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JÉSSICA HENRIQUE DE FREITAS

MicroRNA-21 e microRNA-148a regulam PTEN, NO e ROS na leishmaniose canina

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Tese apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Ciência Animal

Orientadora: Profa. Dra. Valéria Marçal Felix de Lima

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“Entregue seu caminho ao Senhor;

confie nele, e ele agirá”

Salmos 37:5

FREITAS, J. H. **MicroRNA-21 e microRNA-148a regulam PTEN, NO e ROS na leishmaniose canina.** 2023. 97 f. Tese (Doutorado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2023.

RESUMO

A leishmaniose visceral canina (CanL) representa uma grave ameaça à saúde pública em vários países. A progressão da doença depende do grau de supressão da resposta imune. Os microRNAs (miRs) modulam a tradução do mRNA em proteínas e regulam várias funções celulares e vias associadas às respostas imunes. MiR-21 e miR-148a podem alterar a carga parasitária e os macrófagos M1 são as principais células na atividade leishmanicida de cães. Um estudo anterior encontrou aumento de miR-21 e miR-148a em leucócitos esplênicos (LE) de cães com CanL usando a análise de microarranjo, e a análise in *silico* identificou alvos da via PTEN. PTEN está envolvido na regulação imune de macrófagos. Medimos PTEN e a produção de espécies reativas de oxigênio (ROS) e óxido nítrico (NO) após transfecção com mimic e inhibitor de miR-21 e miR-148a em LE de cães com CanL. Os níveis de PTEN aumentaram, NO e ROS diminuíram em LEs de cães com CanL. A inibição do miRNA-21 resultou em aumentos de PTEN; em contraste, o PTEN diminuiu após a inibição do miR-148a. Os níveis de nitrito aumentaram após a transfecção com o inibidor de miR-21, mas diminuíram com o inibidor de miR-148a. O aumento de miR-21 promoveu redução nos níveis de ROS e NO. Esses achados sugerem que miR-21 e miR-148a modulam a resposta imune em cães com CanL por meio de PTEN, NO e ROS.

Palavras-chave: microRNA. ROS. NO. PTEN. Nitrito. *Leishmania infantum*. Leucócitos esplênicos.

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ABSTRACT

Canine Visceral leishmaniasis (CanL) poses a severe public health threat in several countries. Disease progression depends on the degree of immune response suppression. MicroRNAs (miRs) modulate mRNA translation into proteins and regulate various cellular functions and pathways associated with immune responses. MiR-21 and miR-148a can alter the parasite load and M1 macrophages are the principal cells in dog's leishmanicidal activity. A previous study found increased miR-21 and miR-148a in splenic leukocytes (SL) of dogs with CanL using microarray analysis and in silico analysis, identified PTEN pathway targets. PTEN is involved in the immune regulation of macrophages. We measured PTEN and the production of reactive oxygen species (ROS) and nitric oxide (NO) after transfection SL of dogs with CanL with mimic and inhibition of miR-21 and miR-148a. PTEN levels increased, NO and ROS decreased in SLs from dogs with CanL. Inhibition of miRNA-21 resulted in PTEN increases; in contrast, PTEN decreased after miR-148a inhibition. Nitrite levels increased after transfection with miR-21 inhibitor but were decreased with miR-148a inhibitor. The increase in miR-21 promoted a reduction in ROS and NO levels. These findings suggest that miR-21 and miR-148a modulate the immune response in dogs with CanL through PTEN, NO, and ROS.

Keywords: microRNA. ROS. NO. PTEN. Nitrite. *Leishmania infantum*. Splenic leukocytes.

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 Fatores Epidemiológicos da LV

A Leishmaniose Visceral (LV) é uma doença distribuída em diversos continentes e a maior incidência dessa doença estão localizadas em países como Índia, Bangladesh, Brasil, Sudão e Etiópia (ALVAR *et al*., 2012). Trata-se de uma doença zoonótica que apresenta como vetor um flebotomíneo e que pode transmitir algumas espécies de parasitas, como a *Leishmania infantum*. O parasito é capaz de se apresentar em duas formas, sendo a forma promastigota e a amastigota (ALCOLEA *et al*., 2016). Essa doença tem grande destaque em sua expansão e apresenta relevância para o cenário público de saúde em diversos lugares do mundo. Portanto, é fundamental o diagnóstico clínico precoce com base na perspectiva epidemiológica da região acometida e diagnósticos por testes moleculares são um dos métodos de confirmação (ALBUQUERQUE *et al*., 2017).

Os cães infectados podem desenvolver quadros assintomáticos e sintomáticos (MORENO; ALVAR, 2002). Exames laboratoriais são importantes para a confirmação da doença e para identificação da severidade do caso, alterações podem ser observadas em níveis de concentração de enzimas hepáticas, além do quadro clínico de hipoalbuminemia, hiperglobulinemia e anemia (CIARAMELLA *et al*., 1997). Os sintomas que podem surgir são hiporexia, linfoadenopatia, onicogrifose, alopecia, descamação da pele e entre outros. Com o avanço da doença torna-se necessário uma constante vigilância epidemiológica de áreas com o foco da doença, sendo através de testes sorológicos e de inquéritos entomológicos (SAKAMOTO *et al*., 2007). E existem meios de prevenção para minimizar a expansão de novas infecções através da vacinação em cães (SOLANO-GALLEGO *et al*., 2017). O uso de coleiras impregnadas de inseticidas ou dispositivos com imunoestimuladores e a quimioterapia em animais infectados podem também contribuir para o controle da doença (REGUERA *et al*., 2016).

No continente americano, existem 12 países endêmicos e entre o período de 2001-2017 foram relatados o total de 59.769 casos de Leishmaniose visceral em humanos (OMS, 2019). Em alguns países africanos os casos de leishmaniose visceral

podem se agravar pela coinfecção pelo vírus HIV ou se o houver histórico de doenças crônicas (KONE *et al.*, 2019).

A origem da Leishmaniose no Brasil começou na região nordeste e depois se dispersou para as demais regiões no país (MAIA-ELKHOURY *et al*., 2008). E alguns países próximos como Bolívia, Paraguai e Argentina podem ter contribuído para a expansão. A expansão dos vetores têm aumentado em regiões como São Paulo, Minas Gerais, Santa Catarina e Rio Grande do Sul. Diante desse cenário atual, novos estudos e alternativas que contribuem para o controle dessa dispersão é essencial (PASQUALI *et al*., 2019). Um outro fator determinante para a proliferação seria a facilidade de adaptação do vetor *Lutzomiya longipalpis* em regiões urbanas que são diferentes ao meio ambiente de origem e a dificuldade de encontrar os focos de criação do vetor (MAIA-ELKHOURY *et al*., 2008).

O parasito apresenta dois estágios de vida, sendo uma das fases conhecida como promastigota, conhecida como a forma anterior ao processo de fagocitose pelas células de defesa. E a outra fase é a amastigota, no qual reside no interior de macrófagos (LIU; UZONNA, 2012).

A *Leishmania* é caracterizada como um parasito intracelular e sua replicação acontece no interior de macrófagos. Após a infecção, os macrófagos passam a produzir substâncias como as espécies reativas de oxigênio (ROS) como forma de defesa (LIMA-JUNIOR *et al*., 2017). Outras células como os eosinófilos, também já foram encontrados níveis elevados de ROS e óxido nítrico (NO) em cães infectados com a *L. infantum* (COSTA *et al*. 2018). Os neutrófilos polimorfonucleares são os primeiros a serem recrutados e a realizar o processo de fagocitose quando se inicia a infecção (PETERS *et al*., 2008) porém os macrófagos são as células que hospedam de forma definitiva o parasito (PEREIRA *et al*., 2019).

O diagnóstico pode ser feito por meio de testes sorológicos, histopatologia, identificação por microscopia ou testes moleculares, como o PCR que apresenta alta sensibilidade e utiliza o DNA do parasito. É recomendado realizar mais de um teste para confirmação do diagnóstico e também a identificação da espécie para constatar qual tipo de tratamento é o mais indicado ao paciente (ARONSON *et al*., 2017).

No Brasil, os exames mais comuns são para o diagnóstico canino são o TR-DPP®, testes moleculares, sorológicos e parasitológicos. Apesar do amplo uso desses testes, existem limitações e ocorrências de falso-positivo (PESSOA-E-SILVA *et al*., 2019), Portanto, é importante apresentar um bom critério de escolha dos testes

para diagnosticar essa doença e também de novas ferramentas para melhores resultados.

1.2 Resposta imunológica na LV

1.2.1 Resposta Inata

Células como os macrófagos são essenciais para combater o parasito *Leishmania* na resposta imune inata. Porém, esse parasito desenvolveu um mecanismo de defesa capaz de se hospedar dentro dessas células e sobreviver em um ambiente hostil (GUPTA; OGHUMU; SATOSKAR, 2013). Assim como os macrófagos, os neutrófilos também atuam no papel de células hospedeiras da *Leishmania* na fase inicial e as células Natural Killer (NK) podem aparecer após 24 horas da infecção (MÜLLER *et al*., 2001). Os neutrófilos participam na defesa do organismo e através de processos de estresse oxidativo, apoptose e no aumento da atividade da enzima mieloperoxidase, sendo essa enzima importante na produção de espécies reativas de oxigênio (OUALHA *et al*., 2019).

O parasito quando está dentro do organismo do hospedeiro pode entrar em contato com o sistema complemento logo na fase inicial. Após a ativação do complemento, o complexo de ataque à membrana (MAC) C5b-C9 irá contribuir na lise do parasito. Porém, a *Leishmania* tem a capacidade de inibir a atividade da MAC através do Fator de Virulência lipofosfoglicano (LPG) que atua na superfície da *Leishmania*. Além disso, a inativação de C3b e do Complexo MAC também ocorre pela ação da molécula GP63 que fica localizada na superfície do parasito (GURUNG; KANNEGANTI, 2015)

A *Leishmania* para conseguir se manter no hospedeiro é capaz de modular a resposta imune no hospedeiro. O parasito utiliza como alternativa a mudança na produção substâncias antimicrobianas, tais como óxido nítrico. E com isso, a resposta imune inata ficará prejudicada e assim ocorrerá a progressão da doença (OLIVIER; GREGORY; FORGET, 2005). Cães infectados podem expressar níveis altos de IFN-ɣ em locais de maior proliferação, levando a ativação da resposta Th1 que responde contra a infecção. E o aumento de TGF-β no linfonodo de cães, pode ser um indicativo da atuação de uma resposta imune reguladora (SANTOS *et al*., 2019).

Os inflamassomas são compostos por complexos proteicos que são ativados na presença do patógeno (SHIO *et al*., 2015). Esse complexo proteico é formado por três componentes como: molécula adaptadora, caspase inflamatória e uma proteína sensora (ZAMBONI; LIMA-JUNIOR, 2015). Na presença do parasito, a proteína NLRP3 é ativada em resposta à infecção contribuindo na inibição da replicação da *Leishmania* (MENDONÇA *et al*., 2020) Inflamassomas são capazes de controlar a maturação e aumentar os níveis de IL-1β e IL-18, desencadeia o processo inflamatório celular para conter o avanço da doença e promover a piroptose (SCHRODER; TSCHOPP, 2010). A ativação e o reconhecimento dos inflamassomas também estão presentes em infecções de parasitas intracelulares como *Trypanosoma cruzi*, *Plasmodium spp.* e a *Leishmania* (ZAMBONI; LIMA-JUNIOR, 2015).

A expressão de Metaloprotease GP63 é um fator de virulência de espécies do grupo de *Leishmania* e que pode contribuir para inibição da função de macrófagos, e com isso também resultará na diminuição da concentração da citocina IL-1β e na clivação dos componentes do inflamassoma NLRP3. Essa redução inibi a disponibilidade de espécies reativas de oxigênio (ROS) (SHIO *et al*., 2015) que é gerado a partir de NADPH oxidase (LAMBETH, 2004). Além dos macrófagos, os eosinófilos também desempenham um papel importante no controle da leishmaniose através da produção de ROS e NO (COSTA *et al*., 2018).

A polarização de macrófagos para o perfil M1 está relacionada com a geração de substâncias como ROS, NO, IL-12, IL-1β, TNF-α, NADPH oxidase-1 e iNOS que diminuem a carga parasitária. Enquanto a polarização para o perfil M2 pode levar ao aumento da expressão de marcadores como IL-10, CD163, CD206, TGF-β e arginase-1, contribuindo na sobrevivência da *Leishmania* (KUMAR *et al*., 2018). O parasito do gênero *L. donovani* pode exercer influência na inibição da ativação de NF-kB, prejudicar a produção de ROS e interferir negativamente na ativação de inflamassomas NLRP3. E dessa forma, ocorrer a progressão da doença (GUPTA *et al*., 2017).

Durante a infecção pela *Leishmania donovani*, há o importante processo da explosão oxidativa de macrófagos em resposta à fagocitose e levando a produção de ROS (SAHA *et al*., 2019), mas o parasito tenta se adaptar às diferentes condições que dificultam a sua sobrevida como a presença de Oxigênio reativo e espécies de nitrogênio (ROS e RNS) que também são produzidos durante o processo de fagocitose de macrófagos (SARDAR *et al*., 2013). A produção de ROS pode ser

considerada como forma de ameaça à integridade do parasito, e como forma de defesa a *Leishmania* desenvolveu um sistema de reparo do próprio DNA (MISHRA *et al*., 2018).

As espécies de *Leishmania* são afetadas pela presença de óxido nítrico e peróxido de hidrogênio durante a explosão oxidativa. O Óxido nítrico desempenha um papel tóxico e pode dar origem a outros metabólitos como H_2O_2 e $ONOO^-$, e esses metabólitos também apresentam toxicidade. No estresse oxidativo, a ativação de NADPH oxidase e iNOS geram a produção de compostos como ROS e NO (ASSCHE *et al*., 2011).

Na leishmaniose visceral, o sucesso da atividade leishmanicida com a produção de NO por macrófagos (LIEW, 1995) tem sua importância já comprovada no homem (PANARO *et al*., 2001), camundongo (BOGDAN, 2001) e cão (SERARSLAN; YILMAZ; SOGUT, 2005). E em amostras de PBMC de cães com Leishmaniose, os níveis de NO estão aumentados em comparação com o grupo controle (MARTINI *et al*., 2018). Conclui-se que ROS e NO são fundamentais para o controle da doença.

1.2.2 Resposta imunológica Adaptativa

A leishmaniose é uma doença que se não tratada pode levar o animal a morte e levar a progressão devido a diversos fatores, onde o parasito consegue usar como forma de defesa a inibição de citocinas anti-inflamatórias, a exaustão de células T ou prejuízo na estrutura do linfonodo e na resposta humoral. É importante realizar uma compreensão e mais estudos sobre os mecanismos de defesa do hospedeiro e do parasito para realizar uma melhor abordagem no tratamento da doença (RODRIGUES *et al*., 2016)

Macrófagos infectados são capazes de desencadear a resposta imune adaptativa e são consideradas como células apresentadoras de antígenos (APCs). A estimulação do MHC de classe II permite a ligação com o receptor da célula T (ROY *et al*., 2014) e as moléculas coestimuladoras são importantes para ativar a resposta dessas células (GANNAVARAM *et al*., 2016). Moléculas acessórias B7-1 e B7-2 podem se ligar ao CD28 (CAUX *et al*., 1994) resultando em uma regulação positiva, porém quando ligada ao CTLA-4 a ligação é caracterizada como negativa (GREENWALD; FREEMAN; SHARPE, 2005).

No experimento com células de sangue periférico mononucleadas humanas e *in vitro*, foi comprovado a importância de CD80 e CD86 na Leishmaniose. Após o bloqueio de ambas moléculas, houve uma redução de citocinas IL-5 e IFN-ɣ que levou na progressão da doença (BRODSKYN; DEKREY; TITUS, 2001).

O PD-1 conhecido como “Programmed Cell Death protein-1”, pode desempenhar um papel importante em células leucocitárias de cães com Leishmaniose, sendo altamente expressa e participar na regulação da função de leucócitos e na inibição de citocinas. O bloqueio da molécula de PD-1 pode aumentar os níveis de NO e NO2 e levar a redução da carga parasitária (REBECH *et al*., 2020).

Para se obter uma resposta imune protetora é essencial a ativação de Th1 (GRADONI, 2015). Cães infectados e assintomáticos com a ativação do sistema adaptativo devido à *Leishmania,* é observada a predominância da resposta Th1 e aumento dos níveis de IFN-ɣ e TNF-α que colaboram para a morte do parasito da forma amastigota (CARRILLO; MORENO, 2009). E para se produzir uma vacina eficaz, também é utilizado esse modelo de estimulação da resposta Th1 e de forma duradoura em cães (GRADONI, 2015).

O resultado clínico do quadro da Leishmaniose pode variar desde os sintomas como lesões cutâneas (SARIDOMICHELAKIS; KOUTINAS, 2014) até um quadro mais severo com o comprometimento de vísceras e da imunidade mediada por células T (BUNN *et al*., 2018).

Os linfócitos B são células importantes para a proteção do organismo contra diversos patógenos. Em infecções agudas a resposta humoral tem a capacidade de desempenhar um papel importante combatendo o antígeno. E em alguns casos de infecções crônicas, são induzidas ao desequilíbrio do sistema humoral em favor da sobrevivência do agente patológico (HURDAYAL *et al*., 2017). Em um estudo realizado com células B humanas, foi avaliado o perfil específico dessas células na atuação da *Leishmania infantum*, onde revelou a identificação dessa população de células capazes de serem influenciadas pelo parasito a secretarem altos níveis de IL-10. Portanto, foi comprovado que uma das formas de defesa da *Leishmania* é a utilização de células B através da produção de citocinas (ANDREANI *et al*., 2015).

1.2.3 Expressão de mir-21 e mir-148a na LV

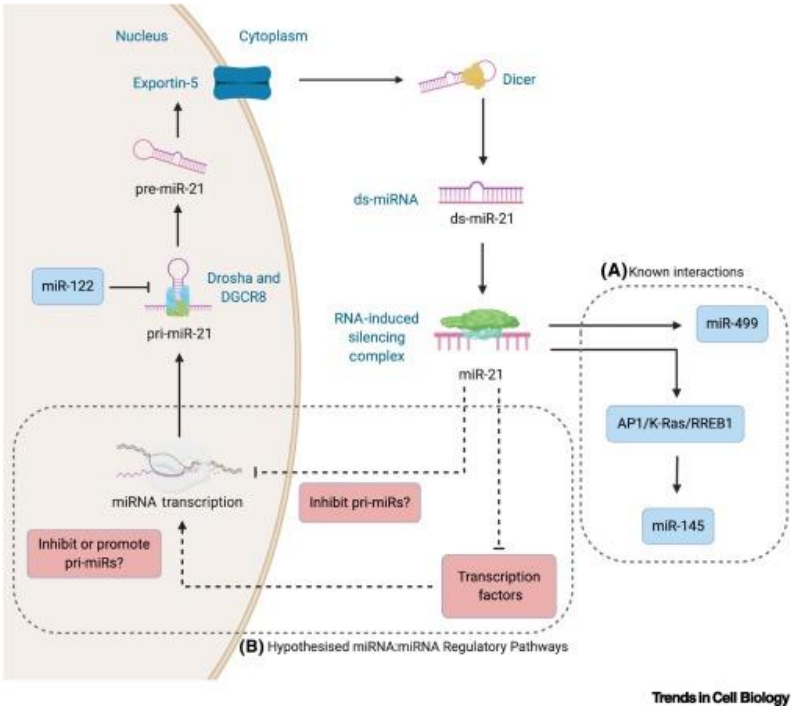
Os microRNAs são responsáveis por agir em diversos processos biológicos realizando um papel importante como mediador intracelular (SHU *et al*., 2017). E por meio de doenças infecciosas, o agente etiológico é hábil a modular a expressão de microRNAs nos processos de regulação de genes específicos na fase pós-transcricional. Na infecção por *Leishmania amazonensis* em camundongos, a modulação de microRNAs feita pelo parasito leva a modificação da expressão da via Toll-like receptors em macrófagos e esse mecanismo resulta em prejuízo para a defesa do sistema imune (MUXEL *et al*., 2018). Do mesmo modo que os microRNAs participam na sobrevivência do patógeno, existem outros que atuam na defesa do sistema imunológico. Em macrófagos de hospedeiros infectados por *Leishmania,* a ativação desses mecanismos de defesa podem induzir ao aumento da expressão de mRNAs que são importantes no controle da progressão da doença (VARIKUTI *et al*., 2019).

Os microRNAs são pequenos RNAs não codificantes que interagem com o mRNA para levar a sua degradação e podem ser usados como alvos terapêuticos para diversas doenças (ZHANG; WANG; GEMEINHART, 2013). O microRNA primário (pri-microRNA) começa a ser processado no núcleo após a sua transcrição, onde é clivado pela ação enzimática de Drosha e DGCR8 formando o pre-microRNA (DAUGAARD; HANSEN, 2017). Em seguida, o pre-microRNA é transportado para o citoplasma pela ação de Exportin-5 para continuar o seu processo de maturação. No citoplasma, sofre clivagem pela ação da Dicer que corta a região do “loop”, formando um microRNA com duas fitas simples. E em seguida, esse microRNA interage com o “complexo de silenciamento induzido por RNA” (RISC) para levar a formação da última forma do microRNA. Na figura abaixo (Fig.1) é demonstrado como acontece a formação do microRNA e com o exemplo envolvendo miR-21:

Após a transcrição da forma primária de miR-21 no núcleo, ocorre a clivagem a partir da Dicer e DGCR8 para formar o pre-microRNA-21 que é transportado para o citoplasma pelo Exportin-5. Em seguida, sofre clivagem da Dicer para originar o microRNA-21 com fita dupla que é incorporado ao complexo de silenciamento induzido por RNA (RISC). (A) Possíveis microRNAs alvos de miR-21 e outras que

miR-21 pode desempenhar, sendo representado em azul. (B) Vias hipotéticas de regulação por miR-21 representadas em vermelho.

Fig. 1. Via canônica da formação de miR-21 e seus possíveis alvos.



Fonte: HILL; TRAN, 2021

Após sua formação, os miRs passam a atuar no alvo promovendo a regulação da expressão gênica e a produção de proteínas, e dessa forma pode desempenhar importantes funções regulatórias no organismo. A expressão de miR-21 conferi um papel protetor em camundongos, pois tem como alvo os genes MARCKS e RhoB que regulam a atividade fagocítica de macrófagos, sendo importante no combate a infecções (JOHNSTON *et al*., 2017). Na leishmaniose, a produção de proteínas, como as citocinas IL-10 e TGF-β, são moduladas pela *Leishmania* através da expressão de miRs para controlar a resposta imune no hospedeiro (HAMIDI *et al*., 2021).

A *Leishmania* também apresenta como mecanismos de evasão a modulação do transporte intracelular de miRs no hospedeiro, como por exemplo, a restrição da entrada de miR-122 provenientes de vesículas extracelulares para reduzir a resposta inflamatória. Contudo, o parasito também atua no processo inverso, realizando a exportação de miR-146 que exerce ação anti-inflamatória em células infectadas, e esse processo acontece através da modulação da proteína HuR. E no momento que esse miR está no meio extracelular, é promovida a polarização de macrófagos

adjacentes para perfil M2, favorecendo a sobrevivência do parasito (GANGULY *et al*., 2021).

Na Leishmaniose canina, alguns miRs apresentam sua expressão aumentada, como o miR-148a e miR-21, e a inibição de miR-21 está associada ao aumento do perfil Th1 e diminuição da carga parasitária (MELO *et al*., 2019). De acordo com o miRBase Sequence Database, cfa-miR-21 e cfa-miR-148a estão localizados no cromossomo 9 e 14 respectivamente (GRIFFITHS-JONES *et al*., 2008). Em humanos, miR-148a é considerado como membro da família miR-148a/miR-152 e pode ser encontrado nos cromossomos 7, 12 e 17 (FRIEDRICH *et al*., 2017); enquanto hsa-miR-21 está no cromossomo 17 (GRIFFITHS-JONES *et al*., 2008).

MiR-21 possui diversos genes alvos envolvidos na imunidade do cão com leishmaniose, como FAS, FASLG, PIK3R1, PTEN e entre outros (MELO *et al*., 2019). O camundongo knockout de miR-21 apresenta resposta protetora em células esplênicas de com *L. donovani,* devido ao direcionamento da polarização de macrófagos para o perfil M1 e com produção de NO, IFN-γ e TNF-α no estágio ativo da infecção (VARIKUTI *et al*., 2021). Além disso, a expressão miR-21 também regula a produção de IL-10 e a expressão de CD69 de linfócitos B em células esplênicas de cães com leishmaniose, sendo sugestivo a modulação desse miR pelo parasito (BRAGATO *et al*., 2022).

Contudo, em células tumorais a expressão de miR-21 pode desempenhar um papel importante no controle da apoptose, e favorecer a progressão da doença através da diminuição da atividade de PTEN (LIU *et al*., 2019). O efeito protetor acontece com a alta expressão de miR-21, pois leva ao direcionamento de macrófagos M2 em pacientes com câncer ovariano (AN; YANG, 2020), e esse efeito protetor pode ser devido à expressão de PTEN.

Em camundongos, o aumento da expressão de PTEN pode promover o controle da progressão do cancinoma hepatocelular e induzir ao estresse oxidativo (KATO *et al*., 2021), mas na leishmaniose favorece a sobrevivência do parasito, pois não há produção de NO e ROS (MAUËL; RANSIJIN; BUCHMÜLLER-ROUILLER, 1991; SAHA et al., 2019). Porém, ainda não foram feitas pesquisas a respeito dessa interação de NO e ROS com o miR-21 na patologia da Leishmaniose canina.

Em células humanas endoteliais, a expressão do miR-21 leva a um processo de estresse, com aumento da síntese de NO e inibição da apoptose (WEBER *et al*., 2010). MiR-21 também pode regular a expressão de KRIT1 que está envolvido na

manutenção da homeostase de ROS (SALA *et al*., 2018), e a expressão do gene SOD3 também modula os níveis de ROS em células epiteliais humanas (ZHANG *et al*., 2012). Portanto, miR-21 tem atuação na regulação de NO e ROS na resposta imune, e uma compreensão desse mecanismo na leishmaniose é necessário para o desenvolvimento de terapias com alvos em microRNAs.

Em processos inflamatórios, a desregulação do miR-21 por meio de fatores como PAMPs ou DAMPs induz a ação anti-inflamatória e imunossupressora. Logo, a atuação do microRNA no sistema imunológico é apto de ser influenciado por agentes infecciosos (SHEEDY, 2015). Em reações inflamatórias excessivas, induzi-lo negativamente poderá conter a inflamação e o dano ao organismo. Em um estudo com sangue periférico humano, comprovou essa capacidade do miR-21 em participar na diferenciação das células T. A diminuição em sua expressão favoreceu o aumento de células de mémoria, pois tem correlação com genes responsáveis pela produção dessas células (KIM *et al*., 2018).

Além dos efeitos regulatórios desempenhados por miR-21 na leishmaniose, miR-148a possui a sua expressão também aumentada (MELO *et al*., 2019). MiR-148a-3p é capaz de promover efeitos regulatórios na diferenciação de monócitos em macrófagos com o uso de um fator estimulante de colônia (GM-CSF). No estudo com células da medula óssea de camundongos, foi demonstrada a ação de macrófagos por meio da expressão de miR-148a-3p, de modo que apresentaram um desempenho melhor na eliminação de patógenos pela alta quantidade produzida de ROS. O aumento na expressão de miR-148a-3p, contribui no aumento da produção de ROS com o uso da via PTEN/AKT que acontece durante o processo infeccioso (HUANG *et al*., 2017).

Essa atuação do miR-148 na regulação imune em macrófagos de camundongos infectados, também foi comprovada em outro estudo com o parasita *Schistossoma japonicum*, onde a resposta também é direcionada aos alvos PTEN e PI3K/ AKT (GIRI; CHENG, 2019).

A sinalização mediada por miR-148a-3p promove a polarização dos macrófagos para o perfil M1 e isso aumenta a sua capacidade bactericida (HUANG *et al*., 2017). Existem estudos sobre esse microRNA que divulgaram o seu importante papel como marcador prognóstico e como alvo terapêutico para diversos perfis de patologias, sendo tumores, doenças autoimunes e doenças inflamatórias crônicas. Esse achados sugerem que a participação do miR-148 na resposta imune inata tem

comprovação, porém foi relatado também a sua atuação na regulação de células T e B (FRIEDRICK *et al*., 2017). Em pacientes com processos inflamatórios como a artrite reumatoide, o aumento da expressão do miR-148 estimulado por T-bet e Twist 1 pode promover uma resposta imune direcionada à resposta Th1 (HAFTMANN *et al*., 2015) Porém, na área da leishmaniose ainda é escasso a quantidade de informações sobre esse microRNA.

MiR-148 é regulador de processos inflamatórios e modula a via de NF-kB e citocinas inflamatórias, como a IL-1β, onde se liga a região 3'UTR do mRNA de IL-1β, inibindo sua expressão e a produção da proteína (DONG *et al*., 2021). A expressão de miR-148a pode exercer um papel na regulação da resposta inflamatória na leishmaniose, contudo a sua inibição leva a redução da carga parasitária, comprovando o seu importante papel no controle da infecção (REBECH *et al.*, 2023).

O tratamento da Leishmaniose canina tem certas limitações porque o arsenal terapêutico contra esta doença é limitado e as drogas mais comumente usadas apresentam alta toxicidade (nefrotoxicidade, problemas intestinais, dores musculares), tem custo elevado e podem ser ineficazes em alguns casos (BANETH; SHAW, 2002). Falhas no tratamento têm implicações epidemiológicas uma vez que, após o tratamento, os cães tornam-se assintomáticos, mas continuam a ser um reservatório para a transmissão do parasito pelo vetor (SOLANO-GALLEGO *et al*., 2009). Devido à supressão imunológica celular ser determinante na progressão da doença, o conhecimento de fatores associados a regulação imunológica é fundamental para a mudança do padrão de resposta.

Portanto, compreender o mecanismo de ação dos miRs na resposta imunológica é fundamental para o desenvolvimento de novos tratamentos para *Leishmaniose,* e assim promover melhores condições de vida ao animal.

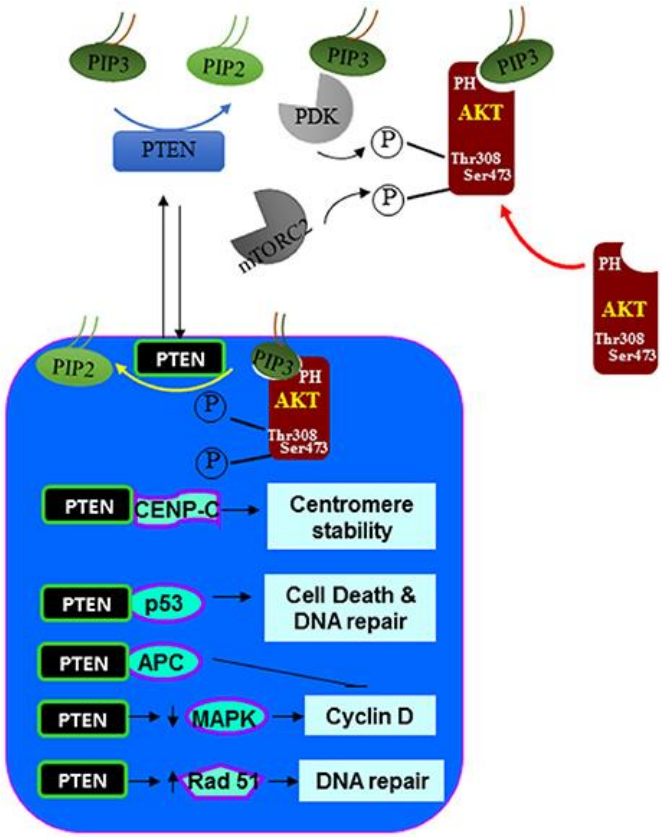
1.2.4 Expressão de PTEN na LV

PTEN é conhecido como um supressor tumoral e tem como alvos substratos lipídicos ou proteicos. A sua importância e o seu papel para tratamentos oncológicos é amplamente discutido em diversos estudos (KATO *et al*., 2021; SHINDE; MADDIKA, 2016) e a sua deficiência pode induzir ao processo de estresse oxidativo em camundongos com carcinoma hepatocelular (KATO *et al*., 2021). A proteína PTEN

sofre uma série de mutações em células tumorais e é considerada importante para o controle celular (FURNARI *et al*., 1997). Contudo, essas mutações decorrentes desses processos podem reduzir a atividade catalítica de PTEN e prejudicar a sua função. O domínio C2 encontrado em PTEN está envolvido nessa atividade e contribui no seu recrutamento para a membrana, e além de ajudar na interação com o seu substrato PI(3,4,5)P3 (WORBY; DIXON, 2014). Na figura 2 está descrito os principais mecanismos de ação de PTEN, incluindo sua interação com PIP3:

PTEN realiza a desfosforilação de PIP3 na membrana plasmática, enquanto no núcleo pode inibir a atividade de AKT, como ocorre de forma semelhante na membrana plasmática. Além disso, PTEN participa na regulação de outras proteínas na região nuclear para regular a reparação do DNA, morte celular e estabilidade do centrômero.

Fig.2. Principais funções regulatórias de PTEN.



Fonte: CHEN *et al*., 2018

A atividade de PTEN pode ser modulada em diversos níveis, como as modificações pós-traducionais (acetilação, fosforilação, SUMOilação, oxidação,

microRNAs, RNA antisense, ou por RNAs longos não codificantes) e interações entre proteínas. Essas modulações contribuem para manter a homeostase celular com níveis regulares de PTEN e PIP3 (WORBY; DIXON, 2014). Outro fator importante para a modulação de PTEN é o processo de metilação. Em camundongos com artrite reumatoide, a metilação de PTEN promove a regulação da atividade inflamatória e a produção de citocinas e quimiocinas, como IL-1β, IL-6, CCL-2 e CCL-3 (LI *et al*., 2021).

A redução de PTEN também é responsável por promover a ocorrência de processos apoptóticos, pois é aumentada a atividade oxidativa, onde é gerado o acúmulo de ROS em células endoteliais (WU *et al*., 2014). Além disso, a falta de PTEN induz o aumento da atividade de PI3K/AKT, gerando falhas na reparação do DNA e comprometimento celular (HO *et al*., 2020). Portanto, é necessário manter concentrações de PTEN/PI3K/AKT equilibradas para evitar o surgimento de doenças ou de processos apoptóticos.

PIP3 é um dos principais substratos encontrados na membrana plasmática, atuando na regulação da atividade de PTEN quando presente na membrana (MASSON; WILLIAMS, 2020). Porém, PTEN também está localizado no núcleo, exercendo papel na manutenção do DNA e evitando danos na sua estrutura (BASSI *et al*., 2013), mas pode interagir na região da membrana quando recrutada (LIU *et al.*, 2005).

PI(3,4,5)P3 é considerado como substrato intermediário de PTEN e após a sua ativação, os níveis de AKT aumentam contribuindo nos processos de sobrevivência, proliferação e migração celular (KOTELEVETS *et al*., 2020). Os níveis de AKT quando aumentados diminui a expressão de PTEN, onde leva a progressão de tumores em cães e gatos (ASPRONI *et al*., 2021), além de exercer importante influência na viabilidade celular e na sobrevivência de macrófagos e monócitos (LINTON; MOSLEHI; RABAEV, 2019). Na leishmaniose, a ativação de AKT permite prevenir o processo de apoptose celular induzido pela *Leishmania* como mecanismo de sobrevivência e evasão (RODRÍGUEZ-GONZÁLEZ; WILKINS-RODRÍGUEZ; GUTIÉRREZ-KOBEH, 2022).

Em células dendríticas, a *Leishmania* pode ter como alvos a via PI3K/AKT e o fator de transcrição NF-kB para manipulação da resposta inflamatória, sendo crucial para a sua multiplicação no hospedeiro (NEVES *et al*., 2010). Além disso, o parasito pode produzir a proteína AKT-like que apresenta similaridades com a AKT humana,

além de ser encontrada em todas as espécies de *Leishmania* e pode facilitar a modulação da resposta imune no hospedeiro (TIRADO-DUARTE *et al*., 2018).

A *Leishmania* apresenta um grande arsenal de mecanismos para promover a sua replicação e conseguir sobreviver no hospedeiro. E fatores epigenéticos podem estar envolvidos nesse processo, como modificações em histonas e alterações na expressão gênica (AFRIN; KHAN; HEMEG, 2019).

Alguns genes têm sua expressão alterada na leishmaniose, como PTEN que pode exercer importante papel durante o estágio da infecção. Em pacientes com leishmaniose visceral, pode ser observado a diminuição de mRNA para PTEN, além do aumento da produção de citocinas, como IFN-γ e IL-10 (SUDARSHAN *et al*., 2016), ambas de perfil Th1 e Th2 respectivamente. Já em camundongos, macrófagos deficientes em PTEN ocorreu a diminuição da capacidade de eliminação do parasito (KURODA *et al*., 2008), porém mais estudos são necessários para confirmar a sua função na modulação da resposta imune na leishmaniose.

A expressão de PTEN pode ser modulada por microRNAs através da interação com o mRNA de PTEN na região 3’ UTR (LIU *et al*., 2019). Na Leishmaniose canina, a expressão de miR-21 e miR-148a estão aumentados, e na análise *in Silico*, mostrou que PTEN participa como alvo desses microRNAs (MELO *et al*., 2019). PTEN é capaz de promover mudanças na polarização do perfil de macrófagos M2 para M1, alterando para um resposta inflamatória (LIU *et al*., 2020). Contudo, macrófagos de perfil M2 podem conter alta expressão de miR-21 que é responsável por modular negativamente a expressão de PTEN (ZHENG *et al*., 2017).

Na leishmaniose, a polarização da resposta para um perfil M1 é importante para a eliminação do parasito, devido a produção de substâncias como ROS e NO (CHOW *et al*., 2022; VAROTTO-BOCCAZZI *et al*., 2020). O efeito da polarização de macrófagos desempenha um papel importante porque exerce influência na atividade microbicida e na função fagocítica (CHOW *et al*., 2022)

No estudo com melanomas humano, mostrou que a presença de NO é capaz de modular negativamente a atividade de PTEN, além de aumentar a expressão da via PI3K/AKT (DING *et al*., 2021). Resultado similar foi encontrado em pacientes com doenças degenerativas, onde NO ocasionou a degradação de PTEN, portanto, NO desempenha um papel importante para regulação da atividade de PTEN (KWAK *et al*., 2010), e sugerimos que tenha participação desse mecanismo na leishmaniose.

ROS também pode levar a inativação da expressão de PTEN e ativar o mecanismo da via PI 3-kinase em macrófagos de murinos que é importante para o controle dos mecanismos de apoptose dos macrófagos e na produção de outros compostos durante a explosão oxidativa (LESLIE *et al*., 2003). Porém, na literatura ainda não há estudos demonstrando o envolvimento na expressão de PTEN com a regulação de ROS na leishmaniose canina e mais estudos são necessários. Assim, o objetivo dessa pesquisa foi de melhorar a nossa compreensão sobre a patogênese da LV no hospedeiro, e como tal, contribuir como alvos terapêuticos para futuros estudos.

2 CAPÍTULO 1 - MICRORNA-21 AND MICRORNA-148A REGULATES PTEN, NO AND ROS IN CANINE LEISHMANIASIS

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2.1 Resumo

A leishmaniose visceral canina (CanL) representa uma grave ameaça à saúde pública em vários países. A progressão da doença depende do grau de supressão da resposta imune. Os microRNAs (miRs) modulam a tradução do mRNA em proteínas e regulam várias funções celulares e vias associadas às respostas imunes. MiR-21 e miR-148a podem alterar a carga parasitária e os macrófagos M1 são as principais células na atividade leishmanicida de cães. Um estudo anterior encontrou aumento de miR-21 e miR-148a em leucócitos esplênicos (LE) de cães com CanL usando a análise de microarranjo, e a análise in *silico* identificou alvos da via PTEN. PTEN está envolvido na regulação imune de macrófagos. Medimos PTEN e a produção de espécies reativas de oxigênio (ROS) e óxido nítrico (NO) após transfecção com mimic e inhibitor de miR-21 e miR-148a em LE de cães com CanL. Os níveis de PTEN aumentaram, NO e ROS diminuíram em LEs de cães com CanL. A inibição do miRNA-21 resultou em aumentos de PTEN; em contraste, o PTEN diminuiu após a inibição do miR-148a. Os níveis de nitrito aumentaram após a transfecção com o inibidor de miR-21, mas diminuíram com o inibidor de miR-148a. O aumento de miR-21 promoveu redução nos níveis de ROS e NO. Esses achados sugerem que miR-21 e miR-148a modulam a resposta imune em cães com CanL por meio de PTEN, NO e ROS.

Palavras-chave: microRNA. ROS. NO. PTEN. Nitrito. *Leishmania infantum*. Leucócitos esplênicos.

2.2 Abstract

Canine Visceral leishmaniasis (CanL) poses a severe public health threat in several countries. Disease progression depends on the degree of immune response suppression. MicroRNAs (miRs) modulate mRNA translation into proteins and regulate various cellular functions and pathways associated with immune responses. MiR-21 and miR-148a can alter the parasite load and M1 macrophages are the principal cells in dog's leishmanicidal activity. A previous study found increased miR-21 and miR-148a in splenic leukocytes (SL) of dogs with CanL using microarray analysis and in silico analysis, identified PTEN pathway targets. PTEN is involved in the immune regulation of macrophages. We measured PTEN and the production of reactive oxygen species (ROS) and nitric oxide (NO) after transfection SL of dogs with CanL with mimic and inhibition of miR-21 and miR-148a. PTEN levels increased, NO and ROS decreased in SLs from dogs with CanL. Inhibition of miRNA-21 resulted in PTEN increases; in contrast, PTEN decreased after miR-148a inhibition. Nitrite levels increased after transfection with miR-21 inhibitor but were decreased with miR-148a inhibitor. The increase in miR-21 promoted a reduction in ROS and NO levels. These findings suggest that miR-21 and miR-148a modulate the immune response in dogs with CanL through PTEN, NO, and ROS.

Keywords: microRNA. ROS. NO. PTEN. Nitrite. *Leishmania infantum*. Splenic leukocytes.

2.3 Introduction [1]

Visceral leishmaniasis occurs in several countries, and approximately 300,000 new cases are registered yearly. The countries with the highest recorded cases are Bangladesh, Brazil, Sudan, India, Ethiopia, and South Sudan (WHO, 2015). In Brazil, there are records of several species of *Leishmania* causing *VL*, including *L. infantum* and *L. amazonensis* in dogs (Tolezano et al., 2007; Sanches et al., 2016) and humans (Grisard et al., 2000; Araújo et al., 2020).

Dogs are reservoirs of *L. infantum* and can develop skin lesions, splenomegaly, weight loss, onychogryphosis, diarrhea, and lymphadenopathy (Tonin et al., 2016). In Leishmania infection, the parasite proliferates in tissues such as the liver, spleen, and bone marrow lead to functional impairment of these tissues. The spleen is where *L. infantum* can be preserved. During infection, the parasite modifies the structure of the spleen and compromises its function to favor its survival, leading to immunomodulation and disease progression (Modabberi et al., 2021).

In the spleen, granulomas can form with macrophage infiltration and rare plasma cells and lymphocytes (Moreira et al., 2016). In dogs with visceral leishmaniasis, the number of macrophages in the spleen is significantly elevated (Martini et al., 2018; Tafuri et al., 1996).

Immunosuppression favors parasite proliferation in several ways. The increased expression of the Th1 response with increased cytokines such as IFN-γ, IL-2, and TNF-alpha might help to control the proliferation (Darrah et al., 2007). However, if the immune response is also directed toward the M2 macrophage profiles, there may be an anti-inflammatory response with increased IL-10 (Azevedo et al., 2021), which is not beneficial for humans and mice, leading to an increased susceptibility to disease (Buxbaum, 2013). The parasite can modulate the immune response through the action of miRNAs and direct the immune profile to a Th2 response (Bragato et al., 2022).

MiRNAs participate in several pathophysiologies and pathways in the immune system; they are essential for developing drugs that target mRNA (Wahid et al., 2014) and can decrease or increase expression of its target gene in transcriptional or post-transcriptional level (Sadakierska-Chudy, 2020; Zhang et a., 2014). A study with miR-

[1] Revista Frontiers in Genetics (Anexo A)

10a reported that miR regulates ribosome biogenesis binding to 5'UTR and increases RP protein production in mice (Ørom et al., 2008). Epigenetic factors or pathologies can deregulate miR expressions and contribute to disease development (Sadakierska-Chudy, 2020).

In CanL, the parasite regulates miRNA expression in splenic leukocytes (SLs) and increases miR-21 and miR-148a expression. *In silico* analysis showed that miR-21 and miR-148a affect several pathways, including PTEN (a tumor suppressor protein) and STAT3 (a signal transducer and transcription activator). The primary direct target genes are PTEN, SOS1, and FASLG, among others (Melo et al., 2019).

MiR-21 can regulate PTEN in several diseases; it was studied in colorectal cancer (Wu et al., 2017), diabetic retinopathy (Lu et al., 2020), and endometrial carcinoma (Zhao et al., 2017). PTEN suppresses tumors, and its absence promotes cell growth (Kirstein et al., 2021). PTEN expression can be modulated by microRNAs via interactions with PTEN mRNA in the 3' UTR region (Liu et al., 2019).

MiR-148a also regulates PTEN expression in macrophages infected by *Schistosoma japonicum* in mice (Giri; Cheng, 2019). In addition, overexpression of miR-148a increases ROS levels in macrophages through the PTEN/AKT pathway in mice (Huang et al., 2017); however, no studies demonstrate this mechanism in CanL.

MiR-21 is involved in *Leishmania infantum* escape mechanisms by inhibiting the Th1 response targeting in dogs with leishmaniasis (Melo et al., 2019). MiR-21 expression in SLs from dogs with CanL also regulates CD69 expression on B lymphocytes and IL-10 levels (Bragato et al., 2022). These findings suggest that *Leishmania* modulates the immune response via miR-21 expression as a survival mechanism (Bragato et al., 2022). In addition, the decreased parasitic load in SL following the use of the miR-148a inhibitor suggests a critical role for this miR in developing microbicidal activity in CanL, and miR148a regulates immune response as demonstrated by reducing iNOS, TNF-α, IL-6, and IL-12 (Rebech et al., 2023).

The PTEN pathway is the target of miR-21 and miR-148a (Melo et al., 2019). PTEN controls the differentiation and activation of several immune cells; PTEN acts downstream from T- and B-cell receptors, costimulatory molecules, cytokine receptors, integrins, and growth factor receptors. PTEN expression is present in M1 macrophages that promote increased inflammatory immune responses (Liu et al., 2020). Loss of PTEN activity in humans and mice is associated with cellular and humoral immune dysfunction, lymphoid hyperplasia, and autoimmunity (Taylor et al., 2019). Decreased

PTEN levels and PTEN/PI3K-related genetic defects are involved in the emergence of autoimmune diseases and inflammatory disorders associated with lymphoid and myeloid cells. PTEN is a PI3K antagonist that converts PI-(3,4,5) triphosphate (PIP3) to PI(4,5) (PIP2) and can control PI3P activity. PI3P is a member of the PI3K family, and its increase can lead to immunodeficiencies (Taylor et al., 2019).

Splenic aspirates from patients with active *Leishmania donovani* infection showed less mRNA PTEN expression than those from treated patients (Sudarshan et al., 2016). PTEN is involved with the immune response in leishmaniasis. PTEN-deficient macrophages showed a reduced ability to kill *Leishmania major* in response to IFN-γ treatment, possibly because the mutant cells exhibited decreased TNF secretion that correlated with reductions in inducible NO synthase expression and production (Kuroda et al., 2008). In CanL, the M1 phenotype is associated with nitric oxide (NO), which is involved in the long-term protection of dogs against natural *L. infantum* infection and the clinical presentation of leishmaniasis (Panaro et al., 2008). NO production depends on the precursor molecule L-arginine (Jorens et al., 1993) and is induced by iNOS (Guzik et al., 2003) to perform microbicidal activity as demonstrated in human leishmaniasis (Panaro et al., 2001), mice (Mauël et al., 1991), and dogs (Vouldoukis et al., 1996). M1 macrophages also produce reactive oxygen species (ROS), which participate in the innate immune response and can be produced during the oxidative explosion from NADPH oxidase (Saha et al., 2019).

This study aimed to evaluate the regulation of PTEN expression and ROS and NO levels by miR-21 and miR-148a in SLs from dogs with CanL.

2.4 Methods

2.4.1 Ethics committee approval

The Ethics Committee on Experimental Animal Research (COBEA) and the Ethics Committee on Animal Use (CEUA) of UNESP – São Paulo State University Júlio de Mesquita Filho, Araçatuba Campus, School of Veterinary Medicine —FMVA— approved the study, according to process 00624-2018.

2.4.2 Animals

We selected 17 adult dogs with CanL naturally infected by *L. infantum* of both sexes and various breeds and weights. The animals were aged between two and five

years. Dogs with leishmaniasis were from the Araçatuba Zoonoses Control Center in the state of São Paulo. Confirmation of seropositivity to *L. infantum* was performed using an immunochromatographic test (DPP) and an indirect enzyme-linked immunosorbent assay (ELISA) (Supplementary Table 1), according to Lima et al. (2003). All dogs had at least three signs of CanL, including onychogryphosis, lymphadenopathy, cachexia, and periocular dermatitis (diffuse skin or ear tip). The dogs used were classified in the moderate stage as proposed by Solano-Gallego et al. (2009) and were classified based on complete blood counts (Supplementary Tables 2 and 3) and biochemical examinations (Supplementary Table 4).

The control group consisted of five healthy dogs of both sexes and various breeds and weights. The animals were aged between two and five years. All dogs in this group had negative serology results according to indirect ELISA and DPP, as proposed by Lima et al. (2003). They also had complete blood counts and serum biochemistries within normal values. The diagnosis of afflicted animals was confirmed using real-time PCR (qPCR) (Ranasinghe et al., 2008) for parasitic DNA amplification and PCR-RFLP for species typing, according to Sanches et al. (2016).

2.4.3 Blood and spleen samples

Five milliliters of blood were drawn by jugular vein puncture and placed in tubes with an anti-coagulant (BD Vacutainer®, Franklin Lakes, NJ, USA) for the complete blood count and DNA extraction. An additional 5 mL of blood was collected and stored in a tube without an anti-coagulant for biochemical analysis and serum for the DPP and ELISA tests.

For the collection of spleen samples from the group of dogs with CanL, barbiturate anesthesia (Thiopental, Cristália Itapira, SP, Brazil) was induced by intravenous introduction, followed by infusion of 19.1% potassium chloride, according to the rules and resolution of No. 1,374, of December 2, 2020, and chapter VIII, article 27 of the Federal Council of Veterinary Medicine (Brazil). Splenic samples were then collected. Samples from Control group were removed by surgical excision per Lima et al. (2012) and processed immediately for analysis.

2.4.4 Obtaining SLs

Total splenic leukocytes (SLs) (1.6×10^5) were obtained from 2 cm^3 fragments, added to 10 mL of RPMI-1640 medium (Sigma®, USA), and supplemented with 10%

heat-inactivated fetal bovine serum, 0.03% L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. After removing debris with a cell strainer (BD Falcon Cell strainer, San Diego, CA, USA), the cell suspensions were processed with 10 mL of red blood cell lysis buffer containing 7.46 g/L of ammonium chloride (NH_4ClO_3) at 4 °C for eight minutes, centrifuged at 2500 rpm for nine minutes, and washed three times with phosphate-buffered saline at pH 7.2. RPMI-1640 medium (Sigma®, USA) was then added.

2.4.5 ELISA and DPP

DPP was performed according to the manufacturer's instructions to perform screening. The sera were analyzed using indirect ELISA from of *L. chagasi* promastigotes (MHOM/BR00/MER02) as the antigen. This technique was performed as described by Lima et al. (2003).

2.4.6 DNA Extraction

DNA extraction from spleen samples from dogs was performed using the commercial DNAeasy® kit (Qiagen, Valencia, California, 91355, USA) according to the manufacturer's recommendations. The extracted DNA was quantified using a 260/280 nm spectrophotometer (NanoDrop Technologies ND 1000 UV/VIS, USA). The degree of purity was evaluated, and samples were stored at –20 °C.

2.4.7 Determining the species of *leishmania*

The determination of *Leishmania* species was performed using PCR-RFLP (Supplementary Figure 1) to confirm *L. infantum* infection as described by Sanches et al. (2016), *L. infantum* (IOC / L0575-MHOM / BR / 2002 / LPC- RPV). The molecular weight marker was 100 bp (Invitrogen, Carlsbad, California 92008, USA)

2.4.8 Transfection of miR-21 and miR-148a

MiR-21 and miR-148a expression was evaluated previously in SL, and there was an increase in the expression of miR-21 (Bragato et al., 2022) and miR-148a (Rebech et al.,2023). SLs were cultured at 1.6 x 10^5 in 24-well plates for 48 h at 37 °C in 5% CO_2. All-Stars Negative control siRNA (Scrambled), miR-21 mimic (5nM), miR-148a mimic (5nM), miR-21 inhibitor (50nM), miR-148a inhibitor (50nM) (miScript miRNA mimic and inhibitor Qiagen, USA) were used. Transfected cells were cultured

using 3 µL of Hiperfect (Qiagen, USA) in each well, following the manufacturer's instructions. Transfection rates were evaluated using a final concentration with 50 nM of AllStars Hs Cell Death Control siRNA reagent (QIAGEN, USA), a blend of highly potent siRNAs that target genes essential for cell survival. Knocking down these genes induces a high degree of cell death. Transfection rates were measured using flow cytometry and the 7-AAD Viability Staining Solution reagent (BioLegend, USA), according to the manufacturer's instructions. The medium transfection rate for both groups was 20% (Supplementary Table 5).

The transfected cells were cultured at 37 °C and 0.5% CO_2. At 48 h after transfection, the cells were stained for NO and ROS, and cell lysates were used for PTEN evaluation. Culture supernatants from SLs were collected, centrifuged at 2500 rpm, and stored at –80 °C for nitrite measurement.

2.4.9 Detection of NO and ROS in SL from infected and control dogs

To quantify NO, cell suspensions were treated with DAF2DA (2 µM) (Sigma-Aldrich, D225, São Paulo, Brazil) and incubated for 1 hour at 37 °C and 5% CO_2. After labeling, samples were stored at 4 °C in the dark until analysis using a flow cytometer (BD Accuri™ C5 flow cytometer), and the data were analyzed using BD Accuri™ C6 software (version 1.0.264.21). Cells (1×10^5 / mL) from various experimental groups were evaluated by fluorescence at 10,000 events. Cells without labeling were used as a negative control to delimit the negative populations. After excluding the debris, the cellular fluorescence of the triazole product (DAF-2T) was collected in the FL1 channel, and myeloid cells were gated separately. The mean values of FL1 were used for NO quantification.

To measure ROS levels, 10 µM H2DCFDA (Introgen-Leiden Molecular Probes) was added to cell suspensions and incubated for 1 hour at 37 ºC and 5% CO_2. Phorbol 12-myristate 13-acetate (PMA, 10 µM) was added 30 minutes before analysis for the positive ROS control. After labeling, the ROS analysis procedure was identical for NO.

2.4.10 Nitrite determination

The Griess method determined nitrite concentrations in supernatant samples collected after 48 hours of culture. For this procedure, we used 50 µL of the culture supernatants and 50 µL of Griess reagent (including 0.1% of NEED and sulfanilamide in 5% phosphoric acid) and maintained the mixture at room temperature for 5 minutes.

Optical density was measured at 540 nm in 96-well plates (Spectra Count, Packard BioScience Company, Meriden, CT, USA). The results were compared with a standard nitrite concentration curve (3 to 200 µM).

2.4.11 Protein concentrations and PTEN analysis

Cell lysates were used to measure PTEN after the equalization of protein levels in samples. Protein concentrations were measured using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). PTEN analysis was performed using the Pathscan® Total PTEN Sandwich ELISA Kit (Cell Signaling Technology); PTEN is highly conserved in humans and dogs, showing 93.5% homology with *Canis lupus* familiaris (BLAST/ XP_546673.2). We performed according to the manufacturer's instructions. The optical density at 450 nm was obtained in a 96-well plate reader (Spectra Count, Packard BioScience Company, Meriden, CT, USA).

2.4.12 Statistical analysis

The data was classified as non-parametric using the Shapiro-Wilk normality test. ROS, NO, and PTEN levels were compared between the CanL and control groups using the Mann-Whitney test. PTEN, NO, ROS, and nitrite with mimic, inhibitor, and scramble treatments were compared using analysis of variance, the Friedman test, and Dunn's multiple comparison test. The data were classified as parametric using the Shapiro-Wilk. Medium ROS levels from untransfected cells were compared with PMA using the t-test. Nitrite and NO levels of miR-148a mimic, inhibitor, and scramble treatments in CanL were compared using analysis of variance with Dunnett´s multiple comparison test. Differences were considered significant when $p < 0.05$. Statistical analyses and graphs were generated using Graph Pad Prism 6 (Graph Pad Software, Inc. CA. USA).

2.5 Results

2.5.1 PTEN concentration in SLs of dogs with CanL.

We measured PTEN from whole cell lysates, and levels were higher in splenic cells from dogs with CanL than from Control (Figure 1A) (p = 0.0270)

Figure 1. PTEN in total cell lysate samples in control group and dogs with CanL

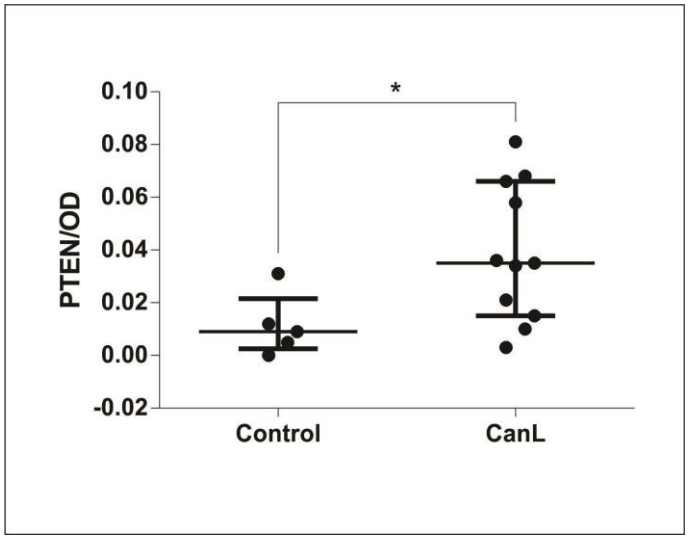

Figure 1. PTEN in total cell lysate samples in control group and dogs with CanL by sandwich ELISA from Pathscan® kit. Data are expressed as median and interquartile range. Mann-Whitney test was used ($p < 0.05$).

Source: Prepared by the author

2.5.2 NO and ROS concentration in SLs of dogs with CanL.

NO production was lower in splenic cells from dogs with CanL than in the Control (Figure 2A) (p = 0.0063). ROS production was lower in splenic cells from dogs with CanL than in the Control (Figure 2C) (p = 0.0246), and PMA (positive control) increased ROS production in the CanL group (Supplementary Table 6). A representative histogram of basal NO and ROS production in splenic cells from dogs with CanL and a Control are shown in Figures 2B and 2D, respectively.

Figure 2. NO and ROS in splenic leukocytes from control group and dogs with CanL.

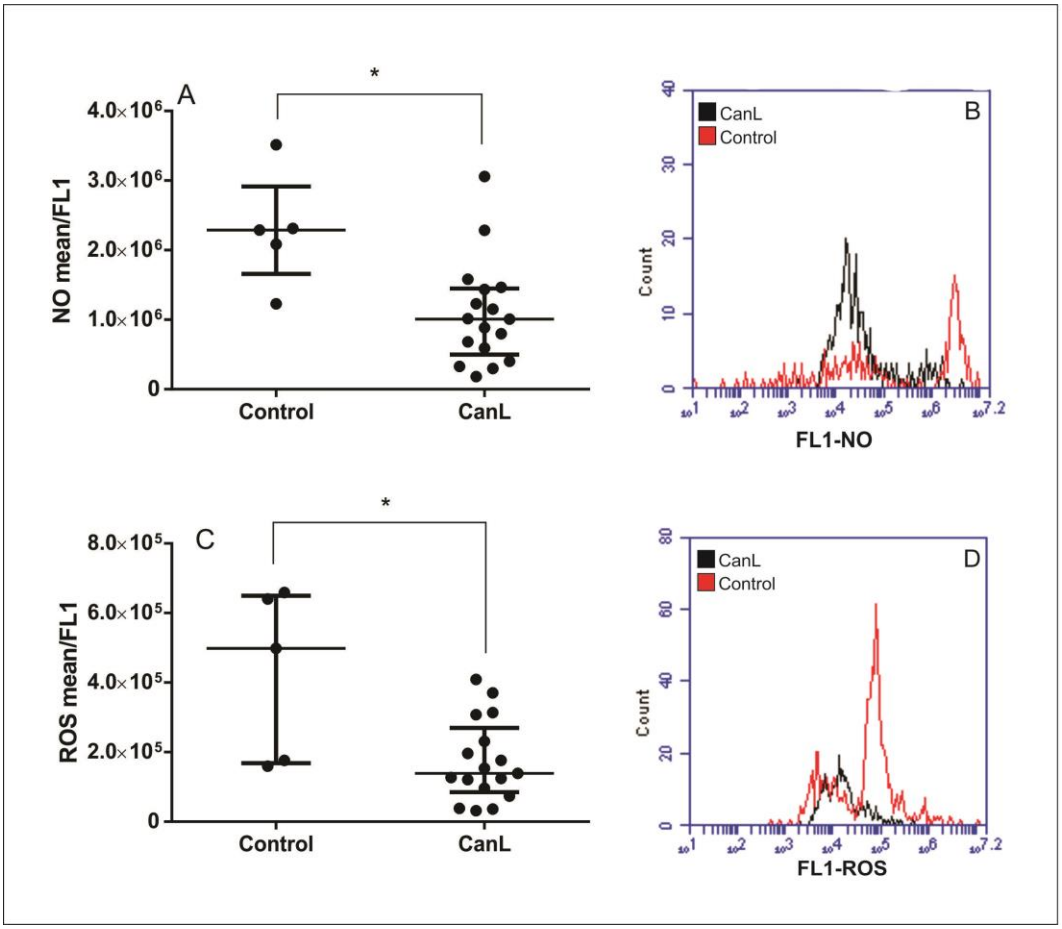

Figure 2. NO (A) and ROS (C) in splenic leukocytes from control group and dogs with CanL. Data are expressed as median and interquartile range. Mann-Whitney test ($p < 0.05$). A representative overlay histogram shows an example of NO (B) and ROS (D) production in splenic leukocytes from control group and dogs with CanL. The black represents dogs with CanL and the red represents control group.

Source: Prepared by the author.

2.5.3 Effect of miR-21 and miR-148a on PTEN concentration in SLs of dogs with CanL

After SL transfection with miR-21 mimic and inhibitor, and miR148a mimic and inhibitor, no significant difference was observed in PTEN level in the group of the Control (Supplementary Figures 2A-2B) ($p > 0.05$). MiR-21 inhibition increased PTEN concentration in cell lysates of dogs with CanL (Figure 3A) ($p = 0.0302$); in contrast, in SL transfected with miR-148a inhibitor, there was less PTEN than in samples from dogs with CanL (Figure 3B) ($p = 0.070$). Based on sequence homology with humans, we can infer that miR-148a binds the 3'UTR region of the PTEN transcript based on TargetScan analysis. We also performed mirDIP analysis, demonstrating target scores of miR-21 and miR-148a (Supplementary Table 7).

Figure 3. PTEN in cell lysates after transfection with miR-21 mimic and inhibitor in dogs with CanL, and miR-148a mimics and inhibitor in dogs with CanL

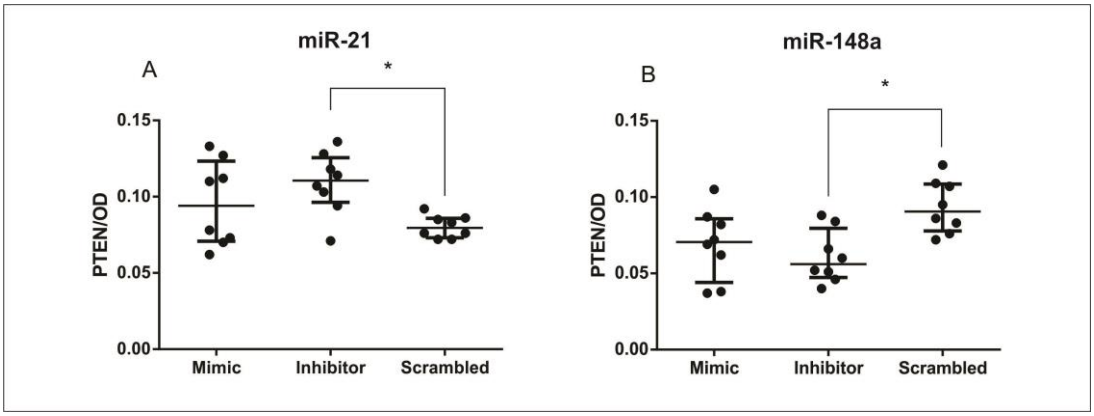

Figure 3. PTEN in cell lysates after transfection with miR-21 mimic and inhibitor in dogs with CanL (A); miR-148a mimics and inhibitor in dogs with CanL (B). Data are expressed as median and interquartile range. Analysis of variance with multiple comparisons ($p < 0.05$).

Source: Prepared by the author.

2.5.4 Effect of miR-21 and miR-148a on NO concentration in SLs of dogs with CanL

We evaluated the effect of miR-21 and miR-148a transfection of SLs with mimic and inhibitor from dogs with the control group and CanL on the regulation of NO production. Transfected SLs were cultured at 37 °C and 5% CO_2 for 48 h, and NO production was evaluated using flow cytometry. We did not find significant differences in the control group following treatment with the miR-21 mimic and inhibitor and miR-148a mimic and inhibitor (Supplementary Figures 3A-3B) ($p > 0.05$). By contrast, SLs from dogs with CanL transfected with miR-21 mimic, there was less NO than when scrambled (SCR) oligonucleotides were used as a transfection control ($p = 0.0469$) (Figure 4A). However, in SLs transfected with the miR-148a inhibitor, there was more NO production in dogs with CanL than when SCR oligonucleotides were used as a transfection control (Figure 4B) ($p = 0.0462$). A representative overlay histogram displays the action of mimic inhibiting NO production by miR-21 (Figure 4C) and the increase of NO production by miR-148a inhibitor (Figure 4D) in SLs from dogs with CanL.

Figure 4. NO in splenic cell leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL (A), and miR-148a in dogs with CanL (B)

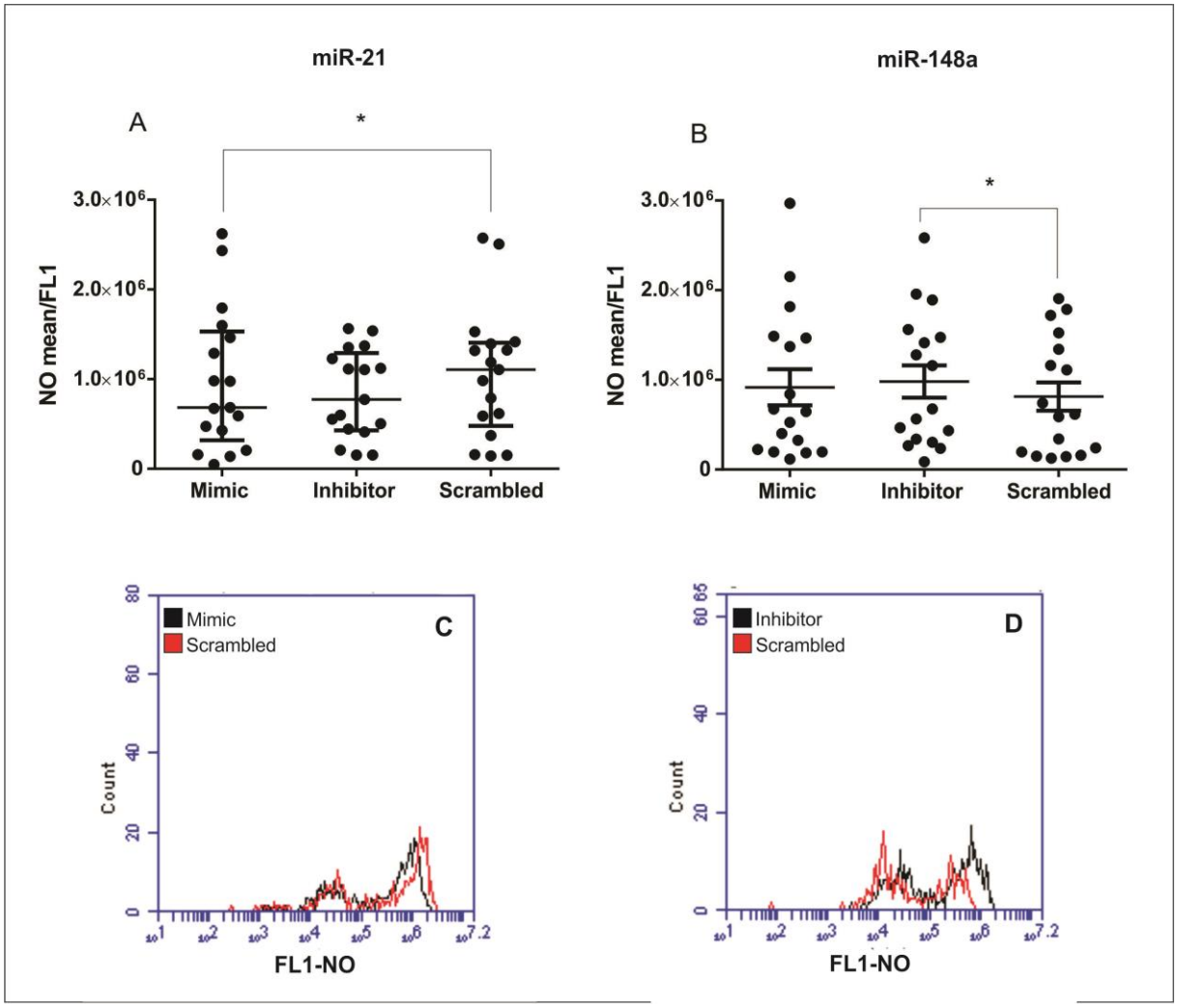

Figure 4. NO in splenic cell leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL (A), and miR-148a in dogs with CanL (B). Data are expressed as the median and interquartile range in (A) and data are expressed as the mean and standard error in (B). The representative overlay histogram of the action of miR-21 mimic in inhibiting NO production (C) and the increased NO production by the inhibitor of miR-148a (D) in splenic cells of dogs with CanL. The treatment with mimic and inhibitor is in black and the scramble is in red. Friedman test and analysis of variance with multiple comparisons ($p < 0.05$).

Source: Prepared by the author.

2.5.5 Effect of miR-21 and miR-148a on nitrite (NO_2) production in SLs of dogs with CanL

We evaluated the effect of miR-21 mimic and inhibitor and miR-148a mimic and inhibitor SL transfection on NO_2 in culture supernatants from SLs from dogs with the control group and CanL. Transfected SLs were cultured at 37 °C and 5% CO_2 for 48h,

and production was measured using the Greiss method. No significant difference was observed in the control group following treatment with the miR-21 mimic and inhibitor and miR-148a mimic and inhibitor in NO2 (Supplementary Figures 4A-4B) ($p > 0.05$). In culture supernatants from SLs of dogs with CanL transfected with miR-21 inhibitor, there was a more significant NO_2 concentration than with SCR oligonucleotides used as a transfection control (Figure 5A) ($p = 0.0359$). After transfection with miR-148a inhibitor in SL, there was less nitrite in culture supernatants in dogs with CanL (Figure 5B) ($p = 0.0069$).

Figure 5. NO_2 in splenic leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL, and miR-148a in dogs with CanL

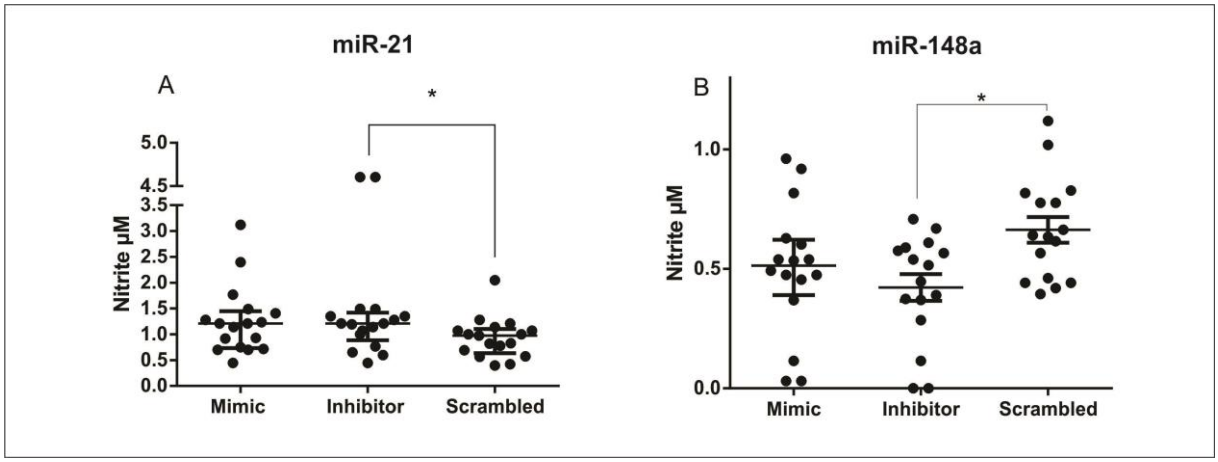

Figure 5. NO_2 in splenic leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL (A), and miR-148a in dogs with CanL (B). Data are expressed as the median and interquartile range in (A) and data are expressed as the mean and standard error of the mean in (B). We used the Friedman test in A, and analysis of variance (with multiple comparisons) and Dunnett's multiple comparison test in B ($p < 0.05$).

Source: Prepared by the author.

2.5.6 ROS concentration and the effect of miR-21 and miR-148a on the regulation of its production in SLs of dogs with CanL

We evaluated the effect of miR-21 and miR-148a transfection on ROS production in SL from dogs with the control group and CanL with mimic and inhibitor. No difference was observed after transfection with miR-21 mimic and inhibitor, and miR-148a mimic and inhibitor in the control group (Supplementary Figures 5A-5B) ($p > 0.005$); however, the transfection with miR-21 mimic gave rise to a decrease in ROS

production in dogs with CanL compared to scrambled (SCR) oligonucleotides used as a transfection control (Figure 6A) (p = 0.0469). After transfection with miR-148a inhibitor in SLs, ROS production was reduced in dogs with CanL (Figure 6B) (p = 0.0468). A representative overlay histogram of the action of mimic on the inhibition of ROS production by miR-21 (Figure 6C) and the inhibition of ROS production by the miR-148a inhibitor (Figure 6D) was shown in SLs from dogs with CanL.

Figure 6. ROS in splenic leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL, and miR-148a in dogs with CanL

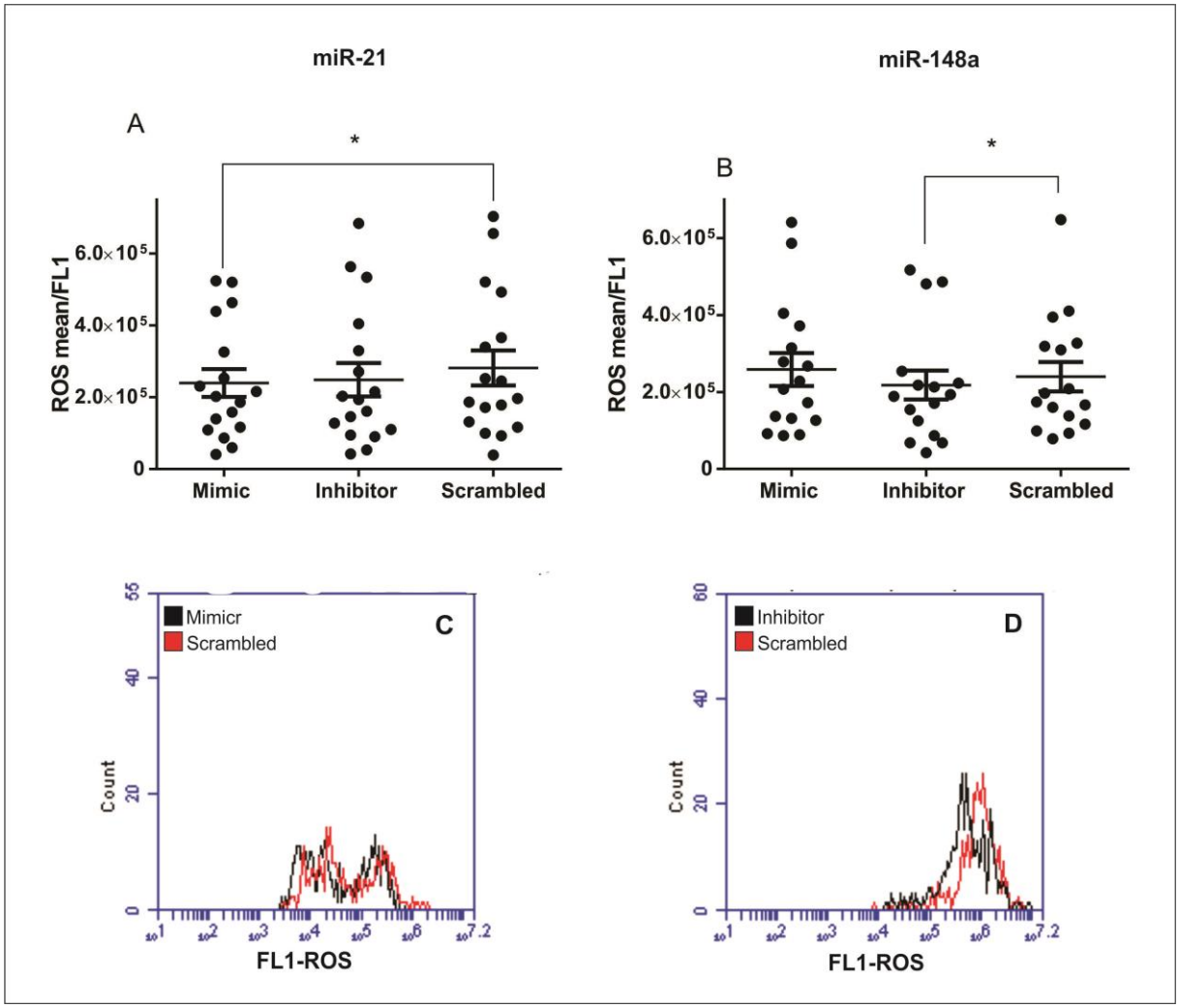

Figure 6. ROS in splenic leukocytes after transfection with miR-21 mimic and inhibitor in dogs with CanL (A), and miR-148a in dogs with CanL (B). Data are expressed as the median and interquartile range in (A) and as the mean and standard error in (B). Representative histogram of the action of mimics on ROS production by miR-21 (C) and ROS inhibition by miR-148a inhibitor (D) in splenic leukocytes from dogs with CanL. The treatment with mimics and inhibitor is represented by black and the scramble by red. We performed the Friedman test and analysis of variance with multiple comparisons ($p < 0.05$).

Source: Prepared by the author.

2.6 Discussion

SLs from dogs with visceral leishmaniasis show increased miR-21 and miR-148 targeting PTEN (Melo et al., 2019). PTEN has several regulatory roles in the immune system, including shifting the macrophage profile from M2 to M1 (Sahin et al., 2014; Liu et al., 2020). To clarify the regulatory role of miR-21 and miR148a targeting PTEN in canine visceral leishmaniasis, SLs from CanL were transfected with mimic and inhibitor of miR-21 and miR-148a, and we measured PTEN, NO, and ROS levels.

PTEN concentrations increased in lysates from SLs from dogs with CanL. In contrast, less PTEN mRNA was observed during the active stage of *Leishmania donovani* infection in human splenic cells (Sudarshan et al., 2016). This difference can be related to mRNA detection instead of protein, as in our study.

High PTEN levels were associated with decreased NO and ROS production by SLs, suggesting a possible inhibition of inflammatory response in CanL. Higher numbers of M2 macrophages were observed in spleens in canine visceral leishmaniasis (Moreira et al., 2016). M2 polarization of macrophages with decreased NO and ROS possibly favors parasite survival. These findings suggest that *Leishmania* modulates PTEN, NO, and ROS levels; nevertheless, more studies are needed to clarify this mechanism.

In our study, NO concentration was lower in splenic cells in the CanL group, confirming a result previously observed in CanL (Martini et al., 2018). NO production was associated with microbicidal activity in CanL (Vouldoukis et al., 1996), and its low production by macrophages could explain the high parasite replication observed in the spleens of the CanL group.

We observed low concentrations of ROS in splenic cells in CanL. Similarly, splenic cells from mice infected with *L. donovani* showed low production of ROS (Ball et al., 2011). In peripheral blood mononuclear cells from humans with visceral leishmaniasis, the parasite decreased the expression of NADPH oxidase, preventing respiratory bursts (Kumar et al., 2002). The parasite expresses lipophosphoglycan, glycoinositol phospholipids, and ceramides that inhibit the protein kinase C activation pathway associated with ROS production (Atayde et al., 2016). In CanL, this low production of ROS might facilitate disease progression. Our findings suggest that *Leishmania* regulates host molecules to decrease microbicidal activity.

Low ROS and NO in SLs from dogs with CanL were associated with high PTEN levels in cell lysates. Upregulation of PTEN causes modulation of PI3K/AKT signaling to reduce ROS generation (Xu et al., 2011). The PI3K/AKT/PTEN pathway is involved in the defense mechanisms against *Leishmania amazonensis*; iNOS downregulation in infected macrophages depends on PI3K/Akt activation (Calegari-Silva et al., 2015), and PTEN negatively regulates the activity of PI3K/AKT signaling by converting PIP3 to PIP2, by which PTEN exerts its tumor-suppressive effect. Notably, the intracellular growth of the parasite was impaired during PI3K/Akt signaling inhibition and in macrophages with knocked-down Akt 1 expression (Calegari-Silva et al., 2015). Our findings suggest that *Leishmania* regulates PTEN in CanL; however, it is necessary to investigate the participation of the PI3K/AKT pathway.

PTEN negatively regulated the expression of pro-inflammatory cytokines such as TNF-alpha in an experimental model of adjuvant-induced arthritis (Li et al., 2019). PTEN was associated with the downregulation of inflammatory cytokines in humans (Yan et al., 2019). In dogs with CanL, TNF is markedly reduced with increased parasite load (Cavalcanti et al., 2015). TNF enhances nitric oxide production in canine macrophages (Pinelli et al., 2000). Therefore, probable PTEN may regulate cytokines; however, this finding must be confirmed in CanL.

Inhibition of mir-21 in SLs increased PTEN concentrations in cell lysate from SLs in CanL; when bone marrow-derived stem cells from dogs were transfected with the miRNA-21 inhibitor, PTEN was upregulated (Yang et al., 2019). Studies with human cancer showed that downregulation of miR-21 increased PTEN expression, inhibited PI3K/AKT activity, promoted apoptosis, and reduced proliferation (Liu et al., 2019, Meng et al., 2007). In human cells, miR-21 modulated gene expression directly at the PTEN 3′-UTR (Meng et al., 2007); this mechanism could occur in dogs with CanL. Molecular studies are necessary to test this hypothesis. We suggest that *Leishmania* has the potential to change immune response through miR-21, which has the PTEN pathway as a target.

We showed an increased PTEN by miR-21 inhibition in SLs occurred while nitrites increased. The miR-21 mimic reduced NO, killing leishmania in dogs (Vouldoukis et al., 1996). Interestingly *in vitro* study shows PTEN-deficient macrophages reduce their ability to kill *Leishmania* in response to IFN-γ treatment, possibly because the mutant cells exhibited decreased TNF secretion that correlated with reductions in inducible nitric oxide synthase expression and nitric oxide production

(Kuroda et al., 2008). In our study, high PTEN associated with inhibited miR-21 induced high nitrite, suggesting a role for PTEN and miR-21 in parasitic control in CanL.

We observed low ROS concentration in splenic cells in CanL after transfection with miR-21 mimic. In a *Leishmania* infection mice model, the spleens of miR-21 knockout mice expressed higher transcripts of M2 macrophages (Varikuti et al., 2021), which are responsible for producing low ROS, impairing a defense mechanism that controls the disease in mice (Gonçalves et al., 2018). Our findings suggest that ROS is regulated by miR-21; however, further studies are needed to clarify these mechanisms and understand how this phenomenon affects immune response in CanL.

In the present study, inhibition of miR-148a in SLs reduced PTEN; inhibition of miR-21 increased PTEN concentration in cell lysates from SLs in CanL. The luciferase assay showed that PTEN is a target of miR-148a in mice, and bioinformatics analyses showed that miR-148a binds the 3' UTR of the PTEN gene (Qingjuan et al., 2016). We performed bioinformatics analysis and observed that miR-21 could regulate the PTEN pathway, while miR-148a has PTEN as a direct target. These findings suggest that PTEN expression can be regulated by more than one miRNA in CanL.

We observed SLs transfected with the miR-148a inhibitor there was more significant NO production, in contrast miR-148a mimic also decrease iNOS in SL in CanL (Rebech et al., 2023), and similar study observed that overexpression of miR-148a in peripheral blood cells of rats was associated with low iNOS (Yin et al., 2021). NO production by macrophages participates in the elimination of *Leishmania* and can be involved in Th1-type immune responses (Reza et al., 2019). These findings suggest that miR-148a participates in NO regulation and that *Leishmania* could use miR-148a expression to favor its survival.

The miR-148a inhibitor decreased ROS concentrations in dogs with CanL. Interestingly, in mice bone marrow, the overexpression of miR-148a-3p in macrophages promoted excessive production of ROS and was responsible for the bactericidal activity of M1 macrophages through the PTEN/AKT pathway (Huang et al., 2017). ROS production from macrophages during *Leishmania* infection is responsible for eliminating the parasite (Das et al., 2021). The PI 3-Kinase pathway promotes oxidative stress with ROS production and can involve PTEN (Leslie et al., 2003). *Leishmania* could use this pathway to decrease ROS levels in CanL as a survival mechanism. We suggest further studies to confirm this finding.

We conclude that miR-21 and miR-148a participate in the immune responses against *Leishmania* by targeting the PTEN pathway. The *Leishmania* parasite can regulate ROS and NO production through miR-21 and miR148a expression for its survival in the host. These findings may contribute to future therapies targeting these microRNAs for treating CanL.

2.7 Conflict of Interest

There are no conflicts of interest.

2.8 Funding

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2.11 Supporting information

Table S1. Serology test for detection of anti-*leishmania sp* antibodies and main clinical signs of dogs with CanL and healthy.

(To be continued)

Animal	D.O. ELISA	Sex	Clinical signs
CanL 1	1.132	F	Onychogryphosis, skin lesions, cachexia, seborrhea.
CanL 2	1.060	M	Lymphadenopathy, onychogryphosis, cachexia, and skin lesions.
CanL 3	0.920	M	Cachexia, seborrhea and skin lesions.
CanL 4	0.473	M	Lymphadenopathy, skin lesions, hepatosplenomegaly.
CanL 5	1.373	F	Onychogryphosis, cachexia, alopecia, skin lesions.
CanL 6	1.207	M	Lymphadenopathy, onychogryphosis, cachexia, alopecia, paw lesions.
CanL 7	1.288	F	Lymphadenopathy, onychogryphosis, seborrhea, alopecia, periocular lesion, hepatosplenomegaly.
CanL 8	1.267	F	lymphadenopathy, onychogryphosis, seborrhea, alopecia, skin lesions, hepatosplenomegaly.

Table S1. Serology test for detection of anti-*leishmania sp* antibodies and main clinical signs of dogs with CanL and healthy.

(Continuation)

Animal	D.O. ELISA	Sex	Clinical signs
CanL 9	1.048	F	Lymphadenopathy, onychogryphosis, periocular lesion, hepatosplenomegaly.
CanL 10	0.968	F	lymphadenopathy, onychogryphosis, cachexia
CanL 11	0.649	F	Onychogryphosis, cachexia, seborrhea, body lesions, ear, skin, snout, paws
CanL 12	1.219	M	Lymphadenopathy, cachexia, seborrhea, lesions on the body, ear, skin, snout, paws, periocular and hepatosplenomegaly
CanL 13	1.192	M	Lymphoadenomegaly, cachexia, seborrhea, ear, snout and periocular lesions
CanL 14	0.666	M	Cachexia, ectoparasites, ear, skin, paws and periocular lesions
CanL 15	1.072	F	Cachexia, skin and periocular lesions
CanL 16	1.007	F	Onychogryphosis, seborrhea, ear and periocular lesions

Table S1. Serology test for detection of anti-*leishmania sp* antibodies and main clinical signs of dogs with CanL and healthy.

(Conclusion)

Animal	D.O. ELISA	Sex	Clinical signs
CanL 17	0.759	M	Onychogryphosis, paw lesions and seborrhea
1	0.062	F	-
2	0.026	M	-
3	0.147	F	-
4	0.028	M	-
5	0.071	F	-

CanL= Canine visceral leishmaniasis.

Source: Prepared by the author.

Table S2. Red blood count in CanL and Healthy dogs groups.

Animal	RBC	Hemoglobin	GV	MCV	MCHC
Reference	**5.5-8.5 x 10^{12}/L**	**12.0-18.0g/dl**	**37-55 %**	**60-77 fL**	**32-36 %**
CanL 1	4,25	10,1	29	68,24	34,83
CanL 2	3,88	8,6	26	67,01	33,08
CanL 3	4,05	7,8	25	61,73	31,2
CanL 4	2,93	6,7	22	75,09	30,45
CanL 5	2,95	6,5	18	61,02	36,11
CanL 6	4,3	9,5	27	62,79	35,19
CanL 7	3,34	6,2	18	53,89	34,44
CanL 8	4,06	10	28	68,97	35,71
CanL 9	2,1	4,1	12	57,14	34,17
CanL 10	3,81	8,7	25	65,62	34,8
CanL 11	5,32	11,4	34	63,91	33,53
CanL 12	4,55	10,5	32	70,33	32,81
CanL 13	4,91	11,6	34	69,25	34,12
CanL 14	5,5	14	39	70,91	35,9
CanL 15	4,55	10,4	32	70,33	32,5
CanL 16	5,77	12,3	37	64,12	33,24
CanL 17	5,33	11,7	35	65,67	33,43
1	7,89	17,8	53	67,17	33,58
2	6,78	16,9	49	72,27	34,49
3	7,89	17,8	52	65,91	34,23
4	6,78	17,3	50	73,75	34,6
5	7,44	17,1	51	68,55	33,53

CanL = Canine visceral leishmaniasis; RBC = red blood cells; GV = globular volume; MCV = mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration.

Source: Prepared by the author.

Table S3: White blood cells and platelets count in CanL and Healthy dogs.

Animals	Leukocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Lymphocytes	Platelets
Reference	6.0-17.0 x 10^9/L	3000-11500 x 10^6/L	150-1250 x 10^6/L	Rare	150-1350 x 10^6/L	1000-4800 x 10^6/L	160-400 x 10^3/L
CanL 1	5,4	4212	108	0	486	594	220
CanL 2	13,3	11172	0	0	133	1995	160
CanL 3	9,9	6831	198	0	396	2475	220
CanL 4	7,1	4686	0	0	284	2130	300
CanL 5	9,3	6417	186	0	837	1860	280
CanL 6	7,2	4320	72	0	288	1800	140
CanL 7	8,1	5022	0	0	81	2997	180
CanL 8	8,5	6035	0	0	425	2040	200
CanL 9	3	2100	90	0	30	780	220
CanL 10	14,6	10950	146	0	146	3358	280
CanL 11	8	6080	160	0	160	1600	400
CanL 12	7,8	5226	234	0	78	2262	280
CanL 13	10,8	8748	0	0	324	1728	380
CanL 14	7,4	5772	222	0	444	962	400
CanL 15	7,1	4047	0	0	142	2911	180
CanL 16	17	12240	1870	0	850	2040	280
CanL 17	6	4020	120	0	60	1800	160
1	16,2	10692	1250	0	1496	2754	200
2	15,7	10048	2041	0	628	2983	300
3	12,2	6588	122	0	610	4800	320
4	16,2	10854	1296	0	324	3726	220
5	10,3	6077	103	0	515	3605	220

CanL = Canine visceral leishmaniasis.

Source: Prepared by the author.

Table S4: Serum biochemical of CanL and Healthy dogs.

Animals	Albumin	Globulin	Total protein	Creatinine	Urea	ALT	Alkaline Phosphatase	GGT
Reference	2.6-3.3 g/L	2.7-4.4 g/L	5.4-7.1 g/L	0.5-1.5 mg/dL	10.03-50.03 mg/dL	21-102 UI/L	20-156 UI/L	1.2-6.4 UI/L
CanL 1	1,6	8,8	10,4	0,6	42	26	66	5,9
CanL 2	1	6,6	7,6	0,7	33	42	243	2,9
CanL 3	1,8	9	10,8	0,7	30	37	75	6
CanL 4	1,9	6,5	8,4	1	45	21	24	1,3
CanL 5	0,94	6,1	7	0,8	70	21	29	4,9
CanL 6	1,1	5,2	6,3	1	60	21	109	1,9
CanL 7	1,5	5,6	7,1	0,5	30	37	22	2,9
CanL 8	1,9	6,8	8,7	0,7	49	92	92	1
CanL 9	2,2	4,6	6,8	0,5	35	70	70	1,5
CanL 10	1,2	7,6	8,8	1,5	120	30	42	1,4
CanL 11	2	8,4	10,4	0,8	42	25	23	2,8
CanL 12	1,6	7,9	9,5	1	51	37	67	2,1
CanL 13	1,8	7,6	9,4	0,9	35,4	15	57	6
CanL 14	3	4,2	7,2	0,7	32	160	42	4
CanL 15	1,2	6,1	7,3	0,8	61	94	268	9
CanL 16	2,1	6,7	8,8	0,9	23	18	97	4
CanL 17	2,4	6,3	8,7	0,7	45	35	38	3,1
1	3,3	3,7	7	0,9	25	80	74	2,6
2	2,9	3,8	6,7	0,7	30	105	148	5,9
3	3,4	3,5	6,9	1	27	40	36	2,9
4	2,9	4,1	7	1	50	32	33	1,2
5	2,7	3,9	6,6	1,1	21	50	85	1,9

CanL= Canine visceral leishmaniasis.

Source: Prepared by the author.

Table S5. Transfection rate.

Animal	Transfection rate %
CanL 1	17
CanL 2	11
CanL 3	41
CanL 4	30
CanL 5	25
CanL 6	42
CanL 7	20
CanL 8	22
CanL 9	20
CanL 10	22
CanL 11	17
CanL 12	26
CanL 13	16
CanL 14	22
CanL 15	16
CanL 16	16
CanL 17	28
1	24
2	13
3	12
4	10
5	48

CanL= Canine visceral leishmaniasis.

Source: Prepared by the author.

Table S6. Comparative analysis between PMA and ROS production in the CanL group.

Animal	Medium	PMA
1	352846,90	156310,70
2	241700,80	503176,30
3	292561,30	545522,10
4	142972,40	252162,70
5	353418,30	468742,60
6	73461,24	117498,50
7	48932,90	50086,47
8	210101,00	571714,30
9	73890,44	191610,10
10	232919,80	121382,80
11	139981,30	393442,60
12	124148,80	258129,50
13	198228,90	263002,40
14	303754,70	318072,90
15	200801,90	448691,90
16	135906,30	444075,30
17	112695,80	257676,70

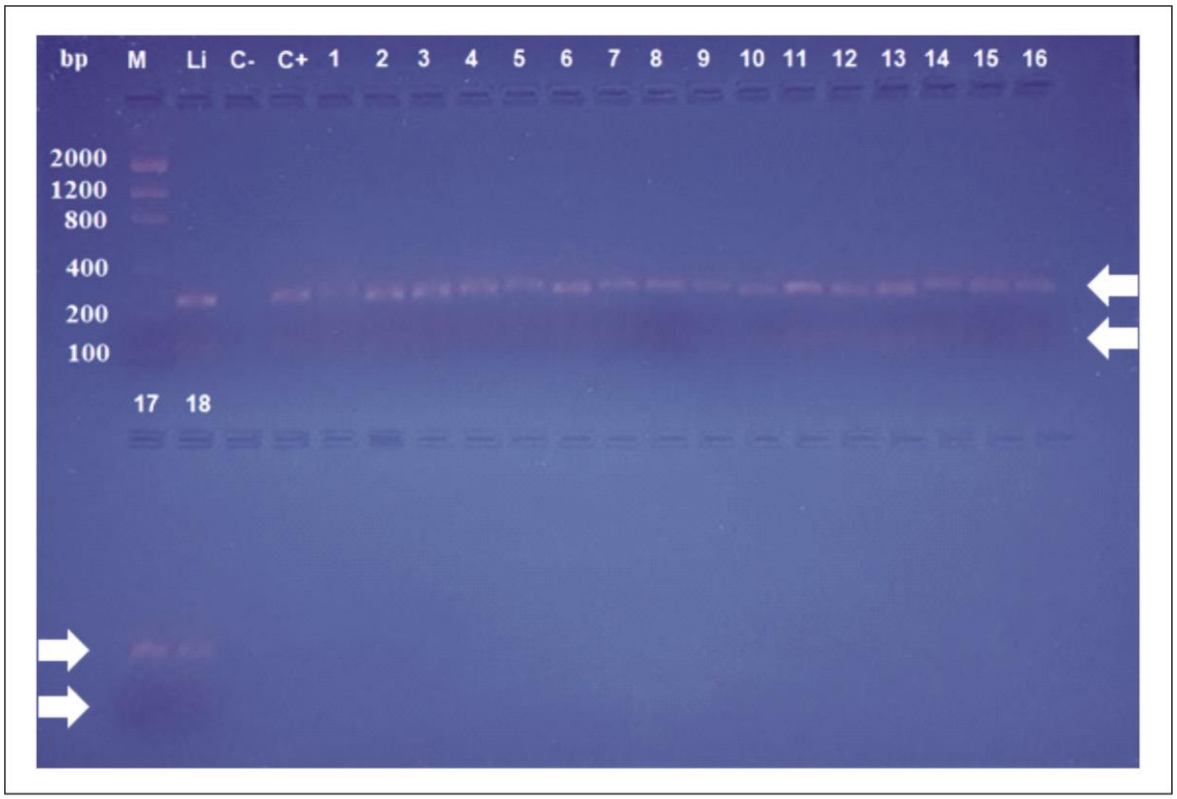
Source: Prepared by the author.

Table S7. MiRDIP analysis.

MiR-21	
Target	**MiRDIP (Integrated Score)**
BMPR2	0.85
FASLG	0.76
PIK3R1	0.85
TGFBR2	0.80
MiR-148a	
Target	**MiRDIP (Integrated Score)**
NRAS	0.84
PTEN	0.89
SOS2	0.92

Source: Prepared by the author.

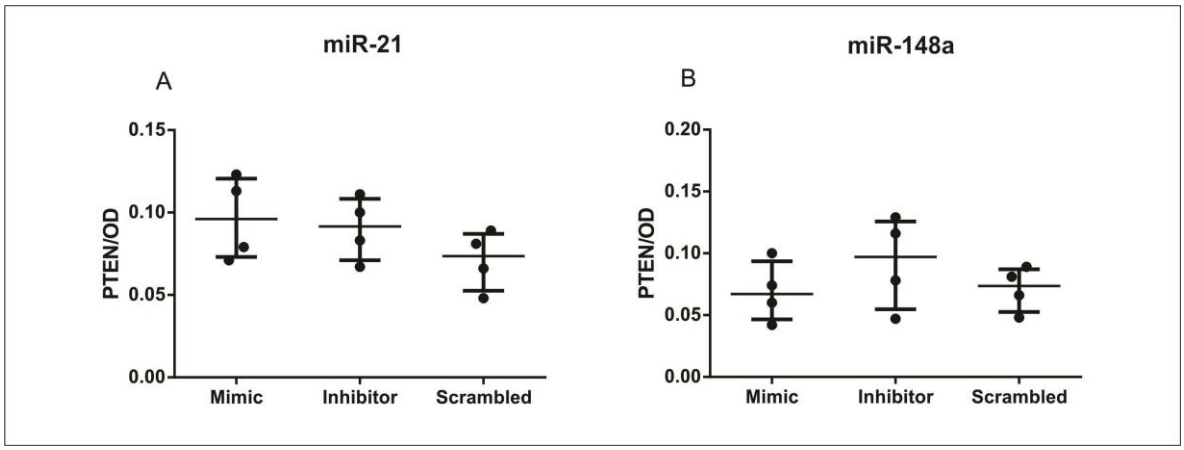
Supplementary Figure 1. PCR-RFLP



Supplementary Figure 1: Restriction fragment length polymorphism analysis with ITS1-PCR fragments amplified from DNA samples using Hae III. M: molecular marker (100 bp); C-: Negative control (water); C+: positive control; Li: *Leishmania infantum* (IOC / L0575-MHOM / BR / 2002 / LPC – RPV); The samples were identified as one to 18 and are identical to the *L. infantum* profile.

Source: Prepared by the author

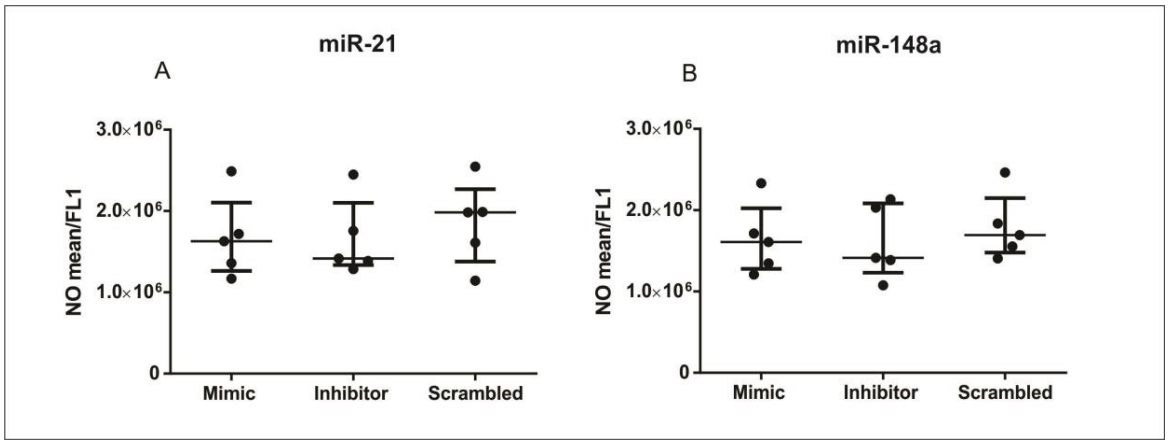
Supplementary Figure 2. Effect of miR-21 and miR-148a on PTEN concentration in SLs of control dogs.



Supplementary Figure 2: PTEN in cell lysates after transfection with miR-21 mimic and inhibitor in control group (A); miR-148a mimic and inhibitor in control group (B). Data are expressed as median and interquartile range. Analysis of variance with multiple comparisons ($p > 0.05$).

Source: Prepared by the author

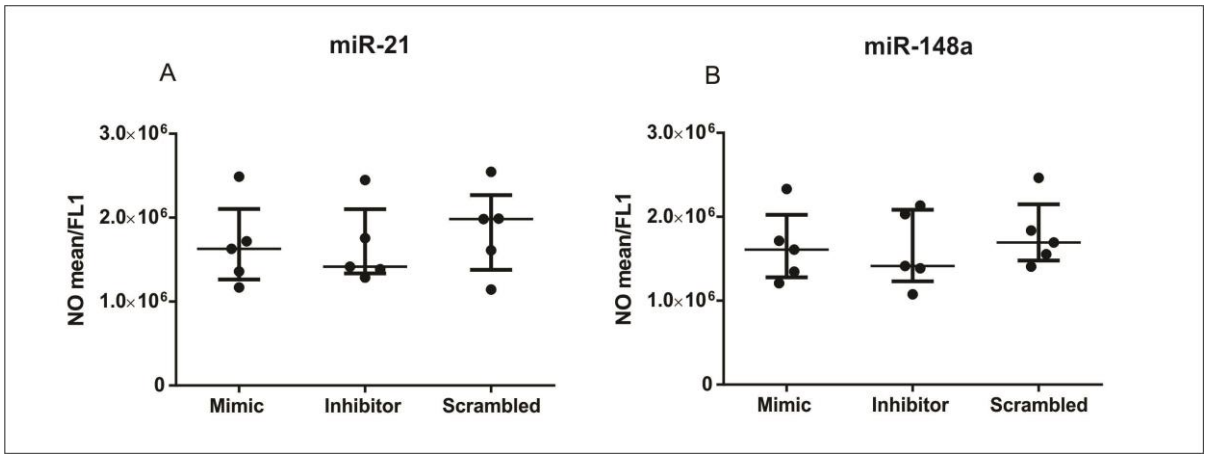
Supplementary Figure 3. Effect of miR-21 and miR-148a on NO concentration in SLs of control dogs.



Supplementary Figure 3: NO in splenic cell leukocytes after transfection with miR-21 mimic and inhibitor in control group (A), and miR-148a in control group (B). Data are expressed as the median and interquartile range in (A) and (B). Friedman test and analysis of variance with multiple comparisons ($p > 0.05$).

Source: Prepared by the author

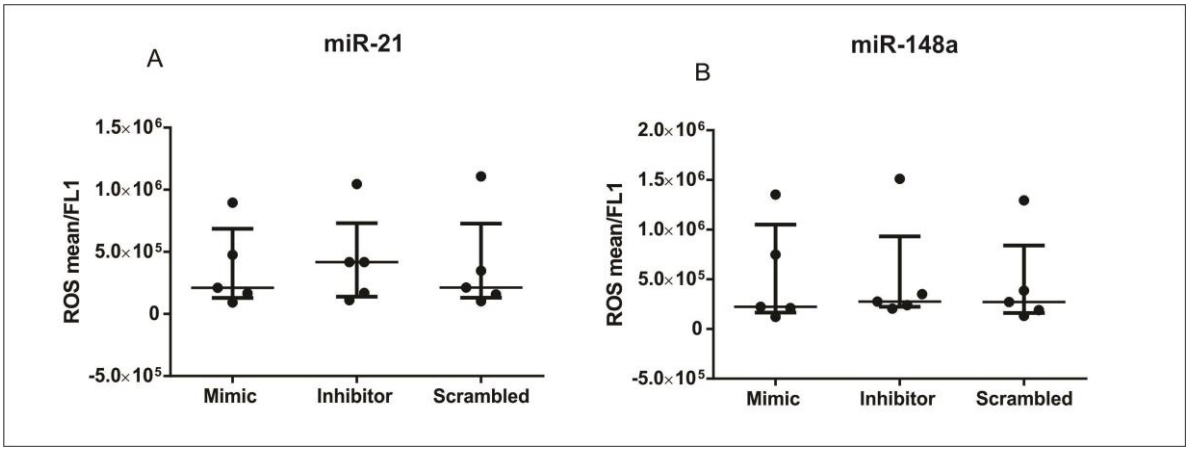
Supplementary Figure 4. The effect of miR-21 and miR-148a on nitrite production in SLs of control dogs.



Supplementary Figure 4: Nitrite in splenic leukocytes after transfection with miR-21 mimic and inhibitor in control group (A), and miR-148a in control group (B). Data are expressed as the median and interquartile range in (A) and (B). The analysis was performed with Friedman test and analysis of variance ($p > 0.05$).

Source: Prepared by the author

Supplementary Figure 5. ROS concentration and the effect of miR-21 and miR-148a on the regulation of its production in SLs of control dogs.



Supplementary Figure 5: ROS in splenic leukocytes after transfection with miR-21 mimic and inhibitor in control group (A), and miR-148a in control group (B). Data are expressed as the median and interquartile range in (A) and (B). We perfomed the Friedman test and analysis of variance with multiple comparisons ($p > 0.05$).

Source: Prepared by the author

APÊNDICE A – REFERÊNCIAS DA INTRODUÇÃO GERAL

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Acknowledgements

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Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

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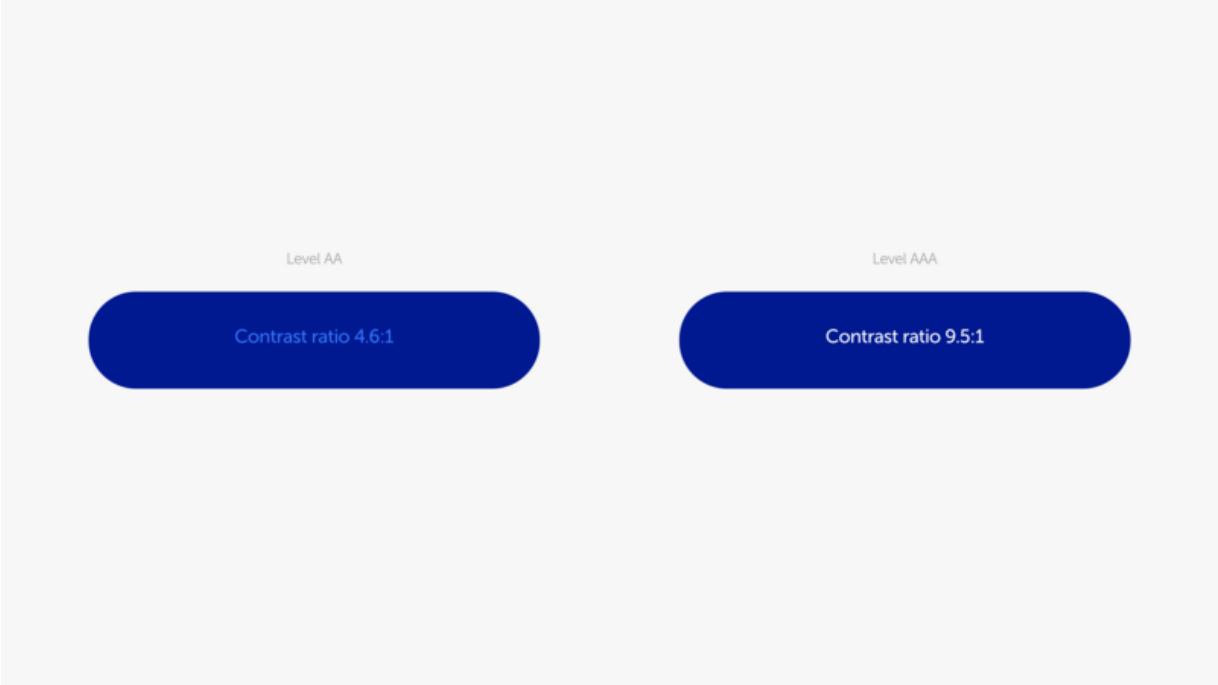

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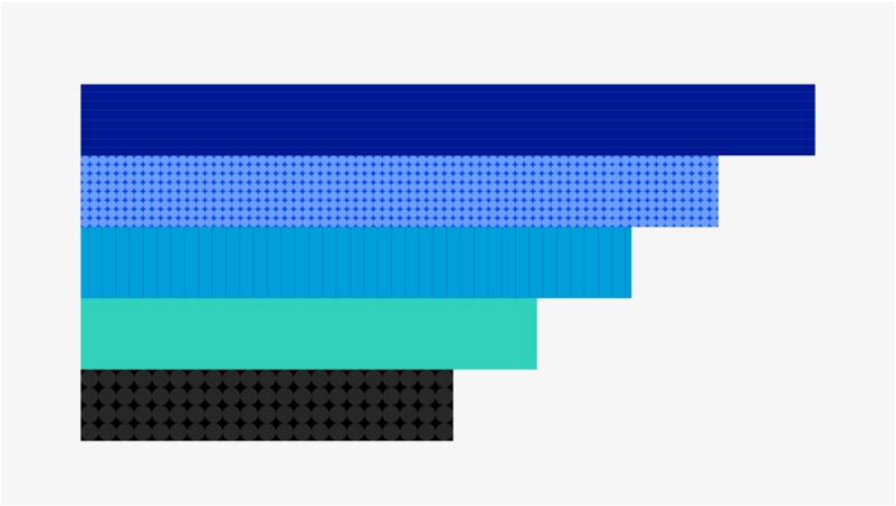

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Website
World Health Organization. (2018). E. coli. https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent
Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and dissertations
Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint
Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

Vancouver reference style (numbered)

Many Frontiers journals use the numbered referencing system; to find the correct reference style and resources for the journal you are submitting to, please visit our help center.

Reference examples are found below, for more examples of citing other documents and general questions regarding the Vancouver reference style, please refer to Citing Medicine.

In-text citations

Please apply the Vancouver system for in-text citations

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis (use square brackets for physics and mathematics articles).

Reference list examples

Article in a print journal
Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal
Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book
Sorenson PW, Caprio JC. "Chemoreception". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book
Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract
Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Website
World Health Organization. E. coli (2018). https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent
Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

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Preprint

Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

ANEXO B – CERTIFICADO DO COMITÊ DE ÉTICA

unesp

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

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CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"miR 21 e miR148a na regulação da resposta imunológica de leucócitos esplênicos na Leishmaniose Visceral Canina"**, Processo FOA nº 00624-2018, sob responsabilidade de Valéria Marçal Félix de Lima apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 14 de Agosto de 2018.

VALIDADE DESTE CERTIFICADO: 01 de Novembro de 2021.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 Dezembro de 2021.

CERTIFICATE

We certify that the study entitled **"miR 21 e miR 148a in regulation of immune response of splenic leukocytes in Canine Visceral Leishmaniasis"**, Protocol FOA nº 00624-2018, under the supervision of Valéria Marçal Félix de Lima presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on August 14, 2018.

VALIDITY OF THIS CERTIFICATE: November 01, 2021.
DATE OF SUBMISSION OF THE FINAL REPORT: December 01, 2021.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
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Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br