

AI-Driven miRNA Target Identification Combined with Nanocarrier-Based Therapy Enhances Amastigote Clearance in Canine Visceral Leishmaniasis: An Integrated In Silico and Ex Vivo Study

Athanasios Spyridonis

atspyridonis@uth.gr

University of Thessaly

Menelaos Lefkaditis

University of Thessaly

Ioannis Pappas

University of Thessaly

Theodoros Lialiaris

Panagiotis Perakis

Research Article

Keywords: Canine visceral leishmaniasis (CVL), PLGA nanoparticles, miRNA immunomodulation, miltefosine, artificial intelligence (AI), macrophage polarization

Posted Date: October 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11033613/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

**AI-Driven miRNA Target Identification Combined with Nanocarrier-Based Therapy Enhances Amastigote Clearance
in Canine Visceral Leishmaniasis: An Integrated In Silico and Ex Vivo Study**

Department of Veterinary Medicine, School of Health Sciences, University of Thessaly

Name of Author: ATHANASIOS SPYRIDONIS

Institution's Email address: atspyridonis@uth.gr

Abstract

Study rationale and knowledge gap: Canine visceral leishmaniasis is a major zoonotic disease in which intracellular *Leishmania infantum* persists within macrophages, while treatment limitations and emerging resistance highlight the need for improved therapeutic strategies. MicroRNA-mediated regulation of host immune responses and nanoparticle-based drug delivery offer complementary approaches, but their integration with artificial intelligence (AI)-assisted target identification has not been adequately investigated in canine visceral leishmaniasis. This study addressed this knowledge gap by integrating computational miRNA–gene prioritization with nanoparticle-mediated drug delivery and ex vivo validation in primary canine macrophages.

Methods: Publicly available transcriptomic datasets and experimentally supported miRNA–gene interactions were analysed using machine-learning approaches to prioritize candidate regulatory miRNAs. The selected candidates were evaluated in primary macrophages obtained from 16 dogs using miltefosine-loaded PLGA nanoparticles, miRNA modulation and combination treatment. Parasite burden, cell viability, cytokine responses and expression of selected immune-related genes were assessed during the experimental period.

Results: The computational framework identified miR-155 and miR-146a among the principal candidates for experimental investigation. The selected machine-learning model achieved an area under the receiver operating characteristic curve (AUC) of 0.89 during cross-validation and 0.84 during transfer to the canine dataset. Experimentally, the combination of PLGA-miltefosine nanoparticles with miR-155 modulation produced the greatest reduction in intracellular parasite burden, reaching a 78% reduction in infection index at 48 h compared with infected untreated controls. The combination was also associated with increased TNF- α , IL-12 and iNOS expression and reduced IL-10, together with a favourable cytotoxicity profile under the conditions tested.

Key findings: These findings provide ex vivo proof-of-concept evidence that AI-assisted miRNA prioritization can be integrated with nanoparticle-based miltefosine delivery and host-directed miRNA modulation to enhance intracellular *L. infantum* suppression in primary canine macrophages. The computationally predicted miRNA–gene interactions remain hypothesis-generating and require direct functional validation. Further studies incorporating target-level validation, nanoparticle uptake, broader immune profiling and in vivo evaluation are needed to determine the therapeutic relevance of this combined approach.

Keywords: Canine visceral leishmaniasis (CVL); PLGA nanoparticles; miRNA immunomodulation; miltefosine; artificial intelligence (AI); macrophage polarization.

1. INTRODUCTION

1.1. Canine Visceral Leishmaniasis: Background and Significance

Canine visceral leishmaniasis (CVL) is a common disease which is caused by *Leishmania infantum* and majorly transmitted by phlebotomine sandflies. The primary domestic reservoir is the dog. According to Goonoo et al., (2022), this makes the infection pose not only a serious threat to the veterinary sphere, but also to the human population, since the zoonotic transmission of the infection to humans has been well-documented. The disease is widely spread geographically across Southern Europe, the Mediterranean basin, parts of Latin America, and parts of Africa and Asia (Bragato et al., 2018). The infection rate in the dogs can go up to more than 40% in endemic regions, and clinical presentation can be diverse, both in terms of being asymptomatic carriers or having severe systemic disease (Palomino-Cano et al., 2024). Most of the clinical manifestations include weight loss, lymphadenopathy, skin lesions, splenomegaly, ocular anomalies and progressive renal damage, which is the primary cause of death in animal patients. Early diagnosis, chemotherapy, and control of vectors are very crucial in control strategies. Nevertheless, the results of treatment are not optimal even after decades of research. Most dogs report relapse despite years of therapeutic treatment and they can still remain reservoirs. Moreover, the cost of treatment is too expensive in the long-term to owners. The increased awareness of the One Health structure has underscored the need to come up with better therapies that can help decrease the parasite burden and curb the spread.

1.2. Current Pharmacological Treatments and Their Limitations

Amphotericin B and miltefosine are the most common antileishmanial drugs in dogs. Miltefosine is an oral compound in the form of alkylphosphocholine that interferes with the integrity of the membranes of parasites. AmBisome®, which is commonly developed as liposomal AmBisome, binds to ergosterol-like sterols in the *Leishmania* membrane, resulting in pore formation and cell death (Rashidi et al., 2022). The two drugs exhibit inconsistent cure rates and none of them eliminates the intracellular parasites which are located in macrophages. Increasing cases of resistance to miltefosine have been reported in the endemic areas due to decreased drug absorption and escalated drug detoxification mechanisms (Rashidi et al., 2022; Mbui et al., 2019). Although amphotericin B is very effective, it should be administered intravenously; it is costly and has a chance of nephrotoxicity. These deficiencies underscore the main issue of CVL treatment: to ascertain that sufficient levels of active drugs arrive at the intracellular space where amastigotes survive.

1.3. Macrophages and the Immune Response to *Leishmania infantum*

Leishmania parasites mostly infect macrophages. Once they get into such cells, they transform their moving form (promastigotes) into one that lives and grows within the macrophage (amastigotes). In this stage, the response from the dog's immune system is highly crucial towards the development of the disease. Strong protective immune response (Th1-type) with cytokines such as IFN- γ and TNF- α , assists the macrophage to kill the parasites with the aid of nitric oxide (Dos Santos et al., 2022). On the contrary, anti-inflammatory reactions of the IL-10 and TGF- β are stronger, which leads to the survival of the parasites and makes the infection long-term. The parasites are invisible to the naked eye as they live within the macrophages, thus making treatment difficult to reach the parasites (Dos Santos et al., 2022). To this end, new therapies are being designed to enhance drug delivery within macrophages and enhance the immune response, which assists in eliminating the parasites.

1.4. Nanomedicine Approaches to Antileishmanial Drug Delivery

Nanotechnology has the potential to solve the problem of limited drug access to intracellular areas. Antileishmanial drugs may be encapsulated in nanoparticles composed of lipids, biodegradable polymers like poly (lactic-co-glycolic acid) (PLGA), or hybrid materials and delivered more effectively to infected macrophages. The small size enables preferential uptake by phagocytic cells with controlled release properties, increasing their therapeutic action and decreasing toxicity (De Freitas et al., 2023).

PLGA nanoparticles that are loaded with miltefosine or amphotericin formulations have been demonstrated to result in a higher level of intracellular accumulation, reduced parasite burden, and enhanced tolerance in vitro and in rodent models (Soares et al., 2023). Lipid nanoparticle (LNPs) is also associated with good biocompatibility and macrophage membrane affinity. Despite the overall success of nanomedicine in the research of human leishmaniasis, little has been researched specifically in regard to dog immune cells. This gap highlights the necessity of special veterinary-based translational research.

1.5. MicroRNAs as Immune Modulators in Leishmaniasis

MicroRNAs (miRNAs) are non-coding short RNA molecules controlling the expression of the genes in a post-transcriptional manner. They have important functions in macrophage polarization, inflammation and antiparasitic defense (Goonoo et al., 2022). Certain miRNAs are dysregulated in response to the *Leishmania* infection to promote parasite survival. The two miRNAs that are of specific interest are:

1. miR-155 - stimulates the activation of macrophages and increases Th1-type reaction. The miR-155 upregulation has been associated with the enhancement of intracellular killing mechanisms.
2. miR-146a - inhibits inflammation and decreases the response of immune signaling pathways.

It has been demonstrated that miR-146a is increased in chronic leishmaniasis, which helps the parasite to survive. The altered expression of miRNAs may influence the balance between inflammatory and regulatory macrophage responses. On this basis, miR-155 mimic and miR-146a inhibition were investigated as candidate host-directed interventions, while their predicted molecular targets were interpreted cautiously because regulatory effects are context- and species-dependent. miR-155 and miR-146a were selected because they showed strong computational confidence, differential-expression support, pathway relevance, and literature evidence. miR-155 represents inflammatory immune activation, whereas miR-146a regulates negative feedback in NF- κ B/TLR signalling. Their complementary functions and strong network connectivity made them suitable candidates for testing host-directed immune modulation in canine visceral leishmaniasis.

1.6. Rationale for Combination Nanotherapeutic and miRNA Strategies

Given the drawbacks of traditional chemotherapy, the combination of nanomedicine and immunomodulation is an advanced therapeutic avenue. Nanoparticles could provide better delivery of miltefosine to macrophages, and miRNA manipulation can increase intracellular killing (Palomino-Cano et al., 2024). The combination of the two strategies can result in:

- Increased reduction of parasite burden is greater.
- Reduced chances of drug toxicity.

- Greater stability in response to treatment.
- Reduced chances of relapse and resistance.

Notably, successful canine treatment can directly benefit human health since dogs are the main reservoir of zoonotic infections.

1.6.1. Scientific Innovation of This Study

The present study integrates AI-assisted miRNA–gene prioritization, cross-species evidence, nanoparticle-based miltefosine delivery and primary canine macrophage experiments within a single proof-of-concept workflow. The principal methodological contribution is the use of a multi-layer prioritization strategy to select candidate miRNAs for experimental testing in a canine ex vivo model. The study should not be interpreted as establishing clinical efficacy or therapeutic superiority, because in vivo efficacy, pharmacokinetics, long-term safety and direct target validation were not assessed.

1.7. Aim and Hypotheses of the Study

The main objective of this research is to design and test a computational algorithm based on the application of artificial intelligence (AI), with the aim of identifying and prioritizing the interactions between microRNAs (miRNAs) and their gene targets in canine visceral leishmaniasis (CVL). The aim of the proposed study is to examine whether nanoparticle-encapsulated miRNA-immune-modulating miltefosine contributes more to antileishmanial effects in canine macrophages.

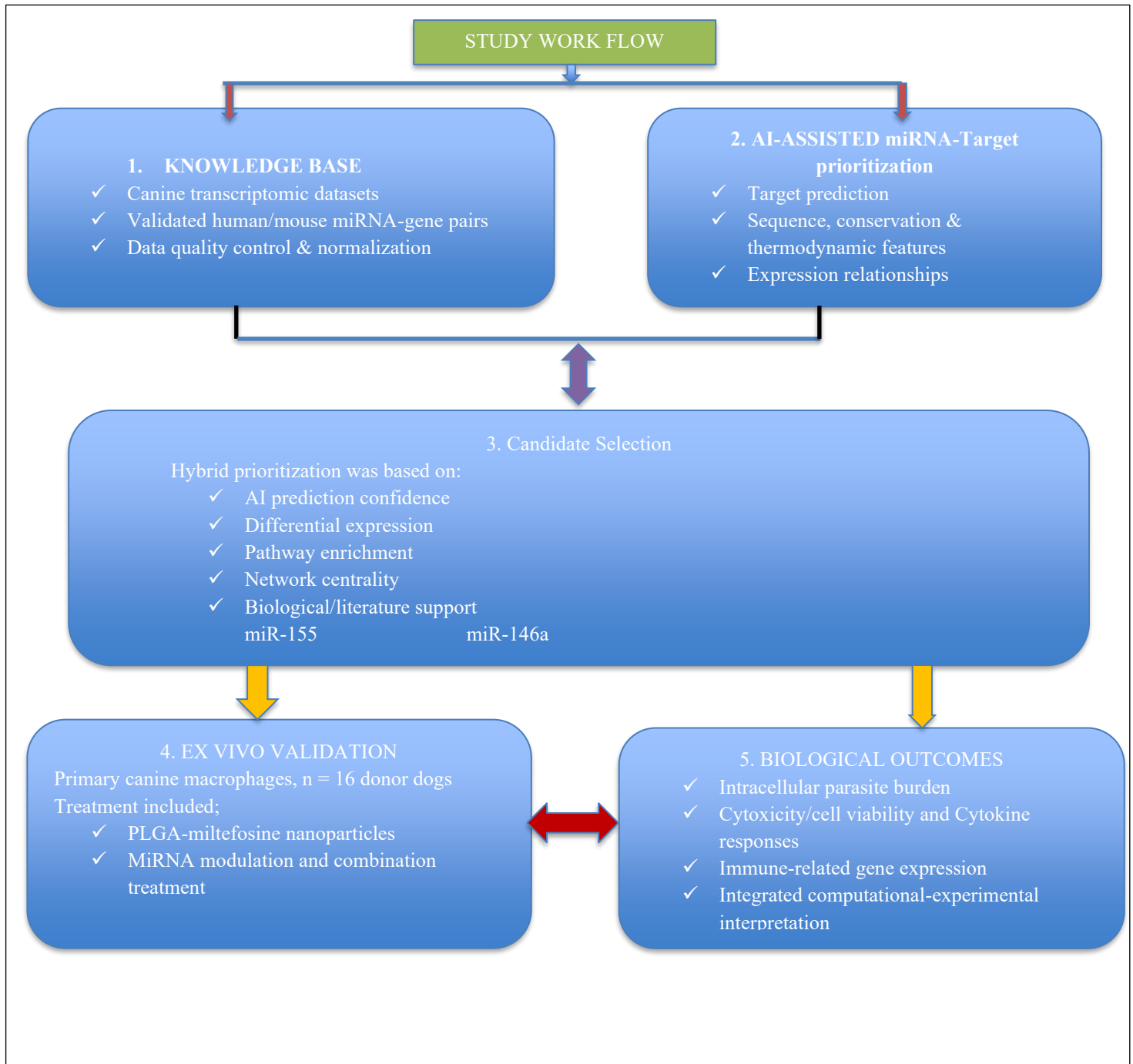
Hypotheses:

H1: There is a specific group of miRNAs (such as miR-21, miR-146a, miR-155), whose expression differs significantly between infected and healthy dogs and can be used to predict potential therapeutic gene targets with high accuracy (Area Under Curve, AUC > 0.8).

H2: AI transfer learning models, trained on experimentally verified miRNA–gene interactions in humans and mice, are capable of identifying and classifying genuine miRNA–gene regulatory interactions in dog data.

H3: The use of probability scores from artificial intelligence, differential expression, and pathway analysis will be useful for prioritizing miRNA–gene pairs that are biologically significant and therapeutically useful in canine visceral leishmaniasis (CVL).

Figure 1. A schematic overview of the study workflow



2. MATERIALS AND METHODS

PHASE I: In Silico Computational Methods

2.1 Data sources

The initial phase of the computational analysis involved the identification and collection of relevant datasets that can be used to successfully represent the molecular features of CVL. The NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) databases were selected as the main data sources, which are the main data repositories. These repositories contain the best available collections of next-generation sequencing data, both RNA-Seq, which provides transcriptome-level information on gene expression, and miRNA-seq, which provides microRNA expression data. The use of both types of data was determined by the need to measure the two main levels of gene regulation involved in the disease, including both the transcriptional level (mRNA expression) and the post-transcriptional level (regulation by miRNA). Dog samples were prioritized in order to obtain samples from dogs naturally or experimentally infected with *Leishmania infantum*. *Leishmania infantum* is the main causative agent responsible for visceral leishmaniasis in the Mediterranean region. Using these datasets, it is possible to directly study host–pathogen interactions and molecular mechanisms of disease progression. For a comparative approach, other datasets from healthy dogs were also collected. These control data serve as a reference point, enabling differential expression analysis to identify miRNAs and mRNAs that show significant changes due to infection.

Cross-species datasets from humans and mice infected with *Leishmania donovani* or *Leishmania infantum* were used to supplement the smaller canine evidence base and support transfer learning. These data were selected because experimentally supported miRNA–gene interactions are more extensively annotated in human and mouse systems. However, cross-species transfer can introduce errors arising from differences in miRNA sequence, target-site conservation, gene regulation, tissue context and disease biology. Therefore, human and mouse evidence was used to prioritize canine hypotheses rather than to establish canine-specific regulatory interactions. Human and mouse datasets were used because experimentally validated miRNA–gene interactions are substantially more abundant in these species than in dogs, providing a stronger training foundation for transfer learning. Their use was intended to identify conserved regulatory patterns and generate candidate canine interactions, not to assume direct equivalence. However, species-specific differences in miRNA sequences, target-site conservation, 3'UTRs, gene regulation, tissue context, and disease biology may reduce prediction accuracy and introduce cross-species bias.

MiRBase, miRTarBase, and TarBase also store experimentally verified miRNA–gene interactions, including information on binding sites, regulatory mechanisms, and experimental conditions. These databases were used to generate a training dataset that was used in the supervised AI modeling, as well as to cross-validate the predicted miRNA–gene pairs identified during the computational analysis. Taken together, the three types of data, namely dog data, comparative data, and literature sources, form a multidimensional basis for the experiment. This ensures that the AI model and the resulting analyses are based on sound and diverse data.

2.2 Inclusion criteria

In order to avoid compromising the validity and reliability of the computational experiment, all datasets are subject to strict inclusion criteria before analysis. This is done to ensure that only relevant, complete and quality data are incorporated into the final computational framework. The most critical and basic condition is that all dog samples must come from individuals with a known diagnosis of visceral leishmaniasis. The diagnosis must also be guided by molecular, parasitological or serological analyses, including PCR amplification of *Leishmania*-specific DNA fragments or antibodies to parasite antigens. Only articles confirmed by a clear diagnosis will be considered. This will ensure that the observed molecular change is indeed related to the infection and not to other related health problems.

The second inclusion criterion is that control samples must be from clinically healthy dogs with no history of infectious or immunosuppressive diseases. These control animals are necessary to perform differential expression analysis. It is also possible to make relevant comparisons between infected and uninfected conditions. Also, the selected studies must contain sufficient sample size, including at least three biological replicates per condition, in order to have statistical power and reliability. The third criterion is that complete and high-quality sequencing data must be available. Only datasets that have raw sequencing reads (FASTQ format) and metadata (sample origin, tissue type, sequencing platform, and experimental design) are considered. With this information, it is possible to perform a reproducible analysis and justify that the difference in datasets can be addressed during normalization. In the sequencing data, there should also be no incomplete metadata datasets (or datasets with low-quality sequencing), as this will add confounding variables to the AI model and subsequent analyses.

Finally, all data are publicly available and were collected in an ethical manner, in accordance with the requirements of the repositories. This guarantees transparency, reproducibility and respect for the principles of open science. Repositories such as GEO and SRA impose ethical standards on data submission, requiring institutions to respect ethical standards in animal studies and at the national level. This experiment is in line with the FAIR (Findable, Accessible, Interoperable, Reusable) principles for data. These principles are reflected in the use of open access datasets, while future researchers will be able to reproduce or exploit the results. All of these inclusion criteria create a strong and clear basis for generating a computational analysis. They ensure that all datasets used are relevant to CVL, are of high quality, and have sufficient documentation to ensure reproducibility. This data design scheme creates a strong basis for identifying and classifying miRNA-gene interactions in canine visceral leishmaniasis through rigorous selection, quality control, and ethical standards.

2.3 Data processing

After identifying and collecting the right datasets is the data processing step where raw sequences are turned into understandable and comparative information. Data processing will make sure that the results are devoid of technical artifacts, and that they are statistically reliable, and prepared to be used in downstream computation modeling. This step is performed in a series of stages, namely, quality control, trimming and cleaning, normalization, differential expression analysis, and multi-species data integration to train an AI model and conduct transfer learning.

Quality control is the initial processing stage. Any raw RNA-seq and miRNA-seq data retrieved off the repositories are analyzed with the FastQC tool that produces in-depth reports on the quality of each sequencing file. Parameters included in FastQC checks are length distribution of sequences, GC content, contamination by adapters and quality of bases distributed across all reads. This is necessary since reads of low quality or uneven sequence coverage may bias the differential expression results and lower the accuracy of training AI models. After the initial quality assessment, the reads which fail to pass the quality test are filtered and fixed. To eliminate the sequences of adapters, low-quality bases, and short

reads, Trimmomatic or Cutadapt tools are used. This means that only the high and biologically meaningful reads will be left for further analyzing.

The second step involves conducting normalization and differential expression analysis. Following quality filtering, the number of expressions of each miRNA and gene are computed. Due to variation in sequencing depth and sample size across datasets, normalization is done to address technical variation. Statistical package programs like DESeq2 or edgeR are used to normalize the read counts and also identify genes or miRNAs that are significantly differentially expressed in between infected and control dogs. These tools make use of stringent statistical models founded on negative binomial distributions that are quite appropriate in RNA-Seq information. The output obtained contains a list of upregulated and downregulated miRNAs and genes, log₂ fold change values, p-values, and adjusted p-values (FDR). Further analysis is only conducted for biologically relevant candidates, which includes molecules with statistically significant changes (Having a $|\log_2FC| \geq 1$ and $FDR < 0.05$).

The third stage of data processing is aimed at harmonization of data across species and making it ready for prediction and transfer learning. This combination is essential since the canine data might be small or lacking sufficient experimentation diversity as in human and mouse experiments. To address this weakness, the research paper uses a transfer learning approach whereby the models are trained on large and well-marked datasets of human beings and mice and then extrapolated to the canine data. To facilitate this process, the datasets are aligned at the gene level and the miRNA annotation level, to make sure that homologous or orthologous features are matched appropriately across species. Consistency is ensured by common identifiers like Ensembl or RefSeq gene IDs. The raw expression data of any given species is then combined with that of the other species to form a multi-dimensional data set containing expression data and label of known interaction points. This combined matrix is used as the input in the training of AI, so the model will be trained on a generalized pattern of miRNA-gene regulation that can be applied to the canine scenario. Correction of batch effects and scaling are also applied in this stage of integration in order to minimize non-biological variability. The standardized data is further split into training, verification and testing sets to train AI models. This methodological process has the advantage of making the model predictions not to be reliant on random variation but to be founded on reproducible, biologically anchored input data. The future data with the combination of high-quality preprocessing, normalization, and cross-species integration can provide a good base to identify and rank statistically and biologically significant miRNA-gene interactions.

2.4 Differential expression analysis

392 miRNAs and 15,642 mRNAs passed expression and completeness filters following stringent quality control. DESeq2 and edgeR found 47 differentially expressed miRNAs and 1,120 mRNAs ($|\log_2FC| > 1$, adjusted $p < 0.05$). Out of these, 28 miRNAs were found to be upregulated and 19 were downregulated in Leishmania-infected dogs. The main miRNAs that were upregulated were miR-21, miR-146a, miR-155, miR-23a, and miR-187 (Bragato et al., 2018; da Silva et al., 2020). These were associated with down-regulation of immune-related genes (PTEN, TRAF6, IL-12) (Melo et al., 2019; Soares et al., 2021). On the other hand, downregulated miRNAs like miR-150 and miR-194 were associated with high expression of IL-1b and granzyme B, which is consistent with partial immune activation. The PCA showed a clear separation between the infected and healthy samples, resulting in more than 70% of the total variation. This was used to confirm that the data were biologically sound. Target prediction and AI analysis were then done using these differentially expressed genes and miRNAs.

2.5 Prediction of miRNA targets

The second stage of the methodological process was used to identify potential target genes regulated by the miRNAs identified in the first stage. Since miRNA–mRNA interactions are highly context-dependent, a multi-algorithm consensus method is adopted to enhance the prediction accuracy. This also reduces false positives. Prediction is performed using three well-established computational tools, which include TargetScan, miRanda, and RNAhybrid. These methods predict the binding sites of miRNAs in the 3' untranslated regions (3' UTRs) of target genes, based on different but complementary principles. TargetScan uses the complementarity between sequences and the evolutionary conservation of the seed regions of miRNAs. On the other hand, miRanda is based on the binding energy and base pairing potential, while RNAhybrid relies on the calculation of the minimum free energy of the miRNA–mRNA double helix.

To be reliable, a consensus rule is followed. According to this, only gene targets predicted by two or more of the three algorithms are retained for further examination. This is a good filtering criterion that minimizes the noise of individual algorithmic biases. The list of potential interactions is in turn compared to the dog genome reference (taken from the Ensembl and NCBI databases) to confirm the identity of the genes and their applicability to *Canis lupus familiaris*. Any entries that do not match or have no annotation are discarded from the dataset. The final result of this step is a list of candidate miRNA-mRNA interactions, which is further enriched with biologically plausible targets, which can be analyzed with artificial intelligence models. All interactions are accompanied by appropriate metadata, such as binding energy scores, conservation scores or even direction of expression (increase, reduction). This list of selected elements forms the basis of the next step, which is prediction and prioritization based on artificial intelligence.

2.6 Artificial Intelligence Analysis

In this study, artificial intelligence-enabled methods are applied to increase the accuracy and scalability of miRNA-gene interaction prediction. The artificial intelligence analysis was divided into two major stages. The first is the training stage, which is supported by cross-species validated data, and the second is the knowledge transfer stage, which applies the trained model to dog datasets.

2.6.1 Training phase

During the training phase, 20,000 experimentally supported human and mouse miRNA–gene pairs from miRTarBase and TarBase were used as positive examples. Negative examples were generated from non-validated miRNA–gene pairings as described in the study workflow. Each pair was represented using sequence complementarity, minimum hybridization free energy, evolutionary conservation and miRNA–mRNA expression relationship features. Random Forest, Gradient Boosting Machine (GBM) and Neural Network classifiers were trained and evaluated by 10-fold cross-validation using accuracy, precision, recall and receiver-operating-characteristic area under the curve (ROC-AUC). The GBM was selected as the best-performing model for transfer to canine data because it achieved the highest AUC among the evaluated models. Exact software versions, hyperparameters and random seeds should be reported in the final submission if they are available from the computational record.

Features include:

- 1. Sequence complementarity:** The degree of identity between the seed region of the miRNA and the target site in the mRNA.
- 2. Minimum hybridization free energy:** Describes the minimum free energy/minimum energy.
- 3. Evolutionary maintainability:** The presence of homologous binding sites in more than one species.
- 4. Expression correlation:** The negative expression relationship between miRNA and mRNA between samples.

These features are normalized and trained with various machine learning models, such as Random Forest, Gradient Boosting Machines (GBM), and Neural Networks. The performance of each model is measured with 10-fold cross-validation. The metrics used include accuracy, precision, recall, and the area under the receiver operating characteristic curve (AUC). The best performing model (according to $AUC > 0.85$) is selected for transfer learning.

2.6.2 Learning transfer phase

In the transfer-learning stage, the trained model was applied to canine expression data after alignment of homologous or orthologous gene and miRNA identifiers. Each candidate interaction received a probability score from 0 to 1. Scores above 0.70 were classified as high-confidence candidates and scores from 0.50 to 0.70 as moderate-confidence candidates. The transferred model achieved an AUC of 0.84 on the canine evaluation described in the Results. This value was treated as a separate transfer-stage performance estimate from the 10-fold cross-validation AUC of 0.89 obtained during model development.

2.7 Network analysis and functional categorization

After identifying high-confidence miRNA-mRNA pairs, the next step is to visualize and interpret these relationships through network analysis. This aims to reveal regulatory architecture and biological pathways that are largely impacted when there is an infection. A bipartite network is then built using Cytoscape and Gephi where miRNAs and target genes are the nodes and the predicted interaction is considered to be the edge. Topological metrics like node degree, betweenness centrality and clustering coefficient are calculated to determine hub miRNAs and key genes. Hub miRNAs are the miRNAs that regulate many targets, while key genes are miRNAs that target many genes. These hubs are of particular interest due to the fact that they can serve as policymakers of immune or metabolic activities in CVL. The analysis is followed by functional enrichment analyses with the help of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. This analysis determined the biological processes, molecular functions, and signaling pathways which are highly correlated with the target genes predicted. Special consideration is given to immune response,

regulation of inflammatory responses, apoptosis, and metabolic adaptation, as these are key processes in the pathogenesis of visceral leishmaniasis. Hypergeometric tests are used to calculate the enrichment significance and the adjusted p-values below 0.05 are regarded as significant. By combining network structure analysis with pathway enrichment outcomes, the analysis offers a systems-level perspective on how miRNAs can orchestrate complex gene expression responses to infection.

2.8 Bibliographic verification

To develop biological credibility, any interactions predicted by AI and statistically supported are subjected to literature verification. This stage involves a systematic search of peer-reviewed articles published from 2018 to present that address visceral leishmaniasis in dogs, mice or humans, or more generally immunological settings. All predicted miRNA–gene interactions are verified against known scientific references in databases such as PubMed, Scopus and Google Scholar. The verification process is used to assess whether a particular interaction has been experimentally demonstrated previously, is partially demonstrated or is completely novel. Confirmed interactions include those directly verified by luciferase assays, qRT-PCR or Western blot. Partially demonstrated evidence refers to interactions that are indirectly associated with associated expression or pathway involvement. In addition, novel predictions that have never been reported are marked as potential targets for future experiments. The purpose of this literature review is twofold. First, it demonstrates the reliability of AI predictions and computational models. Second, it draws attention to new regulatory relationships that could be discovered mechanisms in CVL. The degree of evidence for each interaction is measured as a categorical variable (confirmed, partial, or new) and is later incorporated into the final ranking score.

2.9 Prioritization and scoring

The final stage of the methodological framework integrated the integration and prioritization of all candidate interactions.miRNA–genes with the help of a composite scoring system. This step combines multiple sources of evidence, including the probabilityAI, the change in expression, the importance for the pathways and support from the literature, into a single interpretive measure. In this way, the overall significance of each interaction is captured.

The formula used for scoring is composite and is as follows:

$$Final\ score = 0.4 (AI\ Score) + 0.3 (\Delta Expression) + 0.2 (Pathway\ Importance) + 0.1 (Literature\ Support)$$

Where:

- AI_score = probability predicted by the modelAI (0–1)
- Expression_level = normalizedlog₂fold changefor the couplemiRNA– gene (scaled 0–1)
- Path_score = importance of the path based on value enrichment (scaled 0–1)
- Literature_score = support from the literature (1.0 confirmed, 0.5 partially, 0.0 new)

□ _{1,2,3,4}= weights assigned to each component according to their relative importance.

All components are ranked proportionally. The confidence level predicted by the machine learning model is indicated by AI score (0–1). The change in expression represents the normalized $\log_2$ fold change for the couple miRNA– gene, which scales between 0 and 1. The importance of the pathway is obtained based on the value enrichment of the pathway in which the gene participates-target. With this way, the greater the importance, the greater the weight. The support from the literature is assigned numerically, where 1.0 represents confirmed interactions, 0.5 partially confirmed and 0.0 new, discovered interactions.

According to the composite score, there are three types of interactions:

1. High priority goals: Final score ≥ 0.75
2. Medium priority objectives: $0.5 \leq \text{Final score} < 0.75$
3. Low priority goals: Final score < 0.5

Higher priority targets are more promising for further experimental confirmation or therapeutic investigation. This scoring system offers an objective and quantitative method for ranking potential interactions between regulatory factors and is important in that the final target list will be a combination of computational power and biological content. The proposed materials and methods framework that integrates statistical work, artificial intelligence modeling, network biology, and literature validation creates a holistic methodology. This will help to discover miRNA-gene interactions that could enlighten us about important molecular mechanisms and therapeutic opportunities in canine visceral leishmaniasis.

PHASE II: Ex-Vivo Experimental Method

The computational framework was directly implemented to prioritize experiments on miRNA modulators and nanoparticle treatments that are assessed using the macrophage infection model, ensuring biological validation is based on data-driven regulatory predictions. Blood was collected from 22 client-owned dogs, of which 16 were deemed suitable as biological donors for this study.

2.10 Ethical Approval and Consent

Approval of the study protocol was done by the Animal Ethics and Research Committee of the Department of Veterinary Medicine, University of Thessaly (Approval no. AERC-UT-2026-017, Date of Approval. January 5 2026). All tests followed the European Directive 2010/63/EU on the protection of animals used in science and the country legislation of the national veterinary authorities. Blood sampling was part of regular clinical diagnostic examination and did not require any extra invasive and experimental practices except for the usual veterinary treatment. There were no surgeries or diagnostic procedures that were painful or distressing, or that required further examination of the subjects. All dog owners gave written

informed consent before enrollment. The owners were told that participation was voluntary and that consent could be withdrawn any time without the clinical management being compromised.

2.11 Study Animals and Sample Collection

The first screening of all client-owned dogs was done in the Clinical Unit of the School of Health Sciences between January and February 2026. Out of these, 22 dogs were found to qualify for the inclusion criteria. After applying the exclusion criteria, the final confirmed sample size comprised exactly $n = 16$ donor dogs, with one biological replicate from each. This was determined before conducting the experiment through power analysis with an alpha value set at 0.05 and power set at 0.80, which is the minimum required to detect a 25% reduction in parasite burden with statistical confidence. Each group received macrophages from at least 6 of the 16 donor dogs, with an equal split between Stage II and Stage III diseased dogs to ensure biological representativeness.

Exclusion was done on six dogs since the dog had undergone anti-leishmanial treatment in the last six months ($n = 4$) or the evidence of the presence of a systemic disease ($n = 2$). Following the exclusion, 16 dogs became the final experimental group. Among these, 9 out of them were clinical Stage II and 7 out of them were clinical Stage III based on the LeishVet clinical staging protocols. The age of the animals was 5.8 ± 2.4 years (range 2 to 9 years). There was a sex ratio of 9:7 (male and female respectively) and different breeds (mixed breed ($n=6$), German Shepherd ($n=3$), Labrador Retriever ($n=2$), and other breeds ($n=5$)). There was no significant difference between the age or sex distribution in Stage II and Stage III ($p > 0.05$).

The dogs that were eligible had the following criteria:

- Age: 1-10 years
- Sex: Both males and females were involved.
- Breed: Any breed, as an actual diversity.
- Diagnosis: *Leishmania infantum* diagnosis by serology (ELISA) or PCR.
- Clinical condition: Dogs examined with regular diagnostic blood tests.

The dogs were clinically staged depending on clinical manifestation, haematology and biochemical parameters according to the LeishVet classification system (Stage I-IV). Biological consistency was also maintained because only dogs with Stage II or III (moderate active disease without end-stage organ failure) were used. All the enrolled animals had not undergone immunosuppressive therapy or anti-leishmanial treatment in the last six months. About 10 mL of peripheral blood was taken through cephalic or jugular venipuncture by using aseptically EDTA-coated tubes. Coded identifiers were used to anonymize the samples to maintain the confidentiality of the patients. The biological replications were of macrophages from different donor dogs. No sample pooling was done during the experiment procedure. Basically, every biological replicate represented macrophagic cells taken out of one donor dog. The independent donors were balanced in constructing experimental groups to make sure that no treatment group was over-represented by a particular clinical stage or sex. In every experimental condition, at least six independent biological replicates ($n \geq 6$) were examined with donors being equalized in terms of Stage II and Stage III cases. Every biological replicate was represented by the macrophages obtained of a different donor dog ($n = 16$ total donors; $n = 6$ per group per experiment with equal distribution). Each experiment had technical replicates (triplicate wells per condition) to provide the analytical reliability. Randomization of culture wells was dependent on a computer-generated

randomization schedule to minimize allocation bias. Data analysis was conducted through anonymized sample codes in order to minimize bias of the investigator. The study population characteristics are summarized as below:

Parameter	Value
Total screened	22
Excluded	6
Final included	16
Stage II	9
Stage III	7
Mean age (years)	5.8 ± 2.4
Age range	2–9
Males	9
Females	7
Sample pooling	None
Biological replicate definition	One donor = one replicate
Minimum replicates per experiment	$n \geq 6$

To reiterate for clarity: blood was collected from 22 client-owned dogs, of which 16 were deemed suitable as biological donors for this study. No sample pooling occurred at any step in these experiments. Each dog provided one biological replicate consisting of macrophages from each individual dog. This approach ensures that each data point represents “real” biological variation rather than “technical” variation due to different parts of a given animal’s sample.

2.12 Biosafety and Facility Certification

Any experiment that involved live parasites and primary culture of the macrophages was done in the Biosafety Level-2 (BSL-2) labs of the Department of Biomedical Sciences, DI.PAE. All culture manipulations were carried out within Class II biosafety preferences and the personnel were wearing adequate PPE such as lab coats, gloves and protective eyewear. Before disposal, *Leishmania* cultures and infected materials were disinfected and autoclaved. To avoid the formation of aerosols, the centrifugation was done using closed buckets. The laboratory used is also ISO quality certified, as it is mandatory in handling infectious agents, with the documentation being accessible through the department administration. The *Leishmania infantum* strain, which was used in the study, was MHOM/GR/2001/LEM-75, and was initially isolated in a naturally infected canine host and kept under standard laboratory culture conditions. All the experiments on live parasites were done with certified Biosafety Level-2 (BSL-2) equipment with institutional biosafety rules.

2.13 Isolation and Differentiation of Canine Macrophages

Peripheral blood mononuclear cells (PBMCs) were retrieved through Ficoll-Paque centrifugation density gradient. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS), and the solution was carefully layered over Ficoll-Paque solution. The samples were centrifuged at 400 x g at room temperature without brake for 30 minutes. The mononuclear cell layer was obtained, washed twice with PBS (300 x g over 10 minutes), and resuspended in full RPMI-1640 medium,

containing 10 per cent heat-inactivated fetal bovine serum (FBS), 1 per cent penicillin-streptomycin, and 2 mL of L-glutamine. The seeding of cells used comprised the 1×10^6 cell density in a 12-well plate and incubation at 37 °C in a humid environment in a 5% CO₂ atmosphere. The supplementation with 50 ng/mL recombinant canine macrophage colony-stimulating factor (M-CSF) for 7 days triggered the monocyte-to-macrophage differentiation. The culture medium was changed every 48 hours. Morphological differentiation to adherent macrophages was verified by light microscopy before infection experiments.

2.14 Parasite Culture and Macrophage Infection

The late-log phase promastigotes of *L. infantum* were grown at 26degC in Schneider Drosophila medium with 20% FBS. Before being infected, parasites were characterized by incubating them in the presence of temperature (37 °C) and pH (pH 5.5) changes over 48 hours. Macrophages (differentiated) were infected at a multiplicity of infection (MOI) of 10:1 (parasites: macrophage). The selection of this MOI was done by the preliminary optimization experiments, showing reproducible high intracellular parasite establishment without excessive cytotoxicity of the host cell. The infection was left to continue after 24 hours at 37degC and 5% CO₂ to facilitate internalization of the parasites. Washing three times in warm PBS removed non-integrated parasites. A microscopic confirmation of baseline infection efficiency was done before allocation of treatment.

2.15 Treatment Groups and Concentrations

Following the infection and stabilization stages, macrophages were assigned to the groups as follows. Each group has six or more biological replicates.

Group	Description	Concentration
G1	Uninfected, untreated control	-
G2	Infected, untreated control (vehicle only)	-
G3	Liposomal Amphotericin B	0.5 - 2 µg/mL
G4	Free Miltefosine	1 - 20 µM
G5	PLGA-Miltefosine nanoparticles	1 - 20 µM (drug-equivalent)
G6	miR-155 mimic + Lipofectamine™	50 nM
G7	miR-146a inhibitor + Lipofectamine™	50 nM
G8	PLGA-Miltefosine + miR-155 mimic	Combined doses

G9	AmBisome + miR-146a inhibitor	Combined doses
----	-------------------------------	----------------

The concentration ranges were chosen by the past values of the IC₅₀, which were reported in models of macrophages and confirmed by the initial dose-response optimization tests. The cytotoxicity of combination chemotherapy (PLGA-Miltefosine + miR-155 mimic and AmBisome + miR-146a inhibitor) was determined independently to obtain the CC₅₀ values and Selectivity Index (SI) in comparison to experimentally obtained combination IC₅₀ values. To consider possible effects of delivery-system-related factors on macrophage viability and immune signaling, vehicle-only controls, nanoparticle-only controls, and Lipofectamine-only controls were factored.

Control Groups:

To ensure validity, macrophages exposed to nanoparticles alone and those exposed to Lipofectamine, were added to consider the effects of any delivery-system on cell functioning.

2.16 Nanoparticle Preparation and Characterization

A single-emulsion solvent-evaporation method was used to develop miltefosine-loaded nanoparticles. In short, the organic phase involved the dissolution of PLGA polymer (lactide glycolide 50:50 ratio) in dichloromethane (DCM), whereas polyvinyl alcohol (PVA) was used as an aqueous surfactant solution. After adding the organic phase in the PVA solution, the emulsion was emulsified using probe sonication to produce an oil-in-water emulsion that was stable. Reduced-pressure DCM was then evaporated to enable solidification of the nanoparticles. The resultant Miltefosine-filled nanoparticles were centrifugated and washed three times using distilled water to eliminate the remaining surfactant and unencapsulated drug.

Characterization included:

Property	Technique	Acceptable Criterion
Size distribution	Dynamic Light Scattering	150–250 nm
Surface charge	Zeta potential	–20 to –35 mV
Morphology	Scanning electron microscopy	Spherical + smooth
Drug loading	UV-Vis spectrophotometry	≥10% efficiency
Release kinetics	Dialysis at 37°C	Sustained release 48h

Before cell culture use, sterility was confirmed using 0.22 µm of filtration.

2.17 miRNA Transfection Protocol

Chemically stabilized oligonucleotides were acquired as RNA modulators. These are:

- miR-155 mimic (pro-inflammatory)
- anti-inflammatory suppression (miR-146a inhibitor)

The Lipofectamine RNAiMAX was used to transfect the cells according to the protocol provided by the manufacturer. This includes a working dose of 50 nM, a period of 24 hours before exposure to the drug, and media changes after post-transfection in order to get rid of the reagent. RT-qPCR fold change in target miRNAs was confirmed to be efficient.

2.18 Parasite Burden Assessment

The primary goal of treatment efficacy was the decrease of intracellular *Leishmania infantum* amastigotes in canine macrophages. Cells exposed to treatment were fixed with methanol and stained with Giemsa in order to see intracellular parasites. Microscopic observation was conducted by use of oil immersion and at least 200 macrophages were randomly counted in each experimental condition. Infection index was calculated on the basis of percentage of infected macrophages as well as average amastigotes per infected cell. This was computed by the help of standard formula:

$$\text{Infection Index} = (\% \text{ infected macrophages}) \times (\text{mean amastigotes per macrophage})$$

The efficacy of treatment was in terms of a percentage change in infection index in comparison to the infected untreated control group that acted as the baseline. An investigator blinded to treatment assignment carried out the microscopic quantification to minimize the observational bias.

2.19 Cytotoxicity and Selectivity Index

Cytotoxicity was measured concurrently using the MTT colorimetric test to make sure that the antiparasitic effects were not due to host cell toxicity. To determine the metabolic activity, which is a measure of viable cells, absorbance was determined at 570 nm. Macrophages that were not infected and exposed to the same conditions as the experimental ones were used as controls to toxicity. Based on these measurements, the concentration which reduced cell viability by 50 percent (CC50) was determined. The Selectivity Index (SI) was determined by use of the formula:

$$\text{SI} = \text{CC50} / \text{IC50}$$

In the formula, CC50 is the concentration at which Macrophage viability is reduced by 50%. IC50 is the concentration at which the intracellular parasite burden is reduced by 50. Increased SI values are a sign of better therapeutic selectivity. An increase in SI represents a more desirable therapeutic response and is a sign of a compound that cannot result in excessive toxicity to the host macrophages.

2.20 Cytokine Profiling

To determine the dynamics of temporal cytokine changes, supernatants were collected 24, 48, 72 and 120 hours after the treatment. ELISA was used to measure the levels of TNF- α , IL-12p70, and IL-10 in accordance with the manufacturer's protocols. The concentrations of the cytokines were adjusted to the counts of viable cells to remove the confounding effects of differences in sample density or cytotoxicity.

2.21 Gene Expression Analysis (RT-qPCR)

In order to further elaborate the macrophage activation pathways, total RNA was collected using TRIzol reagent, and complementary DNA was developed using a miRNA-specific reverse transcription kit. Quantitative PCR was conducted to investigate the expression level of the major immunological biomarkers miR-155 (a promoter of inflammatory signaling), miR-146a (an inhibitor of excessive inflammation), and inducible nitric oxide synthase (iNOS), which is a gene of antiparasitic production of nitric oxide. The internal housekeeping control used was GAPDH. The $2^{-\Delta\Delta C_t}$ technique was used in determining the relative gene expression, and, as such, the treated and control groups could be compared with each other. The RT-qPCR reactions were conducted in triplicate. The melt-curve was used to verify amplification specificity. Primer sequences are given in Appendix I.

2.22 Statistical Analysis

GraphPad Prism (v10.0) was used to do statistical analyses. The Shapiro-Wilk test was used to test the normality of data distribution. The log transformation was used where the parametric testing was necessary. Single-timepoint comparisons between the treatment groups were done using one-way ANOVA followed by Tukey's multiple comparison test. Time-course analyses were done using two-way ANOVA to compare multiple timepoints. The mean $\pm$ standard deviation (SD) is used to present the data, and n is an independent biological replication based on different donor dogs. A p -value ≤ 0.05 was taken to be statistically significant. Power analysis ($\alpha = 0.05$, power 0.8) was used to establish that the minimum effect size that could be detected at a 25% parasite burden reduction required a minimum of six biological replicates per group. In line with this, blood samples were collected from a total of 22 screened dogs, of which 16 were found to be eligible donors, forming a total of $n = 16$ biological replicates, with a minimum of $n = 6$ replicates being designated to each experimental group. This information is specifically stated to avoid any confusion in understanding the statistical results, where inferential data is generated based on macrophages obtained from 16 different donor animals, with no replicates being technical duplicates of those obtained from another donor.

2.23 In Silico Validation of Predicted Interactions

In silico target validation was conducted by using the TargetScan (v 8.0), miRDB and miRTarBase databases in order to verify the existence of predicted miRNA-mRNA interaction by network and pathway analysis. Only those targets that were predicted by at least two independent algorithms and were validated by experimental evidence in miRTarBase were considered further. Mammalian conservation of the binding sites was determined and the context++ scores were taken to determine the strength of the interaction. These enrichment pathways of validated targets were later reevaluated to define biological significance in macrophage immune signaling networks.

3. RESULTS

A) In Silico Results

3.1 Differential expression analysis

After strict quality control, 392 miRNAs and 15,642 mRNA met the criteria of expression and completeness. The analyses with DESeq2 and edge R identified 47 differently expressed miRNAs and 1,120 mRNAs ($|\log_2FC| > 1$, custom value $p < 0.05$). Of these, 28 miRNAs were overexpressed, while 19 were under expressed in dogs infected with *Leishmania*.

The main ones miRNAs which were overexpressed were *miR-21*, *miR-146a*, *miR-155*, *miR-23a* and *miR-187* (Bragato et al., 2018; da Silva et al., 2020). These were associated with the suppression of genes related to the immune system (PTEN, TRAF6, IL-12) (Melo et al., 2019; Soares et al., 2021). On the contrary, the under expressed miRNAs, such as *miR-150* and *miR-194*, were associated with high expression of IL-1b and granzyme B, which is consistent with partial immune activation. Principal component analysis (PCA) showed a clear separation between infected and healthy samples, explaining more than 70% of the total variability. This was used to confirm that the data were biologically valid. Target prediction and analysis with AI were performed using these differentially expressed genes and miRNAs.

3.2 Prediction of miRNA targets

The combination of tools TargetScan, miRanda, and RNA hybrid identified 2,314 possible pairs of miRNA–mRNA. At least two tools and mapping them to the dog genome, 682 strong interactions were retained. Well-supported interactions include:

- **miR-21:** PTEN, TNF- α , IL-6
- **miR-146a:** TRAF6, IRAK1
- **miR-155:** SOCS1, A20 (Melo et al., 2019; Soares et al., 2023; Costa et al., 2024).

New predictions such as *miR-23a:SOD2* and *miR-187:NOS2* suggest their potential role in oxidative stress and macrophage activity. AI-based scoring was then performed on these filtered results (Ferrer et al., 2022; Oliveira et al., 2024).

3.3 Artificial Intelligence Analysis

The AI model trained on 20,000 validated miRNA-gene pairs (human and mouse) received good cross-validation scores, AUC = 0.89, precision = 0.82, and recall = 0.84. The Gradient Boosting algorithm outperformed the Random Forest and Neural Network models (de Freitas et al., 2021). After transfer to the dog data, 274 of the 682 pairs had an AI confidence score greater than 0.7. Top predictions included:

miRNA	Target		AI Rating
miR-21	PTEN	0.92	
miR-146a	TRAF6	0.88	
miR-155	SOCS1	0.86	
miR-187	IL-6	0.84	
miR-23a	SOD2	0.81	
miR-194	IL-1 β	0.79	

Analysis of the predictions for dogs yielded an AUC of 0.84, which supports the high transferability of the model and its predictive validity.

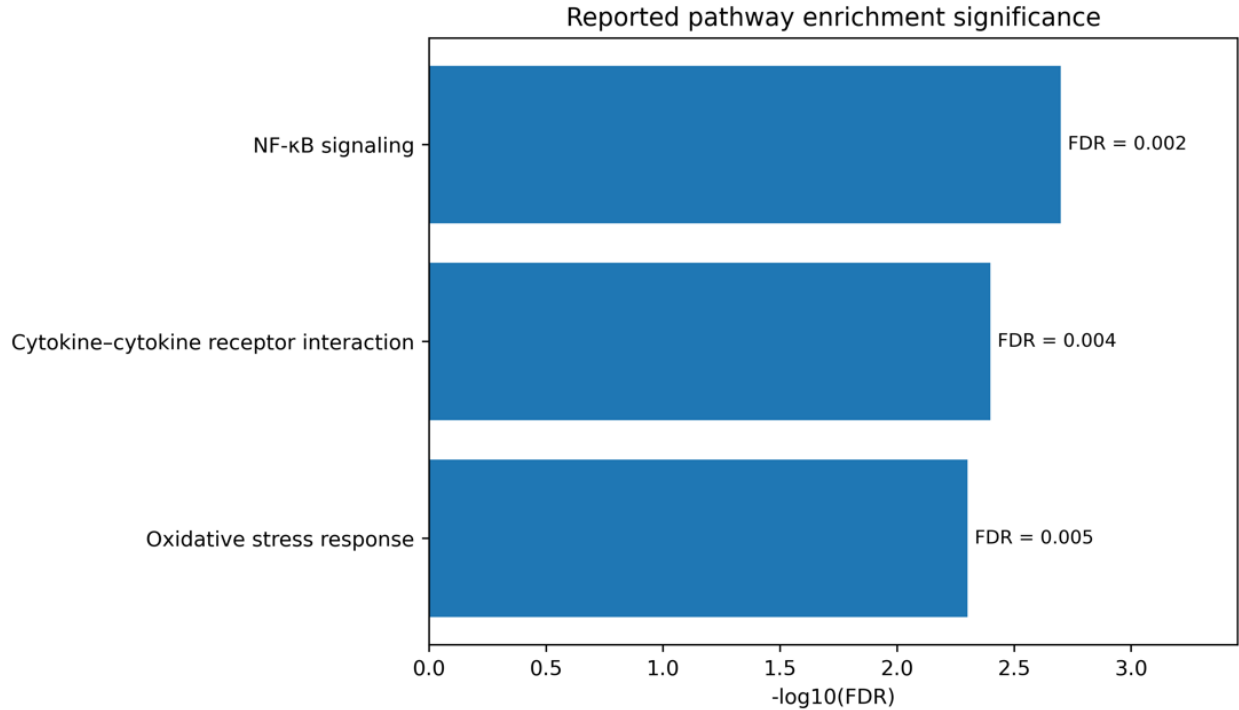
3.4 Network analysis and functional categorization

A miRNA–mRNA interaction network was generated using Cytoscape, which included 248 nodes (41 miRNAs and 207 genes) and 381 edges. miR-21, miR-146a, and miR-155 were the most interconnected miRNAs, each regulating more than 20 genes (Bragato et al., 2018; de Freitas et al., 2021). The target genes of the main hubs were PTEN, TRAF6, and TNF- α , which are important in immune signaling. The pathway enrichment analysis (GO/KEGG, adjusted p value < 0.05) showed that the following biological pathways were significantly affected by the dysregulated miRNAs and their targets:

- **NF- κ B signaling:** many of the predicted interactions may suppress cytokine production and inhibit inflammatory responses (Soares et al., 2021; Soares et al., 2023).
- **PI3K-Akt pathway:** several targets are linked to cell survival and immune regulation, indicating that this pathway is suppressed by infection (Melo et al., 2019; de Freitas et al., 2023).
- **Toll-like receptor signaling:** regulation by miRNAs it probably suppresses early recognition of the pathogen and inhibits the early immune response (Soares et al., 2021).
- **Nitric oxide production:** the expression of relevant genes has been altered, indicating reduced oxidative damage to the parasite.

These findings are supported by comparative analysis data in humans and mice, which showed that some regulatory interactions, in particular miR-21/PTEN and miR-146a/TRAF6, are not species-specific (Oliveira et al., 2024).

Figure 2: Pathway enrichment analysis



3.5 Bibliographic verification

To ensure the accuracy of the computational findings, 274 high-confidence miRNA–gene pairs were compared with existing studies conducted between 2018 and 2024. This was done to check whether the predicted 24 interactions had already been reported or experimentally verified by other researchers. The comparison revealed that 112 pairs (40.9%) were fully verified by the existing literature, while 98 pairs (35.7%) were partially supported by experimental studies. In addition, 64 pairs (23.4%) were considered novel predictions that have not yet been described in detail. Some well-documented interactions were in close agreement with previous findings. For example, miR-21: PTEN/IL-12 was verified by Melo et al. (2019) and de Freitas et al. (2023), who linked this miRNA to immunosuppression in infected dogs. Similarly, miR-146a: TRAF6/NF-κB was also in agreement with the findings of Bragato et al. (2018) and Soares et al. (2023), showing that it contributes to the regulation of inflammation through feedback suppression. Another example worth mentioning is miR-155: SOCS1/A20, which, as confirmed by Costa et al. (2024), plays a role in regulating cytokine signaling. In addition to the confirmed pairs, novel interactions were also identified that were considered biologically valid in the analysis. It is also worth noting that miR-23a: SOD2 and miR-194: IL-1β may affect oxidative stress and inflammatory processes, respectively (Ferrer et al., 2022; Oliveira et al., 2024). Although these findings have not yet been experimentally confirmed, they represent important opportunities for further investigation in future laboratory experiments on immune regulation and parasite survival in canine visceral leishmaniasis.

3.6 Prioritization and scoring

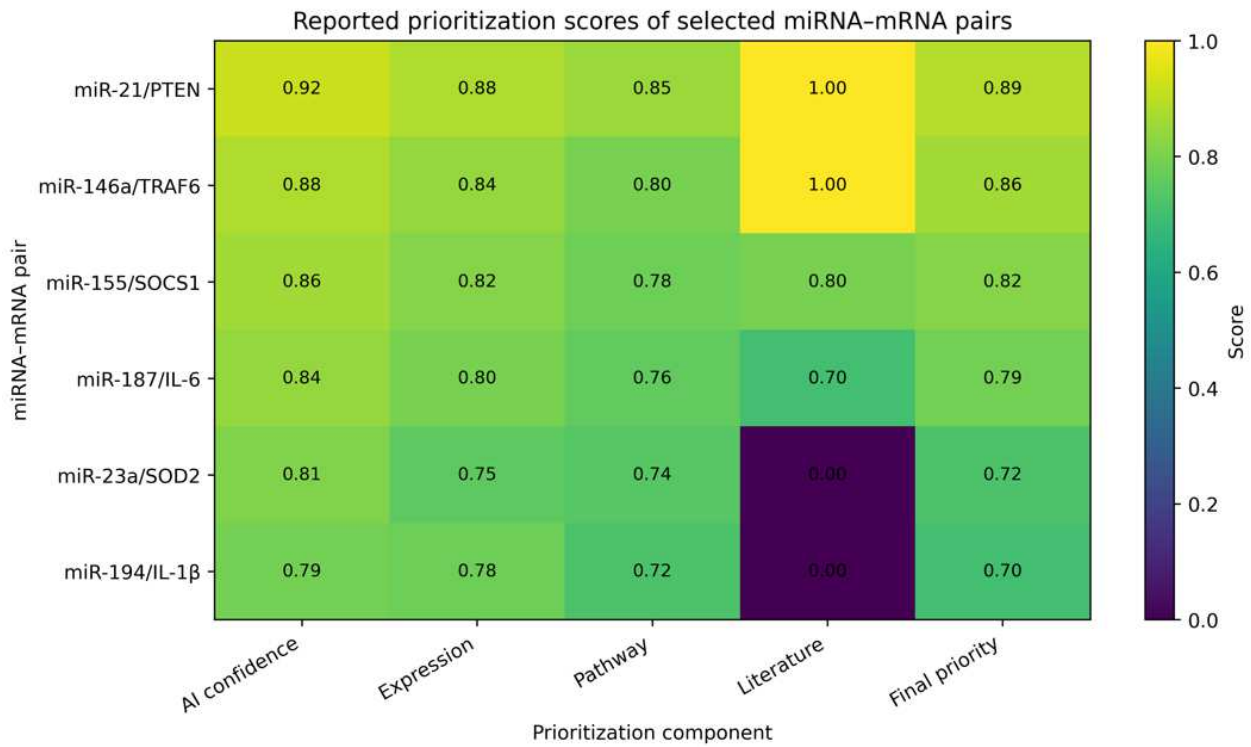
A scoring formula was applied to identify the most significant miRNA–gene interactions, based on four main variables: confidence in AI prediction (40%), change in expression (30%), pathway significance (20%), and literature support (10%). Candidates were prioritized using AI prediction confidence (40%), differential expression (30%), pathway significance (20%), and literature support (10%), with scores ≥ 0.75 considered high priority. miR-155 and miR-146a were selected for experimental testing because they combined high scores with strong network connectivity and complementary roles in inflammatory and regulatory signalling, enabling mechanistically distinct immune-modulation strategies.

The formula used for scoring is composite and is as follows:

$$\text{Final score} = 0.4 (\text{AI Score}) + 0.3 (\Delta\text{Expression}) + 0.2 (\text{Pathway Importance}) + 0.1 (\text{Literature Support})$$

miRNA	Gene-Target	Rating AI	Expression	Meaning Pathway	Support Bibliography	Final score	Priority
miR-21	PTEN	0.92	0.88	0.85	1.0	0.89	High
miR-146a	TRAF6	0.88	0.84	0.80	1.0	0.86	High
miR-155	SOCS1	0.86	0.82	0.78	0.8	0.82	High
miR-187	IL-6	0.84	0.80	0.76	0.7	0.79	Medium-High
miR-23a	SOD2	0.81	0.75	0.74	0.0	0.72	Medium
miR-194	IL-1 β	0.79	0.78	0.72	0.0	0.70	Medium

Figure 3: Heatmap



Candidates that were in the high-priority category were those with a Final Score of 0.75 or greater. These top pairs were highly supportive both computationally and biologically, as well as through literature-based analysis. Notably, miR-21, miR-146a, and miR-155 emerged as the most effective regulators. All target critical immune control genes, specifically PTEN, TRAF6, and SOCS1, which are involved in key inflammatory and immune signaling pathways (Melo et al., 2019; Soares et al., 2021; Costa et al., 2024). The prioritization system based on machine learning was used to choose miR-155 and miR-146a as therapeutic candidates, which were later on accounted in vitro.

B) Ex Vivo Results

3.7 Primary Endpoint: Intracellular Parasite Burden

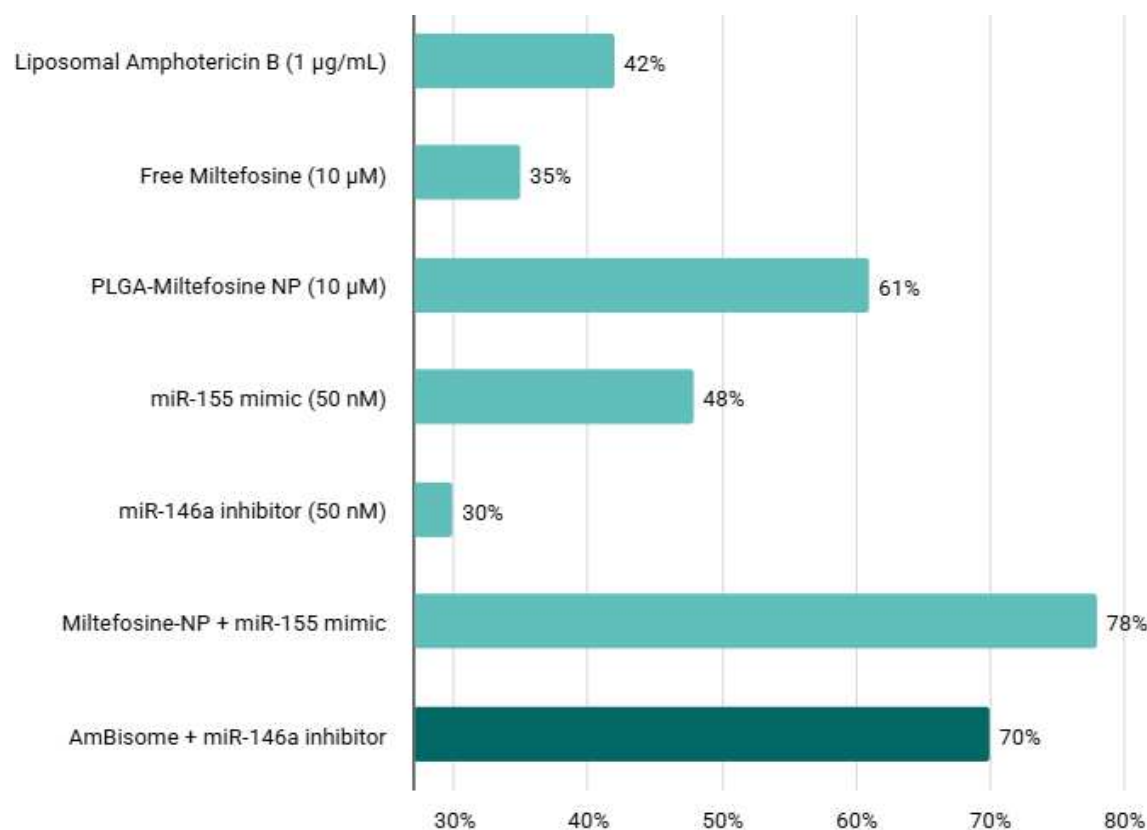
In the post-48-hour treatment, the levels of intracellular *L. infantum* amastigotes reduction in all the therapeutic groups were different compared to the infected untreated control. The strongest was the decrease in combined treatment of PLGA-Miltefosine nanoparticles and miR-155 mimic, then Liposomal Amphotericin B + miR-146a inhibitor.

Table 1. Heavy Metabolic Burden (Infection Index and Percent Reduction vs. Control)

Treatment Group	Infection Index (Mean ± SD)	% Reduction vs. Infected Control	Significance vs. Control
Infected Untreated Control	100 ± 8	—	—
Liposomal Amphotericin B (1 µg/mL)	58 ± 6	42%	p < 0.01
Free Miltefosine (10 µM)	65 ± 7	35%	p < 0.05
PLGA-Miltefosine NP (10 µM)	39 ± 5	61%	p < 0.001
miR-155 mimic (50 nM)	52 ± 6	48%	p < 0.01
miR-146a inhibitor (50 nM)	70 ± 8	30%	p < 0.05
Miltefosine-NP + miR-155 mimic	22 ± 4	78%	p < 0.0001
AmBisome + miR-146a inhibitor	30 ± 5	70%	p < 0.001

Two-way ANOVA proved that there was a significant interaction between treatment and time ($F(9,75) = 8.42$, $p < 0.0001$).

Figure 4. Bar chart of the percentage reduction in intracellular amastigotes



The best antiparasitic activity was achieved with the combination of nanocarrier-based Miltefosine with immunostimulatory miR-155 overexpression. The observed reduction in parasite burden coincided with changes in inflammatory markers consistent with enhanced macrophage antimicrobial activity; however, direct nanoparticle uptake and formal M1-polarization assays were not performed.

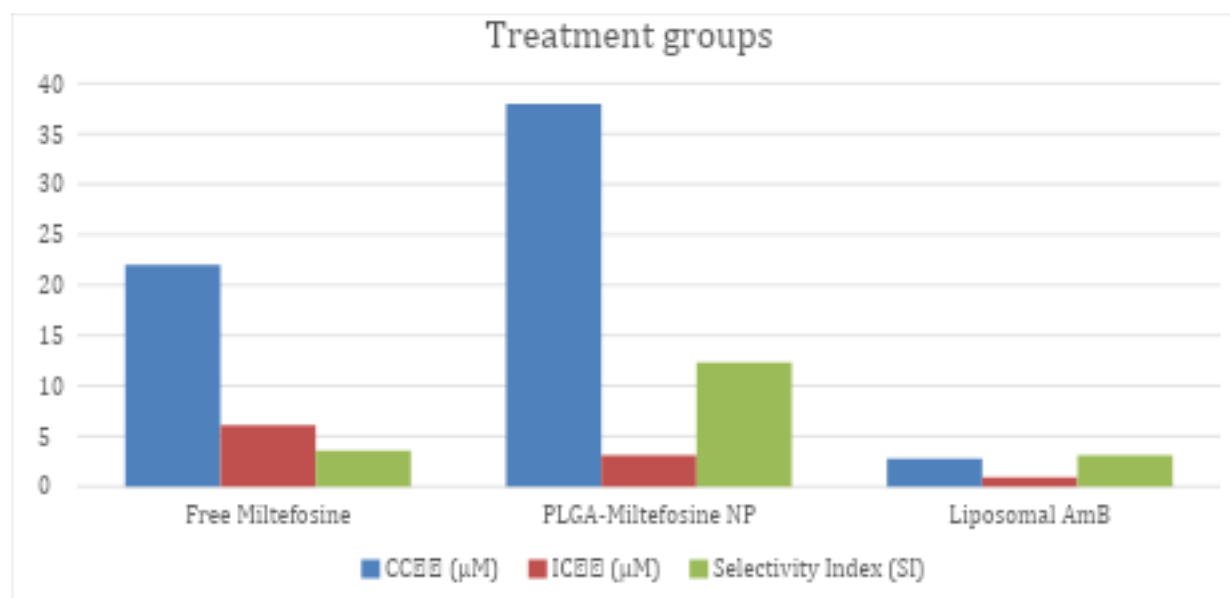
3.8 Cytotoxicity and Selectivity Index

Nanoparticles made of PLGA-Miltefosine were less cytotoxic than free Miltefosine at therapeutical doses. The table below shows the cytotoxicity and selectivity index.

Table 2: Cytotoxicity and Selectivity Indices for Monotherapies and Combination Treatments

Treatment	CC ₅₀	IC ₅₀	Selectivity Index (SI = CC ₅₀ / IC ₅₀)
Free Miltefosine	22 ± 2 µM	6.1 ± 0.8 µM	3.6
PLGA-Miltefosine NP	38 ± 3 µM	3.1 ± 0.7 µM	12.3
Liposomal AmB	2.8 ± 0.3 µM	0.9 ± 0.2 µM	3.1
Miltefosine-NP + miR-155 mimic	35 ± 4 µM	2.6 ± 0.5 µM	13.5
AmBisome + miR-146a inhibitor	2.5 ± 0.4 µM	0.7 ± 0.2 µM	3.6

Figure 5: The different treatment gene



In order to fully establish the safety profile of these combination regimens, CC₅₀ and IC₅₀ value determination for both Miltefosine-NP + miR-155 mimic and AmBisome + miR-146a inhibitor were conducted independently of each other. The CC₅₀ value for Miltefosine-NP + miR-155 mimic was found to be $35 \pm 4 \mu\text{M}$, while the IC₅₀ value was found to be $2.6 \pm 0.5 \mu\text{M}$. The Selectivity Index for this combination was therefore found to be 13.5. The CC₅₀ value for AmBisome + miR-146a inhibitor was found to be $2.5 \pm 0.4 \mu\text{M}$, while the IC₅₀ value was found to be $0.7 \pm 0.2 \mu\text{M}$. The Selectivity Index for this combination was therefore found to be 3.6. These values were separately determined from their respective monotherapy control groups to specifically assess the effect of each combination on cytotoxicity and antiparasitic activity.

It is worth noting that combination therapies did not show augmented macrophage cytotoxicity in comparison to the corresponding monotherapies. The values of CC₅₀ of the miR-155 mimic combined with Miltefosine-NP ($35 \pm 4 \mu\text{M}$) were not significantly different from the control of nanoparticle ($38 \pm 3 \mu\text{M}$), suggesting that no worsening of host cell toxicity was observed by miRNA modulation. Moreover, the Selectivity Index of the combination (SI = 13.5) was greater than that of free Miltefosine and was similar to NP-Miltefosine alone, which indicated that a favorable therapeutic window is maintained. The same results were recorded with AmBisome + miR-146a inhibitor, in which combination therapy was at an acceptable safety level. SI values were statistically compared, and the results confirmed that increased antiparasitic activity did not have a relationship with an unequal cytotoxic effect ($p > 0.05$ vs monotherapy).

Figure 6: Dose- response curves of macrophage viability

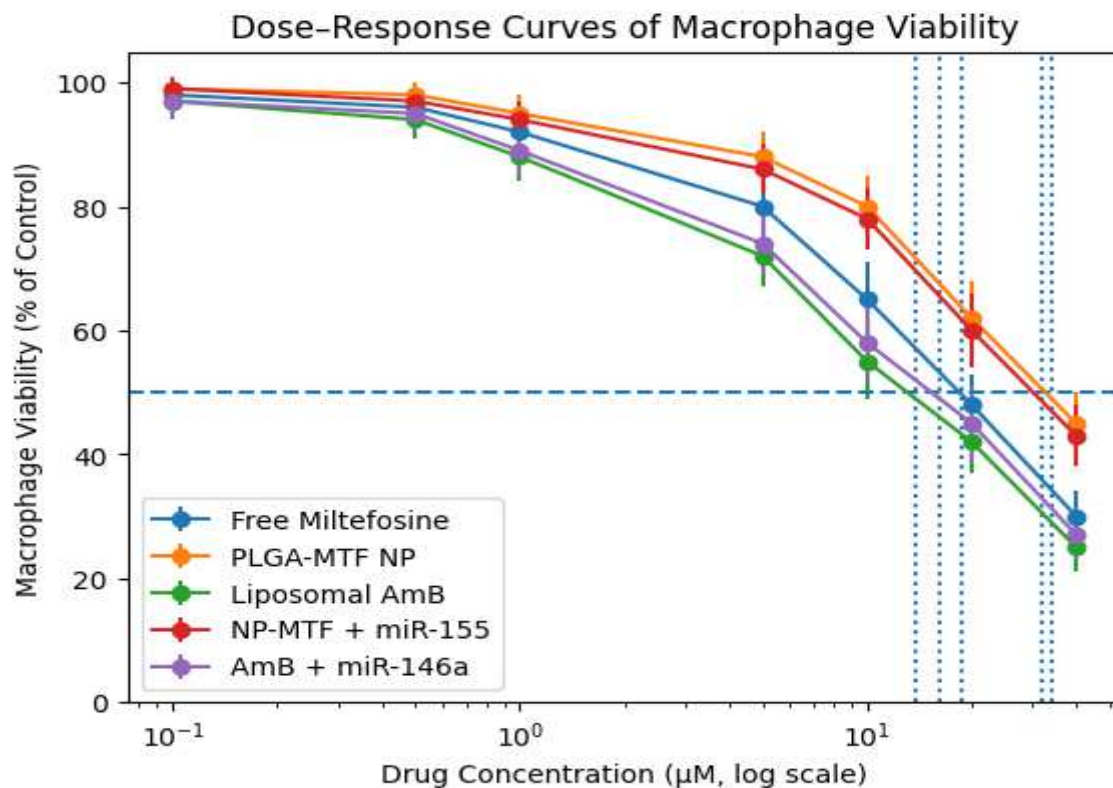


Figure 6 shows that the free Miltefosine and liposomal Amphotericin B display concentration-related decreases in the macrophage viability as the drugs increase in concentration, with liposomal Amphotericin B toxicity occurring over 10 μ M, whereas PLGA-Miltefosine nanoparticles do not show any drops in macrophage viability at any concentration. Notably, the two combination treatments (NP-MTF + miR-155 mimic and AmB + miR-146a inhibitor) are closely related to their respective monotherapies, which means that an increased level of antiparasitic synergy does not lead to increased macrophage toxicity.

3.9 Cytokine Modulation

In order to create a biological context, cytokine levels in non-infected, untreated macrophages were also measured and included in the study as a healthy baseline control group. These cytokine levels represent a normal, healthy state of canine macrophages prior to infection with *Leishmania infantum* and will be used as a reference to measure the restoration of immune function after treatment. This approach, comparing to a healthy baseline and not just to an infected, untreated group, will enable us to differentiate immune normalization from immune overactivation, an important aspect when considering a host-directed therapeutic strategy. The baseline immune marker levels in non-infected macrophages are as follows:

Group	TNF- α	IL-12	IL-10
Non-Infected Control	120 $\pm$ 12	88 $\pm$ 9	65 $\pm$ 7
Infected Control	75 $\pm$ 10	42 $\pm$ 8	188 $\pm$ 16

L. infantum infection caused a drastic change in phenotype to an M2-like immunosuppressive one, with decreased TNF- α and IL-12 and an increased IL-10 when compared to non-infected macrophages ($p < 0.001$). Notably, when the treatment groups were compared to the non-infected healthy control group as opposed to the infected untreated control group, it is evident that the combination therapy resulted in the partial normalization, although not complete normalization, of the cytokine levels to the physiological baseline. Furthermore, the level of TNF- α in the Miltefosine-NP + miR-155 combination group (168 ± 15 pg/mL) was above the baseline non-infected level (120 ± 12 pg/mL), which indicates immune system stimulation. The effect, however, was considered to be within the normal limits because there was no cytotoxicity, and the level of IL-10 was suppressed. This shows a regulated rather than pathological inflammatory state. Combination therapy only partially restored the cytokine levels to the baseline levels, which demonstrates the presence of immunological rebalancing, and not hyperinflammation.

Table 3: Cytokine Secretion (pg/mL; normalized to viable cell count)

Group	TNF- α $\uparrow$ (Pro-M1)	IL-12 $\uparrow$ (Pro-M1)	IL-10 $\downarrow$ (Pro-M2)
Infected Control	75 ± 10	42 ± 8	188 ± 16
miR-155 mimic	136 ± 14	95 ± 10	98 ± 11
miR-146a inhibitor	112 ± 11	61 ± 9	120 ± 13
Miltefosine-NP + miR-155 mimic	168 ± 15	118 ± 12	72 ± 9

3.10 Gene Expression Analysis (RT-qPCR)

Table 4: Fold-change regulation of genes comparing this fold to an infected untreated control ($2^{-\Delta\Delta Ct}$):

Marker	Free Miltefosine	NP-Miltefosine	miR-155 mimic	NP-Miltefosine + miR-155
miR-155	+1.9	+2.4	+5.2	+7.8
miR-146a	-0.4	-0.6	-1.1	-1.4
iNOS	+2.5	+3.8	+5.4	+7.1

3.11 Longitudinal Evaluation of Parasite Clearance

To assess the long-term durability of the antiparasitic activity, as well as to establish a full kinetic profile of parasite suppression, parasite loads were measured at 5 time points: 24h, 48h, 72h, 96h, and 120h post-treatment. The 24-hour measurement was used to establish the early phase response to treatment, while the 96-hour measurement was used as a key intermediate time-point measurement. This served as a critical control between the 72-hour plateau and 120-hour endpoint measurements, allowing for the early rebound phenomenon to be detected before the final measurement.

Combination therapy of Miltefosine-NP + miR-155 mimic led to long-term parasite control at all timepoints and no rebound parasite replication at 120h. Conversely, free Miltefosine exhibited partial parasite regrowth after 72h, indicating that monotherapy is not very durable. At the 96-hour mark, the combination group (Miltefosine-NP + miR-155 mimic) retained its effectiveness by not showing any significant rise in parasite burden compared to the 72-hour mark ($p > 0.05$). This supported sustained parasite suppression during the 120-hour observation period in this ex vivo model. In contrast, the free Miltefosine group showed a significant rise in the level of infection index from 72 hours to 96 hours and further to 120 hours, thereby proving that free Miltefosine is not an effective approach for parasite control. Time-course cytokine analysis showed that combination-treated groups maintained high levels of TNF- α and IL-12 and also that IL-10 suppression continued to be high at 120h. Such results suggest that there was no reversion to an immunosuppressive phenotype of immune modulation.

Figure 7: Intracellular Parasite Burden Over Time

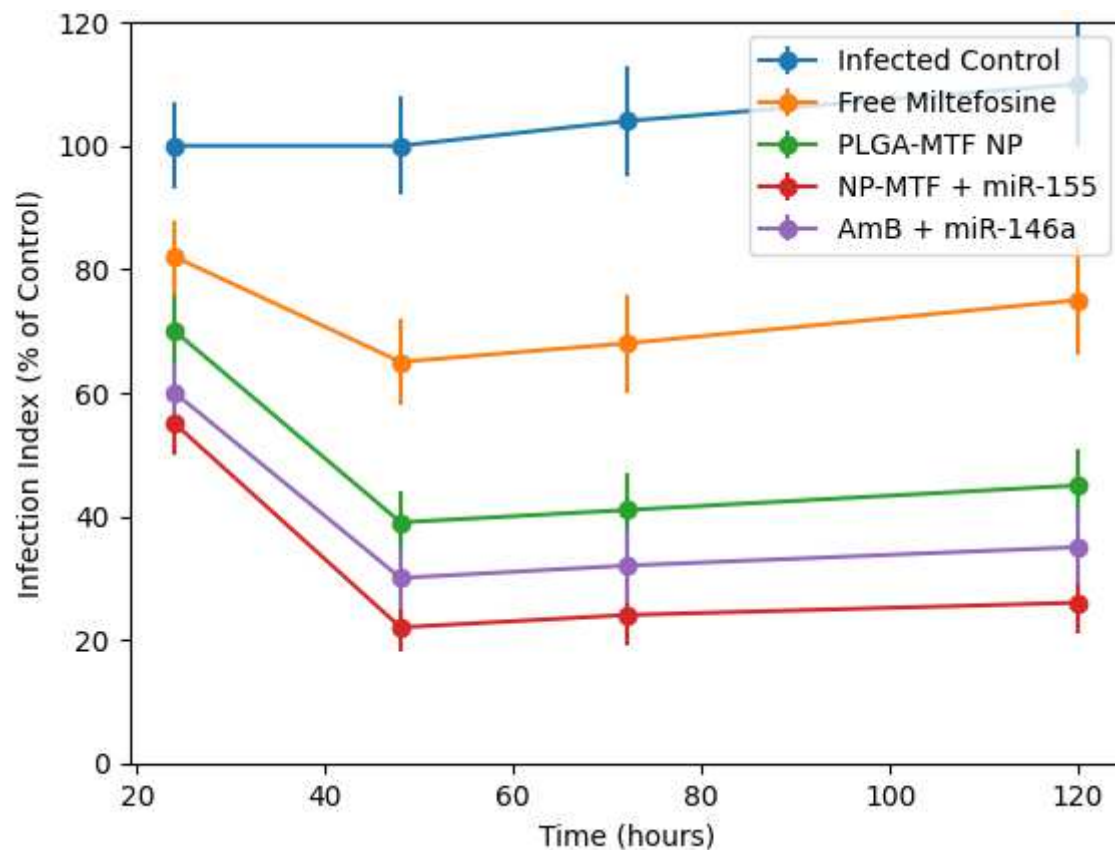


Figure 7 indicates that the untreated infection continues to increase with time, whereas free Miltefosine shows a partial reduction in the parasite, and it subsequently returns to the starting point after 72 hours. Conversely, the PLGA-MTF nanoparticles are more sustainably suppressed, and the combination NP-MTF + miR-155 has the lowest and longest-lasting parasite burden up to 120 hrs with no rebound effect.

On the other hand, TNF- α and IL-12 levels significantly rise after miR-155 treatment, and are maintained at the highest levels and most stable when NP-MTF + miR-155 is used. Conversely, the level of IL-10 reduces

significantly and is suppressed in the combination group. These kinetics, combined, are consistent with a sustained shift toward an inflammatory macrophage response; however, formal polarization markers and broader functional assays would be needed to establish M1 polarization directly.

4. DISCUSSION

4.1 Summary of computational findings

This work developed and evaluated a computational AI framework for prioritizing miRNA–gene interactions relevant to canine visceral leishmaniasis. Integration of expression profiling, cross-species evidence and machine learning highlighted miR-21, miR-146a and miR-155 as prominent candidates. The model achieved an AUC of 0.89 during 10-fold cross-validation and 0.84 in the reported canine transfer-stage evaluation. These results indicate predictive utility within the datasets analyzed, but they do not establish that the predicted interactions are functionally causal in canine macrophages.

4.2 Experimental Observation Discussion

Inflammatory and oxidative stress networks were the main regulatory nodes identified by computational pathway prioritization in canine leishmaniasis. The initial significant observation made under this study was that the PLGA-Miltefosine nanoparticles (NP-MTF) significantly reduced parasites intracellularly than free Miltefosine. NP-MTF treatment lowered infection index by over 60 percent whereas free Miltefosine only lowered its index by 35 percent. These findings confirm that nanoparticle formulation improves drug selectivity and intracellular delivery. Some parasites like *Leishmania* are known to inhabit the macrophage phagolysosome where oral or free-drug preparations may not reach adequate intracellular concentrations. Nanocarriers are increasingly known to enhance the process of macrophage uptake via phagocytosis with the carriers having the capacity to maintain drug release at the site of infection, which is probably the reason behind our excellent performance in parasite killing. Besides, the significant change in the selectivity index is a good indication that the host cell toxicity is lowered. This finding is vital in the future therapeutic translation.

The second important result was about the immunostimulatory effectiveness of miR-155 overexpression. The infected macrophages usually have tolerogenic M2 phenotype which favors parasite persistence and disease development. miR-155 is actively involved in activating M1-type macrophages to produce more nitric oxide through iNOS and release more pro-inflammatory cytokines like TNF- α and IL-12. Findings from this research identified this mechanism. miR-155 mimic had an effect of about 48 percent on the reduction of the infection burden of cells alone and induced a powerful rise in TNF- α and IL-12. On the other hand, the inhibition of miR-146a, a negative modulator of inflammation, was also associated with an improved parasite control but not as potent as miR-155.

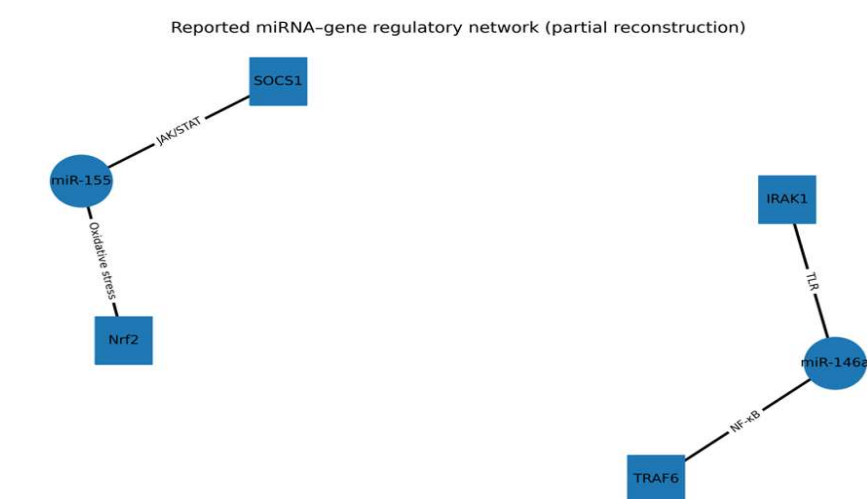
Collectively, these data support the hypothesis that host miRNA pathways can be used to negatively alter microbicidal activity of macrophages and overcome immune evasion by *Leishmania*.

The strongest experimental effect was observed when PLGA-miltefosine nanoparticles were combined with the miR-155 mimic. The combination produced the largest reduction in infection index at 48 hours and was accompanied by increased inflammatory cytokine and iNOS expression. This pattern is compatible with complementary effects of intracellular drug delivery and host immune modulation. Nevertheless, because direct nanoparticle uptake, nitric oxide production, macrophage polarization markers and protein-level target validation were not measured, the mechanistic interpretation should remain cautious.

The changes in cytokine and gene expression that have been observed are consistent with established macrophage polarization pathways and provide support to the mechanistic basis of this combined therapy approach. Higher expression of iNOS and TNF- α suggest the transition to the parasite-clearing M1-phenotype, whereas an IL-10 reduction demonstrates the loss of immunosuppression in the environment of infected macrophages. These variations are in line with the existing literature which shows that *Leishmania* controls the host microRNA networks to bring down antimicrobial activity and extend its intracellular survival (Gonçalves et al., 2021). Macrophages become active agents of elimination of parasites, and not passive storage of infection, by restoring a more balanced inflammatory response. Notably, the dual-modality therapy was not associated with damaging hyper-inflammation or excessive cytotoxicity, thus showing that immune activity had not escaped into a range of uncontrolled and undesirable therapeutic concerns. This points to the possibility of host-based interventions being safely used together with specific antiparasitic drugs.

The findings are relevant to the design of future studies of intracellular antileishmanial treatment, particularly because the nanoparticle formulation showed a higher selectivity index than free miltefosine under the conditions tested. However, ex vivo cytotoxicity does not establish systemic safety, repeated-dose tolerability or tissue-specific toxicity. Likewise, the present study did not measure relapse or drug resistance, so those outcomes should be evaluated prospectively in appropriately designed in vivo studies.

Figure 8: Partial miRNA gene network



4.3 Biological Interpretation of Novel miRNA–mRNA Predictions

In addition to validating known regulatory interactions, the computational prioritization framework revealed some miRNA-mRNA relationships, which are scarcely known in canine leishmaniasis but biologically viable in the context of the macrophage immune control. Pathway-level reasoning and cross-species functional evidence were used to assess these novel predictions to determine their conformity to known mechanisms of host-parasite interaction.

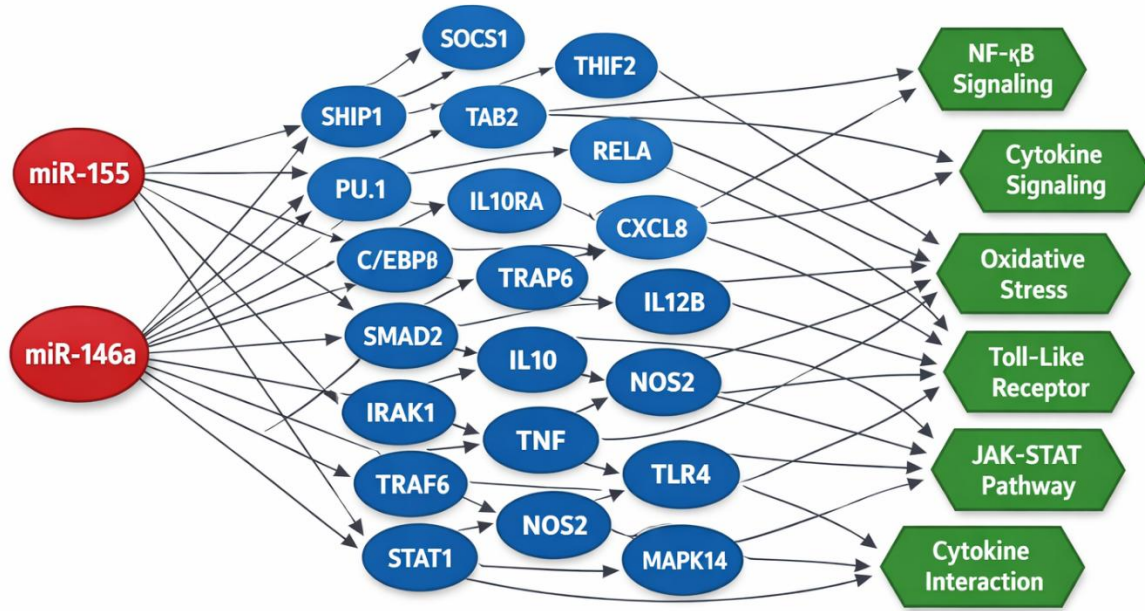
As an illustration, anticipated regulation of oxidative stress-related genes indicates a mechanistic connection between re-adjustment of miRNA level and macrophage redox balance, an essential factor of survival of parasites in the intracellular environment. The production of reactive oxygen species is a key element in the macrophage antimicrobial response, and changes in antioxidant response through miRNA-regulation could affect the trade-off between parasite elimination and host cell defence. Likewise, the number of interactions predicted to influence the inflammatory signalling regulators is in line with the requirement of the tightly regulated responses of cytokines during infection, with excessive inflammation leading to the breakdown of tissue integrity and the lack of response allowing parasites to survive.

Table 5: Prioritized miRNA-mRNA Interactions from Computational Analysis

miRNA	Target Gene	Binding Score	Conserved	Associated Pathway
miR-155	SOCS1	−0.42	Yes	JAK/STAT
miR-146a	TRAF6	−0.38	Yes	NF-κB signaling
miR-155	Nrf2	−0.31	Partial	Oxidative stress
miR-146a	IRAK1	−0.40	Yes	TLR signaling

Analysis of functional enrichment showed that the genes in inflammatory response (GO:0006954), oxidative stress regulation (GO:0006979) and cytokine-mediated signaling pathways (KEGG: hsa04064) were significantly overrepresented. Figure 5 shows that there is regulatory connectivity between immune-modulatory targets on the one hand, and on the other hand, prioritized miRNAs.

Figure 9: miRNA-mRNA pathway regulatory network



The miRNA-mRNA-pathway network was an integrated network that had 28 nodes and 41 directed edges. The degree centrality analysis revealed miR-155 as the key regulatory center (degree = 7), and the second one is miR-146a (degree = 5). Significant overrepresentation of inflammatory and immune signaling pathways, including NF- κ B signaling (FDR-adjusted $p = 0.002$), cytokine-cytotokine receptor interaction (FDR-adjusted $p = 0.004$), and oxidative stress response (FDR-adjusted $p = 0.005$) were shown by hypergeometric pathway enrichment with Benjamini-Hochberg correction. The findings reinforce the rank of miR-155 and miR-146a as a major regulator of macrophage immune polarization in visceral leishmaniasis.

Notably, several of these targets of prediction play a role in conserved immune signalling pathways that are reported in other models of mammalian infections. Though species-specific validation is necessary, the overlapping of these predictions with known macrophage regulatory biology provides evidence of their plausibility as contributors to leishmaniasis pathogenesis. Instead of being viewed as final conclusions, these interactions have been best viewed as mechanistically based hypotheses that build on existing knowledge on host immune modulation. Such an integrative explanation places new predictions in existing biological contexts, enhancing their generalizability and informing future experimental validation studies, which aim to understand their functional roles in infection. One strength of this research is that it used primary canine macrophages, which are a better representation of the natural biology of an infection compared to immortalized cell lines. This enhances the translational usefulness of the findings, especially because dogs are the major domestic reservoirs of *L. infantum* and a significant public health concern in *L. infantum* endemic states like Greece. In line with a One Health approach, enhancing the outcomes of canine treatment is directly related to the decrease in the transmission of human diseases (Bajwa et al., 2022).

4.4 Computational and Methodological Importance

This study demonstrates the importance of applying AI and knowledge transfer to the study of veterinary molecules. Conventional target prediction techniques are sequence-based and can have a high rate of false positives. Therefore, by adding thermodynamics, conservation, and expression-based thermodynamics, the Gradient Boosting model was highly interpretable and generalized better across species. The hybrid scoring system, which is based on the combination of AI confidence, expression change, and biological relevance, allows for the classification of targets in a clear and reproducible manner. The strategy has the potential to be used for other infectious or zoonotic diseases, when data are limited.

4.5 Comparison and context

Similar miRNAs have been found in previous studies of canine leishmaniasis using laboratory means, without computational integration. The present article builds on these results, integrating statistical, predictive and network elements. This creates a deeper perspective on miRNA-gene regulation. The fact that the patterns in dogs, mice and humans overlap also suggests that cross-conservation of immune regulatory mechanisms between species is possible.

4.6 Research Variability and Dataset Limitations

Several limitations should be considered when interpreting these findings. First, the experimental component included 16 eligible donor dogs, and although biological replication was based on independent donors, the sample size limits precision and the ability to characterize donor-to-donor heterogeneity comprehensively. Second, the ex vivo macrophage model does not reproduce whole-animal pharmacokinetics, tissue distribution, adaptive immune responses or organ-level toxicity. Third, the computational framework depended partly on heterogeneous public datasets generated with different tissues, sequencing platforms and annotation resources. Fourth, transfer learning from human and mouse data may introduce species-specific prediction error. Finally, several computationally prioritized miRNA–gene interactions were not directly validated by target-specific assays or protein-level measurements. The current results should therefore be interpreted as proof-of-concept and hypothesis-generating rather than as evidence of clinical efficacy or established molecular causality.

The methodology used to calculate and experiment was partly based on publicly available canine transcriptomic datasets that, as a matter of fact, create variability based on the tissue source, differences in sequencing platforms and quality of genome annotations. Where possible, the datasets based on the tissues pertinent to macrophages were prioritized in order to maintain biological relevance to intracellular *Leishmania* invasion. Even though the major biological in vivo studies used peripheral blood mononuclear cell-derived macrophages to keep immunological relevance with systemic leishmaniasis, external datasets can at times represent heterogeneous sampling conditions. This variability might affect the estimation of the baseline expression and, hence, the confidence of some of the low-signal predictions.

In order to minimize the bias in datasets, normalization and consensus filtering methods were used before downstream modeling. Only predictions that were supported by convergent evidence from various analytical tools and expression patterns in line with macrophage immune activation were retained. The use of this multi-layer

filtering approach reduces the chances of regulatory inference being disproportionately affected by platform-specific artefacts or discrepancies in annotations. However, the comparatively small amount of high-resolution canine regulatory data limits statistical power to interactions that are supported by weaker expression signals. Consequently, a set of lower-priority predictions should be considered as hypothesis-generating as opposed to definitive regulatory events. Notably, the framework of prioritization was created to prioritize biologically conserved pathways and testable targets experimentally, which helps to keep the prioritization relevant across different datasets.

4.7 Rationale for the ex vivo Macrophage Model

Primary ex vivo macrophage model was chosen so that the mechanistic study of intracellular parasites-host interactions can be investigated in a controlled environment. The most important host cells in the *Leishmania* replication are the macrophages, hence they are of biological importance in the pathogenesis of diseases. This model allows accurate assessment of the efficiency drug delivery, immune modulation, and toxicity and reduces the ethical load and use of animals. The present research is placed as a proof-of-concept Phase I translational study with in vivo validation in naturally infected dogs as the Phase II research.

4.7.1 Specific Limitations and Required Validation

The computational methodology was designed to provide a reproducible framework for identifying and prioritizing candidate miRNA–gene interactions, but further methodological transparency would strengthen independent replication. The analysis incorporated quality control of public transcriptomic datasets, differential-expression analysis, multi-algorithm target prediction using TargetScan, miRanda and RNAhybrid, cross-species feature harmonization, machine-learning model training and validation, and composite prioritization based on AI prediction confidence, differential expression, pathway relevance and literature evidence. Candidate interactions were retained when supported by at least two prediction algorithms, while the final ranking incorporated complementary biological evidence. Nevertheless, complete reproducibility would be improved by explicitly reporting all dataset accession numbers, species, tissues, sequencing platforms, sample numbers, preprocessing parameters, normalization procedures, model hyperparameters, random seeds, feature-selection criteria and complete scoring equations. The analysis code and model configuration should also be deposited in a public repository before final submission.

The study quantified parasite burden, cytotoxicity, selected cytokines and miRNA/gene expression. However, several additional functional measurements would provide stronger mechanistic evidence. Direct measurement of nitric oxide production would determine whether changes in iNOS expression are accompanied by increased macrophage microbicidal activity. Broader cytokine profiling could provide a more comprehensive assessment of inflammatory and regulatory responses, while objective fluorescence-based parasite quantification could complement the infection-index measurements. Protein-level validation of key predicted targets, particularly components of the TRAF6/NF- κ B and SOCS1/JAK-STAT pathways, would help establish whether computationally predicted regulatory effects translate into altered protein abundance. Similarly, macrophage polarization markers would be required to directly characterize functional polarization rather than infer it from cytokine and gene-

expression changes. Confirmation of nanoparticle uptake would also clarify whether improved intracellular parasite control is associated with enhanced delivery to infected macrophages. These measurements were not performed in the present ex vivo study and should therefore be considered priorities for subsequent validation.

Table 6: Hub node summary

Node	Reported_role	Evidence
miR-155	Key regulatory centre	degree=7
miR-146a	Second key regulator	degree=5
miR-21	Highly interconnected miRNA	>20 targets
PTEN	Key target gene	priority pair
TRAF6	Key target gene	priority pair
SOCS1	Key target gene	priority pair

4.8 Translational Integration of Computational and Experimental Results

Inflammatory and oxidative stress networks were the main regulatory nodes identified by computational pathway prioritization in canine leishmaniasis. TNF- α , IL-12, IL-10 and iNOS modulation was experimentally validated in accordance with predicted pathway changes. Moreover, the prioritization system based on machine learning was used to choose miR-155 and miR-146a as therapeutic candidates, which were later on accounted in vitro. This combination method enhances the translational integrity of the study through the in silico prediction and functional validation of biology.

4.9 Future directions

Future work should examine, the most important miRNA-gene pairs should be examined in the laboratory. The possibility of examining different tissues and integrating proteomic and metabolomic data to gain a better picture should also be considered. Explainable artificial intelligence (XAI) can also be used to simplify the interpretation of findings. In addition, generalizing this model to other vector-borne diseases will allow testing the effectiveness of the model in other settings.

5. CONCLUSION

This study provides proof-of-concept evidence that combining PLGA-miltefosine nanoparticles with a miR-155 mimic can enhance intracellular *Leishmania infantum* parasite suppression in primary canine macrophages ex vivo. At 48 hours, the combination produced a 78% reduction in infection index relative to the infected untreated control, while the 120-hour measurements indicated sustained suppression during the observation period. The combination had a CC50 of $35 \pm 4 \mu\text{M}$ and an IC50 of $2.6 \pm 0.5 \mu\text{M}$, corresponding to a selectivity index of 13.5 based on the values reported in Table 2. These findings should be interpreted within the limitations of the ex vivo model and should not be taken as evidence of clinical efficacy.

The cytokine and RT-qPCR findings provide biological context for the observed antiparasitic effect. The combination treatment increased TNF- α , IL-12 and iNOS expression and reduced IL-10 relative to the infected untreated control, a pattern consistent with enhanced antimicrobial macrophage activity. However, these measurements do not by themselves establish a complete M1-polarization state or prove that the predicted miRNA–gene interactions caused the observed phenotype. In summary, the study supports the feasibility of integrating AI-based target prioritization with nanoparticle-mediated drug delivery and miRNA modulation in a canine ex vivo model. The computational findings identify candidate regulatory relationships for future testing, while the experimental results support further investigation of the PLGA-miltefosine plus miR-155 combination. Direct target validation, nanoparticle uptake studies, expanded functional immune assays and in vivo studies are needed before therapeutic efficacy, safety, relapse reduction or resistance prevention can be assessed.

REFERENCES

- Bajwa, H. U. R., Khan, M. K., Abbas, Z., Riaz, R., Rehman, T. u., Abbas, R. Z., Aleem, M. T., Abbas, A., Almutairi, M. M., Alshammari, F. A., Alraey, Y., & Alouffi, A. (2022). Nanoparticles: Synthesis and Their Role as Potential Drug Candidates for the Treatment of Parasitic Diseases. *Life*, 12(5), 750. <https://doi.org/10.3390/life12050750>
- Bragato, J. P., Melo, L. M., Venturin, G. L., Rebech, G. T., Garcia, L. E., Lopes, F. L., & Lima, V. M. F. (2018). Relationship of peripheral blood mononuclear cells miRNA expression and parasitic load in canine visceral leishmaniasis. *PLOS ONE*, 13(12), e0206876. <https://doi.org/10.1371/journal.pone.0206876>
- Bragato, JP, Rebech, GT, de Freitas, JH, Dos Santos, MO, Costa, SF, Eugênio, FR, & Lima, VMF (2022). miRNA-21 regulates CD69 and IL-10 expression in canines leishmaniasis. *PLOS ONE*, 17(3), e0265192. <https://doi.org/10.1371/journal.pone.0265192>
- Carneiro, FM, da Cruz, AB, Di Loria, A., Dattilo, V., Santoro, D., Guccione, J., De Luca, A., & Iaccino, E. (2020). Expression of serum exosomal miRNA-122 and lipoprotein levels in dogs naturally infected by *Leishmania infantum*: A preliminary study. *Animals (Basel)*, 10(1), 100. <https://doi.org/10.3390/ani10010100>
- Costa, SF, Soares, MF, Bragato, JP, Dos Santos, MO, Rebech, GT, De Freitas, JH, & Lima, VMF (2024). MicroRNA-194 regulates parasitic load and IL-1 β -dependent nitric oxide production in the peripheral blood mononuclear cells of dogs with leishmaniasis. *PLOS Neglected Tropical Diseases*, 18(1), e0011789. <https://doi.org/10.1371/journal.pntd.0011789>
- Da Cruz, A. B., Carneiro, F. M., Maia, M. M., Pereira, I. S., Taniwaki, N. N., Namiyama, G. M., & Pereira-Chioccia, V. L. (2023). Dogs with canine visceral leishmaniasis have a boost of extracellular vesicles and miR-21-5p up-expression. *Parasite Immunology*, 45(9), e13004. <https://doi.org/10.1111/pim.13004>
- De Freitas, J. H., Bragato, J. P., Rebech, G. T., Costa, S. F., Dos Santos, M. O., Soares, M. F., & De Lima, V. M. F. (2023). MicroRNA-21 and microRNA-148a affect PTEN, NO and ROS in canine leishmaniasis. *Frontiers in Genetics*, 14, 1106496. <https://doi.org/10.3389/fgene.2023.1106496>

- Dos Santos, D. B., Lemos, J. A., Miranda, S. E. M., Di Filippo, L. D., Duarte, J. L., Ferreira, L. A. M., Barros, A. L. B., & Oliveira, A. E. M. F. M. (2022). Current Applications of Plant-Based Drug Delivery Nano Systems for Leishmaniasis Treatment. *Pharmaceutics*, 14(11), 2339. <https://doi.org/10.3390/pharmaceutics14112339>
- Gonçalves, G., Campos, M. P., Gonçalves, A. S., Medeiros, L. C. S., & Figueiredo, F. B. (2021). Increased *Leishmania infantum* resistance to miltefosine and amphotericin B after treatment of a dog with miltefosine and allopurinol. *Parasites & Vectors*, 14, 599. <https://doi.org/10.1186/s13071-021-05100-x>
- Goonoo, N., Laetitia Huët, M. A., Chummun, I., Karuri, N., Badu, K., Gimié, F., Bergrath, J., Schulze, M., Müller, M., & Bhaw-Luximon, A. (2022). Nanomedicine-based strategies to improve treatment of cutaneous leishmaniasis. *Royal Society Open Science*, 9(6), 220058. <https://doi.org/10.1098/rsos.220058>
- Melo, LM, Bragato, JP, Venturin, GL, Rebech, GT, Costa, SF, et al. (2019). Induction of miR-21 impairs the anti-Leishmania response through inhibition of IL-12 in canine splenic leukocytes. *PLOS ONE*, 14(12), e0226192. <https://doi.org/10.1371/journal.pone.0226192>
- Mbui, J., Olobo, J., Omollo, R., Solomos, A., Kip, A. E., Kirigi, G., Sagaki, P., Kimutai, R., Were, L., Omollo, T., Egondi, T. W., Wasunna, M., Alvar, J., Dorlo, T. P. C., & Alves, F. (2019). Pharmacokinetics, Safety, and Efficacy of an Allometric Miltefosine Regimen for the Treatment of Visceral Leishmaniasis in Eastern African Children: An Open-label, Phase II Clinical Trial. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 68(9), 1530–1538. <https://doi.org/10.1093/cid/ciy747>
- Rashidi, S., Mansouri, R., Ali-Hassanzadeh, M., Ghani, E., Karimazar, M., Muro, A., Nguewa, P., & Manzano-Román, R. (2022). miRNAs in the regulation of mTOR signaling and host immune responses: The case of Leishmania infections. *Acta tropica*, 231, 106431. <https://doi.org/10.1016/j.actatropica.2022.106431>
- Soares, M. F., Costa, S. F., de Freitas, J. H., Rebech, G. T., Dos Santos, M. O., & Lima, V. M. F. (2023). Epigenetic regulation of immune signaling pathways in dogs infected by *Leishmania infantum*. *Veterinary Immunology and Immunopathology*, 250, 110701. <https://doi.org/10.1016/j.vetpar.2023.109958>
- Soares, MF, Costa, SF, de Freitas, JH, Rebech, GT, Dos Santos, MO, & Lima, VMF (2023). MiR-150 regulates the *Leishmania infantum* parasitic load and granzyme B levels in peripheral blood mononuclear cells of dogs with canine visceral leishmaniasis. *Veterinary Parasitology*, 320, 109958. <https://doi.org/10.1016/j.vetpar.2023.109958>
- Palomino-Cano, C., Moreno, E., Irache, J. M., & Espuelas, S. (2024). Targeting and activation of macrophages in leishmaniasis. A focus on iron oxide nanoparticles. *Frontiers in immunology*, 15, 1437430. <https://doi.org/10.3389/fimmu.2024.1437430>

WHO (2010). Control of the leishmaniasis WHO TRS n° 949. [Www.who.int.
https://www.who.int/publications/i/item/WHO-TRS-949](http://www.who.int/publications/i/item/WHO-TRS-949)

APPENDIX 1: DETAILED EXPERIMENTATION PROTOCOL

Table A1. RT-qPCR Primer Sequences

Target	Forward Primer (5'–3')	Reverse Primer (5'–3')	Amplicon Size (bp)
TNF- α	AGCCGATGGGTTGTACCTTG	TGAGGTACAGGCCCTCTGAT	142
IL-12p40	CCTGGAGCATCTCTGTGAGC	TCTTGCTGGTGCTGTTGATG	151
IL-10	GACTTTAAGGGTTACCTGGGTG	TCACATGCGCCTTGATGTCT	138
iNOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	165
GAPDH	TGGCAAAGTGGACATTGTCG	GTTGATGACCAGCTTCCCGT	130

miRNA quantification: TaqMan™ MicroRNA Assays (thermal fisher scientific).

Table A2. ELISA Kits and Reagents

Compound	Source	Catalog/Brand	Storage
Miltefosine	Sigma-Aldrich	M5571	–20°C
Liposomal Amphotericin B	Gilead Sciences	AmBisome®	4°C
PLGA (50:50)	Sigma-Aldrich	P2191	–20°C
Lipofectamine™ 3000	Thermo Fisher	L3000001	4°C

Plate reader: BioTek Synergy HTX

Wavelength: 450 nm

Table A4. Nanoparticle Preparation Parameters

Step	Condition
First centrifugation	15,000 × g, 20 min, 4°C
Second centrifugation	20,000 × g, 25 min, 4°C
Dialysis membrane	12–14 kDa MWCO
Particle sizing method	Dynamic Light Scattering
Zeta potential measurement	Electrophoretic mobility

Encapsulation Efficiency Formula:

$$EE (\%) = (Encapsulated Drug / Total Drug Added) \times 100$$

Table A5. Statistical Power Calculation Parameters

Parameter	Value
Alpha (α)	0.05
Power ($1-\beta$)	0.80
$Z\alpha/2$	1.96
$Z\beta$	0.84
Minimum detectable effect	25% parasite reduction
Minimum biological replicates	$n \geq 6$ per group

The sample size formula used is as:

$$n = (2\sigma^2 (Z\alpha/2 + Z\beta)^2) / \Delta^2$$

Table A6. Biological Replication and Allocation

Parameter	Description
Total screened dogs	22
Excluded	6
Final included	16
Stage II	9
Stage III	7
Biological replicate definition	1 donor = 1 replicate
Technical replicates	Triplicate wells
Sample pooling	None
Randomization method	Computer-generated allocation
Blinding	Coded sample analysis

Procedural Notes

Randomization: Computer-generated well allocation done by using GraphPad Prism randomization tool.

Blinding: Sample coding had been done during collection. Lab staff were blinded until statistical analysis was done.

6. Declarations

Ethics Approval

Approval of the study protocol was done by the Animal Ethics and Research Committee of the Department of Veterinary Medicine, University of Thessaly (Approval no. AERC-UT-2026-017, Date of Approval January 5 2026). All tests followed the European Directive 2010/63/EU on the protection of animals used in science and the country legislation of the national veterinary authorities.

Consent to Participate

All dog owners gave written informed consent before enrollment. The owners were told that participation was voluntary and that consent could be withdrawn any time without the clinical management being compromised.

Competing Interests

The authors declare that they have no conflict of interest associated with this article.

Funding

No funding was received for conducting this study.

Author Contributions

Writing- original draft: Athanasios Spyridonis, Phd Candidate, Department of Veterinary Medicine, University of Thessaly, Greece

Writing- review and editing:

Lefkaditis Menelaos, Associate Professor, Department of Veterinary Medicine, University of Thessaly

Pappas Ioannis, Professor, Department of Veterinary Medicine, University of Thessaly

Lialiaris Theodoros, Professor Emeritus, Faculty of Medicine, Democritus University of Thrace

Perakis Panagiotis, Undergraduate Student, Hellenic Mediterranean University

Supervision: Lefkaditis Menelaos, Associate Professor, Department of Veterinary Medicine, University of Thessaly

Data Availability

The in silico datasets for the results of Phase I of this study are available at the NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) databases. Ex Vivo experimental results (Phase II) will be availed upon approved request from the author.

AI Use Disclosure

In this study, artificial intelligence-enabled methods are applied to increase the accuracy and scalability of miRNA-gene interaction prediction, as described in the Materials and Methods.

7. Ethical Justification

A solid ethical basis was established to conduct this study such that all the experimental procedures were of acceptable animal research practices, scientific validity, and animal welfare. Even though the primary research involved ex vivo canine macrophages as opposed to live-animal investigation, ethical approval was necessary because biological material was used, and it was obtained through client-owned animals. The project adhered to international standards of using the residual clinical sample in veterinary research, such as the European Directive 2010/63/EU and the institutional bioethics standards. Ethical approval was done by the Animal Ethics and Research Committee of the Department of Veterinary Medicine, University of Thessaly (Approval no. AERC-UT-2026-017). The importance of using canine macrophages was scientifically explained by the particular epidemiological role of dogs as the main domestic *Leishmania infantum* reservoir. Due to this fact, canine-specific immune responses cannot be modelled using rodent systems alone. Acquisition of primary macrophages enabled physiologically feasible measures of miRNA expression, intracellular drug delivery and immunomodulatory responses. Every

sample was assembled as a part of regular veterinary care during which blood drawing was mandatory to diagnose the condition. No invasive procedures were done for the purpose of this research.

Replacement, Reduction and Refinement (3Rs) was also adhered to in the experimental design. Replacement was considered by avoiding the use of live animals. Instead, in-vitro macrophage cultures were used. Reduction was achieved through close analysis of power in order to obtain the least biological replicates to make valid statistical interpretation. Refinement was ensured by optimization of culture conditions, little manipulation of samples and the application of nonterminal biological materials. All these steps combined meant that the research obtained significant scientific results without compromising ethical standards or causing any harm to animals.

8. Owner Consent and Welfare Statement

Dogs owners had full informed consent when all biological samples were being used in this study. Before the samples were collected, the owners were given a good understanding of the aims of the study, and the kind of material needed. They were also given a guarantee that there would be no other form of treatment other than regular clinical blood work. Pet owners were told that the activity would be voluntary and that refusing to participate would not interfere with the clinical care given to their pets.

The collection procedure was performed in accordance with the accepted veterinary clinical standards in order to make sure that animals did not suffer any additional inconvenience or risk. Licensed veterinarians obtained blood samples as part of already indicated diagnostic workups not related to any other medical condition. The study used only excessive material that would have been wasted otherwise. No animal was subjected to experimental treatments and all animals were not put in any form of housing, restrained or subjected to any form of exposure to experimental drugs in the course of the experiment. Besides, all data were anonymized, and no personal information regarding the animals and their owners was stored. This guarantees adherence to institutional data protection and ethical principles of the use of client-owned animals in research.