary outcome measures were the proportions of dogs that: a) experienced treatment-related side effects; b) were cured (no clinical signs, anemia, hypoalbuminemia, hyperglobulinemia or low AG ratio); and c) were parasitologically

negative (lymph node and bone marrow microscopy and bone marrow RT-PCR) at the end of the trial. Secondary outcome measures were the proportions of dogs that a) were clinically cured (no clinical signs, irrespectively of the clinicopathological abnormalities); b) had no anemia, hypoalbuminemia, hyperglobulinemia, low AG ratio or increased C-reactive protein concentration; c) were seronegative; and d) were LST positive.

There were no differences between groups for the primary outcome measures (treatment related side effects: p=1; cure: p=0.074; negative parasitological examinations: p=0.485). However, there were differences in some secondary outcome measures favor of group B: prevalence of clinical cure (p=0.044), hyperglobulinemia (p=0.008) and prevalence of seronegative dogs (p=0.02). On the other hand, the prevalence of anemia (p=0.189), hypoalbuminemia (p=1), decreased AG ratio (p=0.09), increased C-reactive protein concentration (p=0.074) and LST positivity (p=0.097).

The aminosidine-allopurinol combination resulted in a significant improvement of clinical signs and clinicopathological abnormalities, a reduction of antibody titers and parasitic load and an increased prevalence of positive LST. The time and the probability to achieve these goals of treatment were not generally different between the groups.

No significant nephrotoxic or ototoxic adverse events were observed in the dogs of the trial. Increases of creatinine concentration >0.3 mg/dl were recorded in both groups at a similar frequency.

Based on these results aminosidine-allopurinol concentration should be considered a safe and effective treatment for CanL. Due to some differences in secondary outcome measures, it cannot be considered as equally effective with the meglumine antimoniate-allopurinol combination and, thus, it is proposed a second-line drug combination for the treatment of CanL.

ΠΕΡΙΛΗΨΗ

ΣΥΓΚΡΙΤΙΚΗ ΜΕΛΕΤΗ ΤΗΣ ΑΜΙΝΟΣΙΔΙΝΗΣ (ΠΑΡΟΜΟΜΥΚΙΝΗΣ) ΚΑΙ ΤΗΣ ΑΝΤΙΜΟΝΙΑΚΗΣ ΜΕΓΛΟΥΜΙΝΗΣ ΓΙΑ ΤΗ ΘΕΡΑΠΕΙΑ ΤΗΣ ΛΕΪΣΜΑΝΙΩΣΗΣ (*LEISHMANIA INFANTUM*) ΤΟΥ ΣΚΥΛΟΥ

Δημήτρης Ν. Κασαμπαλής

Διδακτορική Διατριβή 2020

Οι λεϊσμανιώσεις του ανθρώπου και του σκύλου είναι νοσήματα που οφείλονται στα πρωτόζωα του γένους *Leishmania* και είναι ενδημικές σε πολλές χώρες του κόσμου, μεταξύ των οποίων και η Ελλάδα. Στο σκύλο, η μόλυνση από *L. inafntum* μπορεί να προκαλέσει συστηματικό νόσημα που θα οδηγήσει στο θάνατο αν δεν αντιμετωπιστεί έγκαιρα. Οι πρώτης γραμμής θεραπευτικές επιλογές είναι οι συνδυασμοί της αντιμονιακής μεγλουμίνης ή της μιλτεφοσίνης για 4 εβδομάδες με την αλλοπουρινόλη για τουλάχιστον 12 μήνες. Σε μεγάλο αριθμό μελετών έχει διαπιστωθεί ότι με τα παραπάνω σχήματα επιτυγχάνεται συνήθως κλινική ίαση, βελτιώνονται ή εξαφανίζονται οι εργαστηριακές διαταραχές, μεταβάλλεται προς την κατεύθυνση της κυτταρικής ανοσίας η αντίδραση του σκύλου στο πρωτόζωο και μειώνεται το παρασιτικό φορτίο, ενώ οι παρενέργειες δεν είναι ιδιαίτερα συχνές. Επιπλέον, η μακροχρόνια (συχνά εφ' όρου ζωής) χορήγηση της αλλοπουρινόλης μειώνει σημαντικά τη συχνότητα των υποτροπών.

Ωστόσο, τις τελευταίες δεκαετίες, διαπιστώνεται, σε πολλές περιοχές του κόσμου, μια δραματική αύξηση της ανθεκτικότητας των *Leishmania* spp. του ανθρώπου στα φάρμακα πρώτης επιλογής. Παράλληλα, τα τελευταία χρόνια πληθαίνουν οι αναφορές απομόνωσης ανθεκτικών στελεχών της *L. inafntum* από σκύλους με λεϊσμανίωση. Το πρόβλημα αφορά κυρίως την αντιμονιακή μεγλουμίνη και την αλλοπουρινόλη. Μάλιστα, οι επαναλαμβανόμενοι θεραπευτικοί «κύκλοι» με την πρώτη και η μακροχρόνια χορήγηση της δεύτερης αποτελούν πλέον γνωστά αίτια εμφάνισης ανθεκτικών στελεχών. Δεδομένου ότι η λεϊσμανίωση του σκύλου είναι πολύ συχνή στην κλινική πράξη και ότι ο σκύλος αποτελεί δεξαμενή του παρασίτου που μπορεί να μολύνει τον άνθρωπο, γίνεται επιτακτική η έρευνα για εναλλακτικές

θεραπευτικές επιλογές, κατά προτίμηση με δραστικές ουσίες που δε χρησιμοποιούνται για την αντίστοιχη νόσο του ανθρώπου.

Η αμινοσιδίνη (παρομομυκίνη) είναι αμινογλυκοσιδικό αντιβιοτικό δραστικό έναντι της *L.infantum* που έχει χρησιμοποιηθεί κυρίως για τη θεραπεία των λεϊσμανιώσεων του ανθρώπου. Η αποτελεσματικότητα και η ασφάλειά της στη λεϊσμανίωση του σκύλου έχει διερευνηθεί σε περιορισμένο αριθμό μελετών, οι οποίες, παρά τις μεγάλες διαφορές που έχουν μεταξύ τους σε ότι αφορά το σχεδιασμό, καταλήγουν ότι βελτιώνει την κλινική εικόνα και τις εργαστηριακές διαταραχές αλλά παράλληλα προκαλεί σοβαρές παρενέργειες ενώ οι υποτροπές μετά τη διακοπή της είναι πολύ συχνές. Πρόσφατα η χορήγηση της αμινοσιδίνης στη δόση των 15 mg/kg σωματικού βάρους, υποδόρια, κάθε 24 ώρες είχε ως αποτέλεσμα την επίτευξη θεραπευτικών συγκεντρώσεων στο πλάσμα του αίματος, τη σημαντική υποχώρηση των συμπτωμάτων και των εργαστηριακών διαταραχών και τη μείωση του παρασιτικού φορτίου.

Μέχρι σήμερα δεν υπάρχουν δημοσιευμένες μελέτες που να αξιολογούν την αποτελεσματικότητα και την ασφάλεια της ταυτόχρονης χορήγησης αμινοσιδίνης και αλλοπουρινόλης σε σκύλους με λεϊσμανίωση. Ωστόσο και οι δύο δραστικές ουσίες πρώτης επιλογής για τη θεραπεία της λεϊσμανίωσης του σκύλου (αντιμονιακή μεγλουμίνη, μιλτεφοσίνη) χορηγούνται απαραίτητα μαζί με την αλλοπουρινόλη, ώστε να βελτιωθεί η αποτελεσματικότητα και κυρίως να μειωθεί η συχνότητα των υποτροπών.

Στόχος αυτής της τυφλής, τυχαιοποιημένης και με θετικό μάρτυρα μελέτης ήταν η σύγκριση του συνδυασμού αμινοσιδίνης-αλλοπουρινόλης με το συνδυασμό αντιμονιακής μεγλουμίνης-αλλοπουρινόλης, σε ότι αφορά την ασφάλεια, την κλινική αποτελεσματικότητα, τη συχνότητα των υποτροπών, τη βελτίωση των εργαστηριακών διαταραχών, τη μείωση του παρασιτικού φορτίου και τη μεταβολή της ειδικής έναντι του παρασίτου χυμικής και κυτταρικής ανοσίας.

Στη μελέτη χρησιμοποιήθηκαν 40 ιδιόκτητοι σκύλοι με ένα ή περισσότερα συμπτώματα λεϊσμανίωσης χωρίς νεφρική νόσο σταδίου III-IV, που τυχαιοποιήθηκαν στην ομάδα Α [n=20, αμινοσιδίνη (15 mg/kg, υποδόρια, κάθε 24 ώρες για 28 ημέρες) και αλλοπουρινόλη (10 mg/kg, από το στόμα, κάθε 12 ώρες για 6 μήνες)] και στην ομάδα Β [n=20, αντιμονιακή μεγλουμίνη (100 mg/kg, υποδόρια, κάθε 24 ώρες για 28 ημέρες) και αλλοπουρινόλη (ίδιο δοσολογικό σχήμα με την ομάδα Α)]. Μετά την

ένταξη τους στη μελέτη (χρόνος 1) εξετάζονταν κλινικά και λαμβάνονταν τα βιολογικά υλικά (αίμα, ούρα, υλικό παρακέντησης λεμφογαγγλίου και μυελού των οστών). Οι επανεξετάσεις ήταν προγραμματισμένες στις 15 (χρόνος 2), τις 30 (χρόνος 3), τις 60 (χρόνος 4) και τις 180 ημέρες (χρόνος 5) από την έναρξη της θεραπείας. Οι εξετάσεις που πραγματοποιήθηκαν ήταν οι: α) κλινική εξέταση με βαθμολόγηση της παρουσίας και της έντασης των συμπτωμάτων (χρόνοι 1-5), β) γενική εξέταση αίματος και λευκοκυτταρικός τύπος (χρόνοι 1-5), γ) πλήρης βιοχημική εξέταση στον ορό του αίματος (χρόνοι 1-5), δ) μέτρηση της C-αντιδρώσας πρωτεΐνης και της c-συστατίνης στον ορό του αίματος (χρόνοι 1-5), ε) γενική ανάλυση ούρων με μέτρηση του UPC (χρόνοι 1-5) και καλλιέργεια για αερόβιους μικροοργανισμούς (χρόνος 1), στ) μικροσκοπική εξέταση και καταμέτρηση του παρασιτικού φορτίου σε επιχρίσματα λεμφογαγγλίου και μυελού των οστών (χρόνοι 1 και 3-5), ζ) μέτρηση του τίτλου των ειδικών κατά της *Leishmania* αντισωμάτων με τη μέθοδο IFAT (χρόνοι 1 και 3-5) η) RT-PCR σε δείγμα μυελού των οστών για τη μέτρηση του παρασιτικού φορτίου (χρόνοι 1 και 3-5) θ) LST για τον έλεγχο της ειδικής έναντι του παρασίτου κυτταρικής ανοσίας (χρόνοι 1 και 3-5) και ι) BAER για τον έλεγχο της ακοής (χρόνοι 1-4). Όλες οι παραπάνω εξετάσεις πραγματοποιήθηκαν χωρίς να είναι γνωστή η ομάδα του κάθε σκύλου. Στη διάρκεια του πρώτων 4 εβδομάδων (ενέσιμη θεραπεία) όλα τα ζώα νοσηλεύτηκαν στις εγκαταστάσεις της Παθολογικής Κλινικής του Τμήματος Κτηνιατρικής του Π.Θ. και ελέγχονταν κλινικά σε καθημερινή καθημερινά από ερευνητή που γνώριζε την ομάδα του κάθε σκύλου και ήταν υπεύθυνος για την τυχαιοποίηση και τη χορήγηση της θεραπείας.

Τα πρωτογενή μέτρα έκβασης της μελέτης περιλαμβάνονταν το ποσοστό των σκύλων που: α) εμφάνισε παρενέργειες από τα φάρμακα β) ιάθηκε (απουσία συμπτωμάτων, αναιμίας, υπολευκωματιναιμίας, υπερσφαιριναιμίας και μειωμένου λόγου AG στο τέλος της μελέτης, και γ) ήταν παρασιτολογικά αρνητικοί στη μικροσκοπική εξέταση των λεμφογαγγλίων και του μυελού των οστών και στη RT-PCR στο τέλος της μελέτης. Στα δευτερογενή μέτρα έκβασης περιλαμβάνονται το ποσοστό των σκύλων που: α) εμφάνισε κλινική ίαση (απουσία συμπτωμάτων), β) δεν είχε αναιμία, υπολευκωματιναιμία, υπερσφαιριναιμία, μειωμένο λόγο AG και αυξημένη C-αντιδρώσα πρωτεΐνη, γ) ήταν ορολογικά αρνητικό και δ) εμφάνισε θετική LST στο τέλος της μελέτης.

Δεν παρατηρήθηκαν στατιστικά σημαντικές διαφορές μεταξύ των δύο ομάδων για τα πρωτογενή μέτρα έκβασης, δηλαδή το ποσοστό των σκύλων που εμφάνισε παρενέργειες ή πέθανε (p=1), που ιάθηκε (p=0,074) ή που ήταν παρασιτολογικά αρνητικό (p=0,485). Ωστόσο υπήρχαν μερικές διαφορές υπέρ της ομάδας Β σε ορισμένα δευτερογενή μέτρα έκβασης και συγκεκριμένα τη συχνότητα της κλινικής ίασης (p=0,044), της υπερσφαιριναιμίας (p=0,008) και του αρνητικού αποτελέσματος της ορολογικής εξέτασης (p=0,02). Αντίθετα δεν υπήρχαν διαφορές μεταξύ των ομάδων αναφορικά με τη συχνότητα της αναιμίας (p=0,189), της υπολευκωματιναιμίας (p=1), του μειωμένου λόγου AG (p=0,09), της αυξημένης συγκέντρωσης C-αντιδρώσας πρωτεΐνης (p=0,074) και της θετικής LST (p=0,097).

Η χορήγηση του συνδυασμού αμινοσιδίνης και αλλοπουρινόλης είχε ως αποτέλεσμα της σημαντική μείωση της συχνότητας και της έντασης των περισσοτέρων συμπτωμάτων και εργαστηριακών διαταραχών, τη μείωση του παρασιτικού φορτίου και του τίτλου των αντισωμάτων και την αύξηση του αριθμού των σκύλων με θετική δοκιμή LST. Ο χρόνος για να επιτευχθούν οι παραπάνω στόχοι της θεραπείας και ο ρυθμός βελτίωσης αυτών των παραμέτρων κατά κανόνα δεν διέφεραν μεταξύ των δύο ομάδων.

Σε κανένα σκύλο της μελέτης δεν παρατηρήθηκε σημαντικές παρενέργειες σε ότι αφορά τη νεφρική λειτουργία ή συμπτώματα ωτοτοξικότητας (μείωση ακουστικής ικανότητας, περιφερικό αιθουσαίο σύνδρομο). Μικρές αυξήσεις της συγκέντρωσης της κρεατινίνης (> 0,3 mg/dl) παρατηρήθηκαν και στις δύο ομάδες χωρίς σημαντικές διαφορές μεταξύ τους.

Με βάση αυτά τα αποτελέσματα, ο παραπάνω συνδυασμός αμινοσιδίνης-αλλοπουρινόλης είναι ασφαλής και αποτελεσματικός για τη θεραπεία της λεϊσμανίωσης του σκύλου. Οι διαφορές σε μερικά από τα δευτερογενή μέτρα έκβασης δεν επιτρέπουν να χαρακτηριστεί ισοδύναμος με το συνδυασμό αντιμονιακής μεγλουμίνης-αλλοπουρινόλης και κατά συνέπεια προτείνεται ως αγωγή δεύτερης επιλογής για τη θεραπεία της λεϊσμανίωσης του σκύλου

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