**UNIVERSIDADE ESTADUAL PAULISTA**

**"JÚLIO DE MESQUITA FILHO"**

**FACULDADE DE MEDICINA VETERINÁRIA**

**CÂMPUS DE ARAÇATUBA**

# CARACTERIZAÇÃO DO MICROTRANSCRIPTOMA DE MACRÓFAGOS INFECTADOS POR CEPAS SAPRÓFITA, ATENUADA OU VIRULENTA DE *Leptospira* spp.

**Leandro Encarnação Garcia**

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**Leandro Encarnação Garcia**

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Tese apresentada à Faculdade de Medicina Veterinária – Unesp, Campus de Araçatuba, como parte das exigências para a obtenção do título de Doutor em Ciência Animal (Medicina Veterinária Preventiva e Produção Animal).

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## DADOS CURRICULARES DO AUTOR

**LEANDRO ENCARNAÇÃO GARCIA –** Nascido em 18 de março de 1986, na cidade de Pelotas, interior do Rio Grande do Sul. Graduado em Ciências Biológicas em 2012 pela Universidade Federal de Pelotas. No segundo ano da graduação, comecei a lecionar no curso Pré-Vestibular gratuito da universidade, onde fiquei até 2013. Durante a graduação, trabalhei com o Ensino de Ciências, projeto que concluí com artigos publicados. Além disso, realizei projeto de Iniciação Científica na área de Biodiversidade, na qual realizei também o Mestrado, concluído em 2015, na Universidade Federal de Pelotas. Em seguida, ingressei no doutorado no Programa de Pós-graduação em Ciência Animal da FMVA-UNESP, na área de Epigenética, sob a orientação da Dra. Flavia Lopes. Contei com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) durante o desenvolvimento do doutorado. Acredito que nesse período de doutoramento, cresci como professional e aprendi muito sobre Ciência, Pesquisa e práticas laboratoriais, juntamente com professores e colegas. O projeto desenvolvido durante o doutorado contou com auxílio financeiro da Fapesp, permitindo a publicação dos resultados em revistas internacionais antes da defesa do doutorado.

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# SUMÁRIO

Página

**CARACTERIZAÇÃO DO MICROTRANSCRIPTOMA DE MACRÓFAGOS INFECTADOS POR CEPAS SAPRÓFITA, ATENUADA OU VIRULENTA DE *Leptospira* spp.**

**RESUMO -** Leptospirose é considerada uma zoonose de importância global causada pela bactéria *Leptospira* spp. A incidência da doença chega a um milhão de casos severos no mundo, atingindo uma mortalidade de 60.000 óbitos/ano. Apesar de considerados avanços na pesquisa sobre a doença, há muito ainda a se descobrir sobre sua patogenicidade. A influência de mecanismos epigenéticos em processos patológicos, particularmente da regulação pós-transcricional mediada por RNAs não-codificadores, é fundamental. Uma gama de estudos vem relatando a influência desses mecanismos na resposta imune do hospedeiro em uma variedade de infecções bacterianas. O objetivo desse estudo foi identificar e caracterizar, pela primeira vez, a influência de miRNAs na regulação da resposta de macrófagos frente a infecção por *Leptospira*. Foi realizado um microtranscriptoma, através da técnica de microarranjo, em macrófafgos murinos J774A.1 desafiados por cepa virulenta, atenuada e saprófita de *Leptospira* após 8h de infecção. Após análise dos dados, identificamos 29 miRNAs modulados pela infecção comparados ao controle, com fold change ± 1.5 e p-valor<0,01. Na análise de enriquecimento funcional, encontramos um grande número de alvos para os miRNAs modulados participando em processos-chave da resposta imune. Podemos sugerir que a regulação pós-transcricional por miRNAs possui papel na resposta do hospedeiro em infecção por Leptospirose, e que essa resposta é dependente da espécie, bem como da virulência da bactéria.

**Palavras-Chave:** leptospirose, macrófagos, microRNA

# MICROTRANSCRIPTOME CHARACTERIZATION OF MACROPHAGES INFECTED WITH VIRULENT, ATTENUATED OR SAPROPHYTE STRAINS OF *Leptospira* spp.

**SUMMARY -** Leptospirosis is a bacterial zoonosis of global importance, caused by *Leptospira*. The incidence of disease leading to one million severe cases and 60,000 deaths per year. Despite considerable advances in research, much is yet to be discovered about disease pathogenicity. The influence of epigenetic mechanisms, particularly RNA-mediated post-transcriptional regulation of host immune response has been proven vital following a variety of bacterial infections. The aim of this study was to identify and characterize, for the first time, the influence of miRNAs on the regulation of the immune response to *Leptospira* infection. We examined the microtranscriptome, trough microarray technique, of macrophages J774A.1 following an 8h infection with virulent, attenuated and saprophyte strains of *Leptospira*. Microarray analysis revealed that 29 miRNAs were de-regulated by the *Leptospira* strains compared to control with fold change ±1.5 and p-value <0.01. Enrichment analyses of the targets of these differentially expressed miRNAs suggest that several processes involved in immune response are directly regulated by miRNAs. We suggest that post-transcriptional regulation by miRNAs may play a role in host response to infection in leptospirosis, and that this response is dependent on species and bacterial virulence.

**Keywords:** leptospirosis, macrophage, miRNA

# CAPITULO 1 – CONSIDERAÇÕES GERAIS

## 1 Contextualização do Problema

Todos os organismos devem ter a capacidade de reconhecer estímulos ambientais e responder de forma apropriada a estes estímulos, e muitas destas respostas são mediadas por fatores de transcrição que sinalizam o DNA (ácido desoxirribonucleico) e RNA (ácido ribonucleico) para a produção de proteínas (MATTICK, 2001; EDWARDS & BATEY, 2010). Há algumas décadas, regiões de RNA não codificadores foram descobertas com função de regular a transcrição e/ou tradução (HOUMAN et al., 1990; AYMERICH; STEINMETZ, 1992; ARNAUD et al., 1996). Desde então, mecanismos regulatórios baseados no RNA, como os microRNA (miRNAs), vêm sendo investigados (MATTICK, 2001; COSTA, 2007).

Os miRNAs são pequenas moléculas de ácidos nucleicos, com tamanho aproximado de 20-22 nucleotídeos, que contribuem no controle pós-transcricional de expressão gênica (BARTEL, 2004; HE AND HANNON, 2004; FILIPOWICZ et al., 2008; KROL et al., 2010; CALDELARI et al., 2013), tendo importante papel em quase todos os organismos eucariotos (BARTEL, 2004; AMBROS, 2004). Basicamente, essas pequenas moléculas (miRNAs) regulam a síntese de proteínas através do pareamento de bases com sequências parcialmente complementares na região 3’ UTR (regiões não-codificantes) de alvos RNA mensageiros (RNAm), favorecendo sua degradação ou reprimindo sua tradução (BARTEL, 2009; HUNTZINGER AND IZAURRALDE, 2011; FABIAN AND SONENBERG, 2012).

Células de organismos expressam diversos miRNAs que participam de muitos processos biológicos como desenvolvimento embrionário, diferenciação celular e apoptose (HE AND HANNON, 2004; AMBROS, 2004), e a sua importância no sistema imune de mamíferos já foi previamente reportada

(LODISH et al., 2008; O'CONNELL et al., 2012). Experimentos recentes indicam que vários patógenos, como vírus, parasitas e bactérias, conduzem mudanças no perfil de expressão de miRNAs de células hospedeiras (CULLEN, 2011; EULALIO et al., 2012). Por essa razão, a identificação dos alvos da expressão diferencial de miRNAs após infecção por patógenos são de grande importância para a compreensão da resposta imune do hospedeiro. A patogenicidade de bactérias requer do hospedeiro um alto controle de genes envolvidos na resposta à infecção. Como parte da resposta, fatores de transcrição dos hospedeiros podem ativar ou desativar um grupo de genes (PAPENFORT & VOGEL, 2010) e os miRNAs envolvidos na regulação da tradução possuem também importantes funções em infecções bacterianas.

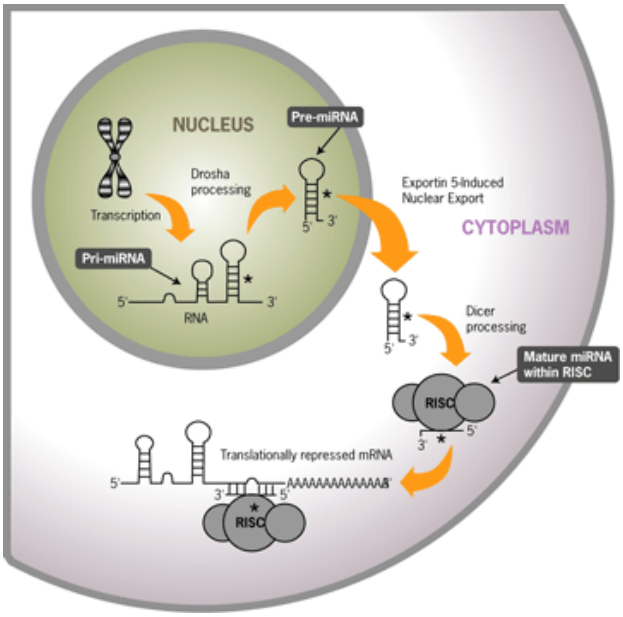
Bactérias fazem parte do grupo de organismos mais diverso do planeta, desenvolvendo processos-chave no meio ambiente (EMBLEY et al., 1994). A maioria das bactérias são inócuas e até favoráveis aos mamíferos, no entanto, existe um considerável número de bactérias que causam doenças de grande relevância e prevalência no mundo todo, como clamidíase (*Chlamydia trachomatis*), a tuberculose (*Mycobacterium tuberculosis*), a listeriose (*Listeria monocytogenes*), a salmonelose (*Salmonella*) e a leptospirose (*Leptospira interrogans*) (MCBRIDE et al., 2005; EULALIO et al., 2012; MAUDET et al., 2014). Dessas, somente a leptospirose não possui trabalhos relacionando sua infecção e a expressão diferencial de miRNAs do hospedeiro. Um dos objetivos deste trabalho é investigar o papel de miRNAs em células infectadas pelo patógeno causador da leptospirose.

## 2 Objetivos Específicos

1) Analisar o microtranscriptoma (expressão de miRNAs) de macrófagos murinos controle e infectados após 8h de exposição às cepas virulenta, atenuada e saprófita de *Leptospira*
2) Buscar os RNA codificadores alvo dos miRNAs diferencialmente regulados pela cepa patogênica e identificar vias de atuação dos alvos

## 3 Revisão de Literatura

De acordo com o último lançamento de dados sobre miRNAs no site miRBase, base de dados pública sobre miRNAs, estão descritos 38.589 miRNAs maduros em mais de 200 espécies (miRBase 21, outubro 2018, www.mirbase.org). A maioria dos miRNAs funcionais são produzidos em várias etapas, essenciais para a sua biogênese, que tem início no núcleo celular e se completa no citoplasma (Figura 1). Na primeira etapa, os genes que dão origem aos miRNAs através da ação da RNA polimerase II, produzem um transcrito de miRNA primário (pri-miRNA), possuindo capeamento 7 metil guanosina na ponta 5' e que são poliadenilados na ponta 3'(CAI et al., 2004; LEE et al., 2004). A transcrição de pri-miRNAs pode acontecer através de genes independentes ou a partir de sequências intrônicas de genes que codificam proteínas (CAI et al., 2004; LEE et al., 2004). Dentro do núcleo, a enzima Drosha e a proteína (DGCR8) de ligação processam o pri-miRNA em um precursor chamado pré-miRNA (hairpin de 70 nucleotídeos), que é a seguir exportado para o citoplasma. Após este transporte, a enzima RNAse III (Dicer) cliva o pré-miRNA em um duplex de aproximadamente 20 nucleotídeos (revisado em MAUDET et al., 2014). Este miRNA duplex se associa a proteínas como Argonauta, GW182 e TRNC6 formando o complexo miRISC (miRNA- "induced silencing complex"), um complexo de ribonucleoproteínas que buscam a repressão, deadenilação e consequente degradação de RNAm alvos (EULALIO et al., 2008; HUNTZINGER AND IZAURRALDE, 2011; FABIAN AND SONENBERG, 2012).




Fonte: Adaptado do blog all about RNA silencing, 2010.

**Figura 1.** Processamento do miRNA.

A leptospirose é uma doença causada por bactérias espiroquetas gram-negativas do gênero *Leptospira*, presente em humanos e em muitos animais domésticos, sendo uma das zoonoses de maior incidência no mundo, principalmente em países em desenvolvimento de climas tropicais e subtropicais (LEVETT, 2001; BHARTI et al., 2003; KO et al, 2009). No entanto, ela também pode ser encontrada em países da Europa e nos Estados Unidos (GSELL, 1990; HOLK et al., 2000; KATZ et al., 2001). Essas bactérias colonizam os túbulos renais do seu hospedeiro. Os animais infectados excretam as leptospiras pela urina e a exposição direta ou indireta à urina contaminada causa a infecção por *Leptospira* em humanos (BHARTI et al., 2003). A doença pode se apresentar como assintomática, levar a sintomas gripais leves ou sintomas graves (CHIU et al., 2016), tais como diarreia, hemorragia, comprometimento renal ou meningite asséptica (MCBRIDE et al., 2005).

Durante décadas, considerou-se a imunidade humoral como a única responsável pela resposta imune na leptospirose. Porém, nos últimos anos, os

papéis da imunidade inata e da adaptativa, vem sendo descritos (ADLER AND FAINE,1976; PEREIRA et al., 1998; WERTS et al., 2001; BARBOSA et al., 2009). Estudos recentes demonstram que a resposta imunológica inata pode apresentar diferenças de acordo com o hospedeiro. XUE et al., (2013), estudando a resposta à infecção por *Leptospira interrogans* em macrófagos murinos e humanos, através da técnica do microarranjo para análise transcriptômica, verificaram diferença na expressão gênica de citocinas entre animal resistente e susceptível à *Leptospira*. Essas diferenças refletiriam parcialmente os resultados encontrados na infecção por leptospira durante a forma aguda e crônica com relação a resposta do hospedeiro (VERNEL-PAUILLAC AND MERIEN, 2006; VERNEL- PAUILLAC AND GOARANT, 2010).

O gênero *Leptospira* compreende 12 espécies, entre patogênicas e não patogênicas, com mais de 230 sorotipos descritos (SMYTHE et al., 2002). *Leptospira interrogans* possui maior patogenicidade e prevalência, sobrevivendo a ambientes naturais e reservatórios animais como roedores e animais domésticos. Dos casos de infecção, cerca de 5-15% podem evoluir para sintomas mais graves, como falência renal e hemorragia pulmonar (ADLER et al., 2009; LEVETT, 2001). Diversos componentes da *Leptospira interrogans,* como lipopolissacarídeos, peptideoglicanos, lipoproteínas, glicoproteínas, proteínas membranárias, podem induzir resposta imune do hospedeiro (CINCO et al., 1996; WERTS et al., 2001; DIAMENT et al., 2002; YANG et al., 2006).

Agentes microbianos como a *Leptospira* estão envolvidos em alterações na expressão gênica das células do hospedeiro (HUANG et al., 2001; CHAUSSABEL et al., 2003; CHEVRIER et al., 2011; GAT-VIKS et al., 2013; FAIRFAX et al., 2014; LEE et al., 2014). A função dos miRNAs na regulação do sistema imune tem sido foco de inúmeros estudos, que demonstram sua participação na regulação de diversas vias do sistema imunológico (CHEN et al. 2004; JOHNNIDIS et al. 2008; LODISH et al. 2008; O'CONNELL et al. 2012; ARKATKAR et al. 2015; GUPTA et al. 2015).

Os miRNAs foram primeiramente elucidados após infecções bacterianas em plantas (*Arabidopsis thaliana*), onde foi demonstrada sua contribuição para

resistência contra o patógeno extracelular *Pseudomonas syringae* (NAVARRO et al., 2006). O primeiro estudo com mamíferos demonstrou em células humanas a regulação de vários miRNAs em resposta ao estímulo com LPS (via dependente de TLR4) (TAGANOV et al., 2006). RODRIGUEZ et al., (2007) demonstrou que a deleção de um único miRNA, o miR-155, altera a resposta do sistema imune contra *Salmonella*. *Helicobacter pylori* também induz mudança de expressão neste mesmo miRNA (XIAO et al., 2009; FASSI FEHRI et al., 2010).

Um miRNA pode controlar diversos alvos RNAm, devido ao seu reduzido tamanho (20-22 nucleotídeos), e ao fato de que a complementariedade não precisar ser total para que o alvo seja reconhecido. FRIEDMAN et al. (2009) estimou que 60% do transcriptoma mamífero seja regulado por miRNAs. No entanto, a regulação desta importante classe de RNAs não-codificadores ainda não foi investigada após a infecção por *Leptospira*, patógeno responsável por uma importante zoonose no mundo.

A descrição das alterações no transcriptoma e microtransciptoma do hospedeiro em resposta a esta infecção abre novas possibilidades diagnósticas, prognósticas e de tratamento como, por exemplo, o desenvolvimento de elementos que simulem ou inibam este RNAs não-codificadores para promover a eliminação eficiente do patógeno. No presente estudo, foi aplicado a técnica de microarranjo associada a ferramentas de bioinformática para identificar o papel dos miRNAs na resposta de macrófagos murinos à infecção com *Leptospira interrogans*, bem como na diferença de resposta celular frente à infecção com espécies saprófitas e atenuadas de *Leptospira*.

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## CAPITULO 2 - Data Descriptor: microRNA profile datasets of murine macrophages infected with different strains of *Leptospira* spp


### ABSTRACT

MicroRNAs play an important role in the regulation of immune responses. The influence of epigenetic mechanisms, particularly RNA-mediated post-transcriptional regulation of host immune responses has been proven vital following infections by different pathogens, and bacteria can modulated host miRNAs. Global miRNA expression analysis from macrophages infected *in vitro* with different strains of *Leptospira* spp was performed using miRNA 4.1 microarray strips. Leptospirosis is a bacterial zoonosis of global importance, responsible for significant morbidity and mortality worldwide. Despite considerable advances, much is yet to be discovered about disease pathogenicity, particularly in regards to host-pathogen interactions. We present here a high-quality dataset examining the microtranscriptome of murine macrophages J774A.1 following 8h of infection with virulent, attenuated and saprophyte strains of Leptospira. Metadata files were submitted to the Gene Expression Omnibus (GEO) repository.


**Key-words:** Leptospira; microRNA profiling assay; macrophage cell line.

## 1 Background & Summary

It is known that during bacterial infection, miRNAs play an important role in the regulation of host immune response[1,2]. These small molecules of 21 nucleotides have a vital function in the posttranscriptional regulation of gene expression. Through base pairing, miRNAs bind to complementary sequences of their target mRNAs leading to degradation or translational repression[3–5]. Each miRNA can control hundreds of target genes[6]. A range of pathogens (viruses, parasites and bacteria) can affect expression of host miRNA[1,2,7]. For this reason, pathogen-induced gene modulation of host cells is essential to understanding the pathophysiology of diseases. In this study, we evaluate, for the first time, global miRNA expression in macrophages infected with different strains of *Leptospira* spp.

*Leptospira interrogans* is a highly invasive gram-negative spirochete that leads to development of leptospirosis. These bacteria can occur in urban and rural environments, surviving mainly in water. Currently, the rate of mortality can reach 60.000 deaths per year [8,9]. In the literature 12 species and more than 250 serotypes of *Leptospira* are described, varying in pathogenicity [10,11]. During early infection, antibiotics are effective, however most vaccines available for veterinary use provide limited protection against more than 250 pathogenic *Leptospira* serovars [12,13].

Macrophages are responsible for bacterial phagocytosis in mammals and are important cells in leptospiral infection [14,15]. Host/pathogen interaction can modify gene expression profiles in the host with leptospirosis [16]. Our goal was to contribute to the understanding of pathogen-mediated control of host gene expression by identifying miRNAs modulated by saprophyte, attenuated or virulent strains of *Leptospira* in macrophages compared to non-infected control cells. Through the use of microarray technology, we generated microtranscriptome datasets following 8 hours of infection. We worked with the hypothesis that *Leptospira* infection modulates macrophageal expression of miRNAS, and that bacterial virulence affects this modulation. Our study suggests

that post-transcriptional regulation by miRNAs plays a role in host response to infection in leptospirosis. Here, we describe detailed information on the experimental design (Fig. 1) and generation of our datasets (Data Citation 1). This data descriptor is an extend version of the methodology described in a chapter 3, with the objective of disseminating the raw data produced in this experiment. These raw data can be a valuable resource for further bioinformatics investigation of biological pathways associated with pathogenicity, leading to the identification of novel targets for therapy.

## 2 Methods

### 2.1 Cell culture

Murine macrophage cell line J774A.1 was provided by the Paul Ehrlich cell bank, Rio de Janeiro, Brazil. This cell lineage was maintained in RPMI-1640 media (Sigma, USA), supplemented with 10% heatinactivated fetal bovine serum (Gibco, USA), 100 ug/mL streptomycin (Sigma, USA), 0.03% L-glutamine solution (Sigma, USA) and 100UI/mL of penicillin. Cells were incubated at 37 °C, 5% CO2 until formation of a confluent monolayer in 6-well cell culture plates (3 cm/well).

### 2.2 Bacterial culture

All strains of Leptospira used in this study, *Leptospira interrogans* serovar Copenhageni (FIOCRUZ L1-130) as a virulent strain, L. interrogans serovar Copenhageni M20 as an attenuated strain and Leptospira biflexa serovar Patoc (FIOCRUZ -Patoc I) as a saprophyte strain, were kindly provided by the Laboratory of Preventive Veterinary Medicine of University of São Paulo (USP). Attenuation of M20 strain was done by successive passages (>200), according with reference18–20. All strains were maintained in Fletcher's semi solid medium, and incubated at 30 °C. Virulence of L. interrogans L1-130 was preserved by intraperitoneal inoculation in hamsters (Mesocricetus auratus) with kidney recovery, following previously published guidelines19. Before infection, all strains

were counted in a Petroff–Hausser counting chamber (Fisher Scientific)21. Project had the approval of the Ethics Committee for Animal Use (FOA-FMVA Unesp), under protocol number 2015-00895.

### 2.3 Infection of Macrophages

After the formation of monolayers with >90% confluency, cells were washed with sterile phosphate buffer solution (pH 7,2) for removal of antibiotics and non-adherent cells. *L. interrogans* and *L. biflexa* were centrifuged, for removal of their growth media, and resuspended in RPMI-1640 medium (Sigma), and added to macrophages (100:1 bacteria:cell). Experimental groups were devised as follows: infection of macrophages with a virulent strain (L. Interrogans; n=3), infection with attenuated strain (L. interrogans; n=3), infection with saprophyte strain (L. biflexa; n=3) and non-infected macrophages (control; n=3). All treatments were incubated in fresh RPMI medium, without antibiotics, for 8h at 37 °C, 5% $CO_2$. Following this period, RNA extraction was immediately performed.

### 2.4 miRNA extraction and Quantification

Extraction of total RNA from macrophages was conducted using a miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. RNA samples were immediately stored at −80 °C. Quantification was performed using a spectrophotometer (Nanodrop ND-20, Thermo Scientific, Wilmington, DE, USA) and quality of samples was assessed with capillary electrophoresis (Bioanalyzer 2100 Agilent, Santa Clara, CA, USA).

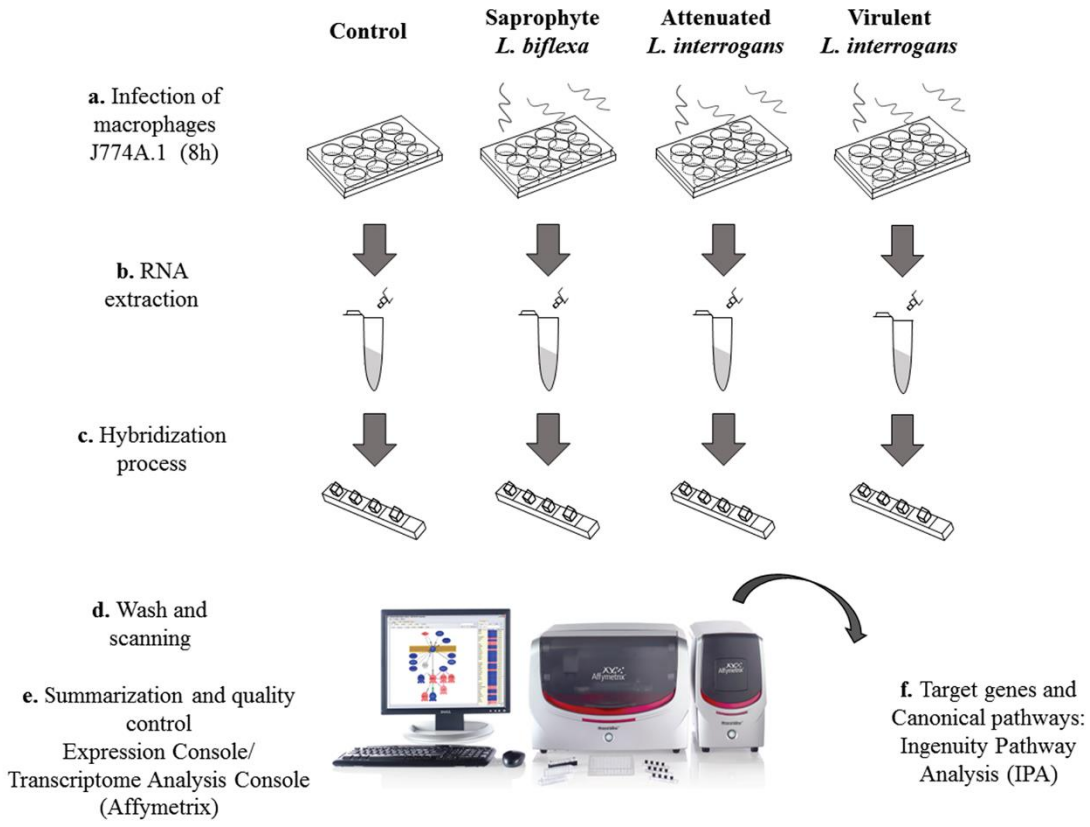


**Figure 1.** Illustration of experimental design. Cell lineage J774A.1 of murine macrophages was cultured to a confluent monolayer. Infection was performed adding 100:1 bacteria:cell to the macrophages. (a) Treatments, analyzed in triplicate, were carried as follows: infection of macrophages with a virulent strain of *L. interrogans*, with an attenuated strain of *L. interrogans*, and with an saprophyte strain *L. biflexa* and non-infected macrophages as controls. All treatments were incubated in fresh RPMI medium, without antibiotics, for 8h at 37 °C, 5% $C0_2$. (b) Following this period, total RNA was immediately extracted, (c,d) hybridization of samples to the strips was carried at 48 °C for 20h, strips were then washed, stained and scanned using the GeneAtlas® System (Affymetrix). (e) Raw intensity values were background corrected, log2 transformed and then quantile normalized by the software Expression Console (Affymetrix) using the Robust Multi-array Average (RMA) algorithm. Statistical analysis was performed in the TAC software (Affymetrix) and cel files were submitted to Gene Expression Omnibus repository (GEO). (f) Target genes and Pathway analysis was performed in the Ingenuity Pathway Analysis (Qiagen).

## 2.5 Microtranscriptome Array

FlashTag Biotin HSR RNA Labeling Kit and the Affymetrix miRNA 4.1 Array strip (Affymetrix, Santa Clara, California, EUA) were used to analyze the expression of 3195 murine specific probes in the strips. ELOSA (enzyme-linked oligosorbent assay) quality control assay was run for all samples, and hybridization to the strips was carried at 48 °C for 20h. Strips were processed and scanned in the GeneAtlas System (Affymetrix). Raw intensity values (cel files) were background corrected, log2 transformed and then quantile normalized by the software Expression Console (Affymetrix) using the Robust Multi-array Average (RMA) algorithm. Figure 2c shows the relative signal of probes. Also in expression console software, we performed a correlation with linearized signal intensity values for the samples within treatment groups, showing a strong correlation coefficient between samples (Fig. 2d). Figure 3 shows plots of p-values for miRNAs (p-value <0.05) up- and downregulated by the different leptospires in comparison to non-infected control samples.

## 2.6 Data Records

The cel and arr (strip information) files produced by microarray were deposited at the Gene Expression Omnibus repository (Data Citation 1). Through the Expression Console (Affymetrix), a free software, the cel files were normalized and chp file were generated for the identification of differentially expressed miRNAs. Samples used in the study are shown in Table 1.

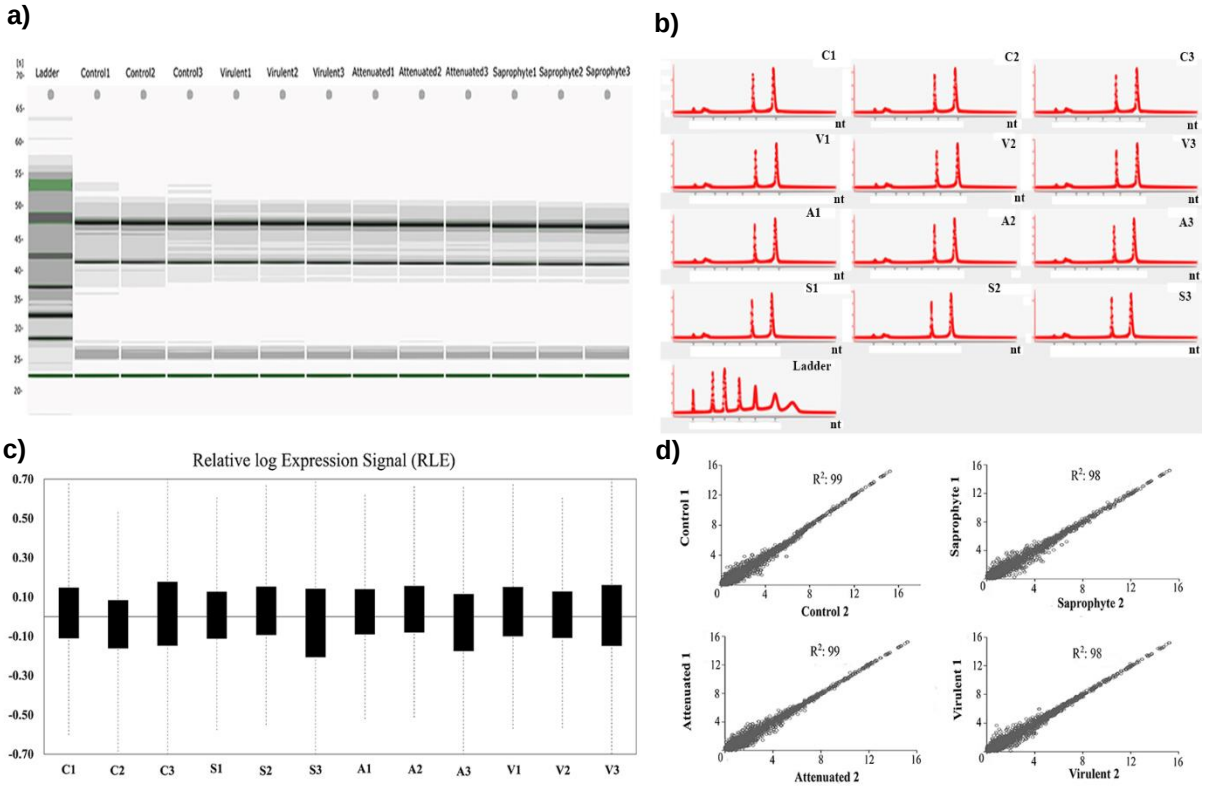


**Figure 2.** RNA and microarray quality control and summarization. (a,b) Bioanalyzer gel image and graphic of all RNA samples; Control (Ctrl); Virulent (Vir); Attenuated (Att) and Saprophyte (Sap), used for microarray analysis. The 28S and 18s distinctive ribosomal RNA bands are observed for all samples. (c) Values of relative log expression signal RMA-DABG between treatments. (d) Signal intensity correlation analysis within groups.

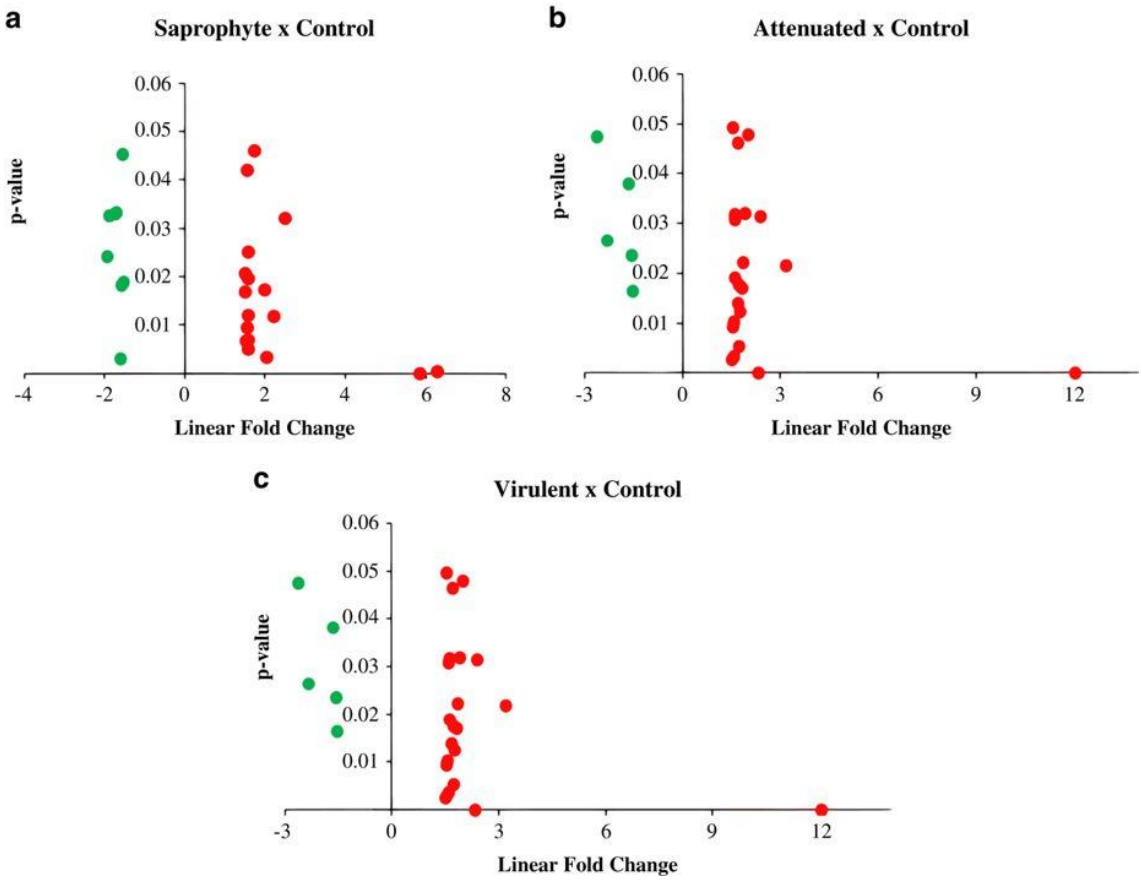


**Figure 3.** Distribution of differentially expressed miRNAs. (a-c) Upregulated (red) and downregulated (green) miRNAs in macrophages infected with virulent strains compared to control non-infected, plotted by p-value <0.05 and linear fold change. (n =3/treatment).

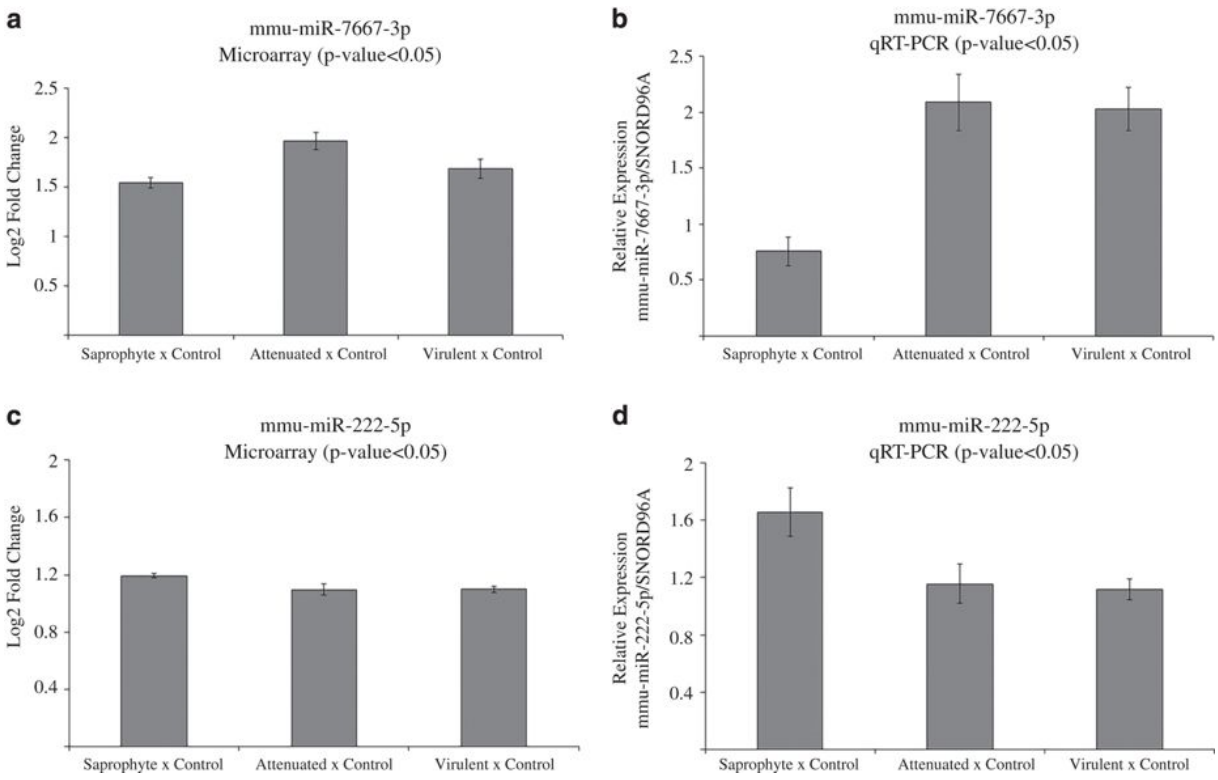


**Figure 4.** Microarray and qPCR fold change values of validated miRNAs. (a) mmu-miR-7667-3p microarray fold change. (b) mmu-miR-7667-3p qPCR fold change. (c) mmu-miR-222-5p microarray fold change. (d) mmu-miR-222-5p qPCR fold change. Quantification based on a standard curve and adjusted to SNORD96A (housekeeping). Statistical analysis between two groups (infected vs. control) was performed with ANOVA (p-value <0.001) followed by Tukey´s Range Test.

## 2.7 Technical Validation

### 2.7.1 RNA quality control

Quality control of RNA samples was performed prior to microarray experiment using capillary electrophoresis (Bioanalyzer 2100 Agilent, Santa Clara, CA, USA), using the Eukaryote Total RNA Nano kit. All samples used for microarray analysis had a RNA integrity number (RIN) of 10 (Fig. 2a,b).

Table 1. Dataset and sample description across treatment groups.

| GSM-ID | Sample Name | Oganism | Treatment description | Molecule | Technology |
|---|---|---|---|---|---|
| **GSM2817991** | **Control 1** | Mus musculus | Macrophages_non-infected_8h_rep1 | miRNA | Microarray |
| **GSM2817992** | **Control 2** | Mus musculus | Macrophages_non-infected_8h_rep2 | miRNA | Microarray |
| **GSM2817993** | **Control 3** | Mus musculus | Macrophages_non-infected_8h_rep3 | miRNA | Microarray |
| **GSM2817994** | **Saprophyte 1** | Mus musculus | Macrophages infected with saprophyte strain_8h_rep1 | miRNA | Microarray |
| **GSM2817995** | **Saprophyte 2** | Mus musculus | Macrophages infected with saprophyte strain_8h_rep2 | miRNA | Microarray |
| **GSM2817996** | **Saprophyte 3** | Mus musculus | Macrophages infected with saprophyte strain_8h_rep3 | miRNA | Microarray |
| **GSM2817997** | **Attenuated 1** | Mus musculus | Macrophages infected with attenuated strain_8h_rep1 | miRNA | Microarray |
| **GSM2817998** | **Attenuated 2** | Mus musculus | Macrophages infected with attenuated strain_8h_rep2 | miRNA | Microarray |
| **GSM2817999** | **Attenuated 3** | Mus musculus | Macrophages infected with attenuated strain_8h_rep3 | miRNA | Microarray |
| **GSM2818000** | **Virulent 1** | Mus musculus | Macrophages infected with virulent strain_8h_rep1 | miRNA | Microarray |
| **GSM2818001** | **Virulent 2** | Mus musculus | Macrophages infected with virulent strain_8h_rep2 | miRNA | Microarray |
| **GSM2818002** | **Virulent 3** | Mus musculus | Macrophages infected with virulent strain_8h_rep3 | miRNA | Microarray |

### 2.7.2 Microarray summarization and quality control

The Gene Atlas equipment scans hybridized strips and converts the readings into raw intensity values (cel files). These files were background corrected, log2 transformed and then quantile normalized by the software Expression Console (Affymetrix) using the Robust Multi-array Average (RMA) algorithm. Figure 2c shows the relative signal of probes. Also in the expression console software, we performed a correlation with linearized signal intensity values for the samples within treatment groups, showing a strong correlation coefficient (R2) between samples (Fig. 2d). Figure 3 shows plots of p-values for miRNAs up and downregulated (p-value <0.05) by the different strains in comparison to non-infected control samples.

### 2.7.3 Validation of microarray results by qRT-PCR

Validation of miRNAs in infected macrophages (saprophyte, attenuated and virulent strains) and noninfected controls was done using the miScript miRNA PCR System (Qiagen-Valencia, CA, USA) for preparation of cDNA and realtime PCR, according to the manufacturer's instructions. Validated primers were purchased from Qiagen. PCR was performed using a Stratagene QPCR Systems Mx3005P (Agilent Technologies, Santa Clara, CA, USA) Expression levels were determined using standard curves at each individual run, and the expression of candidate miRNAs is presented as a ratio to the control miRNA SNORD96A. Real time PCR data were analyzed using least-squares analysis of variance and the general linear model procedures of SAS (SAS Institute, Cary, NC, USA; p<0.01). Comparison of means was done using Tukey's range test, and significance was set at po0.05, (Fig. 4a-d).

### 2.8 Usage Notes

Raw data (cel files) can be normalized by Expression Console and Transcriptome Analysis Console(TAC), both are free softwares from Affymetrix. They perform statistical testing and present results as fold change for differentially expressed miRNAs. For miRNAs, TAC software can also identify target genes reported by Affymetrix (NetAffx).

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**Data Citations**

1. Garcia, L. E. et al. Gene Expression Omnibus GSE105104 (2018).

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## CAPÍTULO 3 - CHARACTERIZATION OF THE MICROTRANSCRIPTOME OF MACROPHAGES INFECTED WITH VIRULENT, ATTENUATED AND SAPROPHYTE STRAINS OF *Leptospira* spp.


### ABSTRACT

Leptospirosis is a bacterial zoonosis, caused by Leptospira spp., that leads to significant morbidity and mortality worldwide. Despite considerable advances, much is yet to be discovered about disease pathogenicity. The influence of epigenetic mechanisms, particularly RNA-mediated post-transcriptional regulation of host immune response has been described following a variety of bacterial infections. The current study examined the microtranscriptome of macrophages J774A.1 following an 8h infection with virulent, attenuated and saprophyte strains of Leptospira. Microarray analysis revealed that 29 miRNAs were misregulated following leptospiral infection compared to control macrophages in a strain and virulence-specific manner. Pathway analysis for targets of these differentially expressed miRNAs suggests that several processes involved in immune response could be regulated by miRNAs. Our data provides the first evidence that host miRNAs are regulated by Leptospira infection in macrophages. A number of the identified miRNA targets participate in key immune response processes. We suggest that post-transcriptional regulation by miRNAs may play a role in host response to infection in leptospirosis.


**Key-words**: leptospirosis, macrophage, miRNA

## 1 Introduction

Leptospirosis is a zoonosis of global importance, particularly in developing countries with tropical climates [1], and is caused by a highly invasive gram-negative spirochete known as Leptospira. This genus is comprised of 12 species and 250 serotypes between pathogenic and non-pathogenic strains [2–3], with Leptospira interrogans being the most common pathogenic species. Mortality rate is around 60,000 deaths per year and the annual number of severe cases can reach 1 million, placing leptospirosis as a major player in morbidity, and number of deaths, by zoonotic causes [4–5].

Rodents are natural reservoirs for these bacteria and they shed the pathogen in their urine, contaminating water and soil in urban and rural environments. Humans can be infected by skin contact, mainly in areas lacking sanitation [6]. During early infection, antibiotics are effective, however most vaccines available for veterinary application provide limited protection against more than 250 pathogenic Leptospira serovars [7–8]. Advances in research have been made through the use of conserved leptospiral proteins, aiming at better vaccine candidates for leptospirosis [9].

Macrophages play a central role in leptospirosis by phagocytizing bacteria in humans and other mammals [10–11]. Cinco et al. [12] suggest that Leptospira inside macrophages are fully capable of replication, and Li et al. [13] showed that *Leptospira* are capable of escaping host defense responses, however only in human macrophages.

*Leptospira interrogans* has higher pathogenicity due to components such as lipopolysaccharides, peptideoglycans, lipoproteins, glycoproteins and membrane proteins, which induce a robust inflammatory response [14–17]. Activation of innate immunity by toll-like receptors (TLRs 2/4) in macrophages is essential for host defense [18]. TLR activation and MyD88 recruitment, signaling through several pathways like mitogen-activated protein (MAP) kinases, NF-κB and pro-inflammatory cytokines, lead to B and T cell activation [19–21]. Another rapid response induced by *L. interrogans* is apoptosis in macrophages and

hepatocytes. This pathway is activated by caspase 3 and 6 through a FADD–caspase-8-dependent pathway [22] involving intracellular free calcium ion (Ca2+) [23].

It is known that the pathogen/host interaction can significantly modify gene expression profiles in an infected host, and it was suggested that post-transcriptional regulation might be acting in this interaction [24]. MicroRNAs (miRNAs) are small non-coding RNAs spanning 20–22 nucleotides, and have a major role in posttranscriptional regulation of gene expression. These small RNAs negatively regulate protein synthesis through base pairing with partially complementary sequences in the 3´UTR region of target mRNAs, favoring their degradation or translational repression [25–27]. Each miRNA has the potential to target, thus controlling, hundreds of genes [28]. An extensive body of research indicates that several pathogens (viruses, parasites and bacteria) can affect miRNA expression in host cells [29–31]. Further, miRNAs associated with disease can act as biomarkers or therapeutic targets [32–33].

For this reason, miRNAs and their targets, modulated by the pathogen, are of great importance to comprehend the pathophysiology of leptospirosis. Based on the identification of targets of differentially expressed miRNAs, following infection of murine macrophages with different types of *Leptospira*, we report several canonical pathways that could be affected by infection. We have established, for the first time, that modulation of miRNAs is present in *Leptospira* infection, and obtained potential miRNA signatures for different strains, varying in virulence. We suggest that posttranscriptional regulation by miRNAs may play a role in host response to infection in leptospirosis. These findings add to the growing list of infectious diseases that involve miRNAs regulation by the host.

## 2 Results

### 2.1 Expression profile of miRNAs in macrophages infected with saprophyte, attenuated and virulent strains of *Leptospira* spp.

In total, we identified 29 miRNAs that were modulated in macrophages after 8h of infection with different strains of *Leptospira* spp (fold change ± 1.5; p <0.01). When compared to non-infected control cells, 17 miRNAs were significantly altered (15 upregulated and 2 downregulated) following infection with the virulent strain, 16 miRNAs were modulated (12 upregulated and 4 downregulated) as a response to the attenuated strain, and 9 miRNAs were altered by infection with the saprophyte strain (5 upregulated and 4 downregulated) (Fig 1). The intersection of treatments in the Venn diagram shows that three miRNAs are modulated by all strains. MiRNAs were also modulated in a strain-specific manner, where 7 miRNAs were modulated specifically by the virulent strain, 7 by the attenuated and 5 only by the saprophyte bacteria (Fig 2).

Average signals (log2) of samples were hierarchically clustered using Pearson´s correlation and complete-linkage, we observed a clustering of samples based on species and virulence, with the virulent and attenuated strains clustering closer together, followed by the saprophyte strain (Fig 3), and all infected samples clearly separating from non-infected controls. Validation of chosen regulated miRNAs by quantitative realtime PCR (miR-155-5p; miR-7667-3p; miR-203-3p and 222-5p), corroborated the microarray results with respective correlation coefficients between techniques of 0.99, 0.79, 0.92 and 0.99 (Fig 4). The highest fold change was observed for miR-155-5p with an upregulation of >12 fold for *L. interrogans* and >5 fold for the saprophyte *L. biflexa*, when compared to non-infected cells. In Fig 5, we depict the significant canonical pathways for miR-155-5p targets, identified in the virulent treatment compared to noninfected control cells.

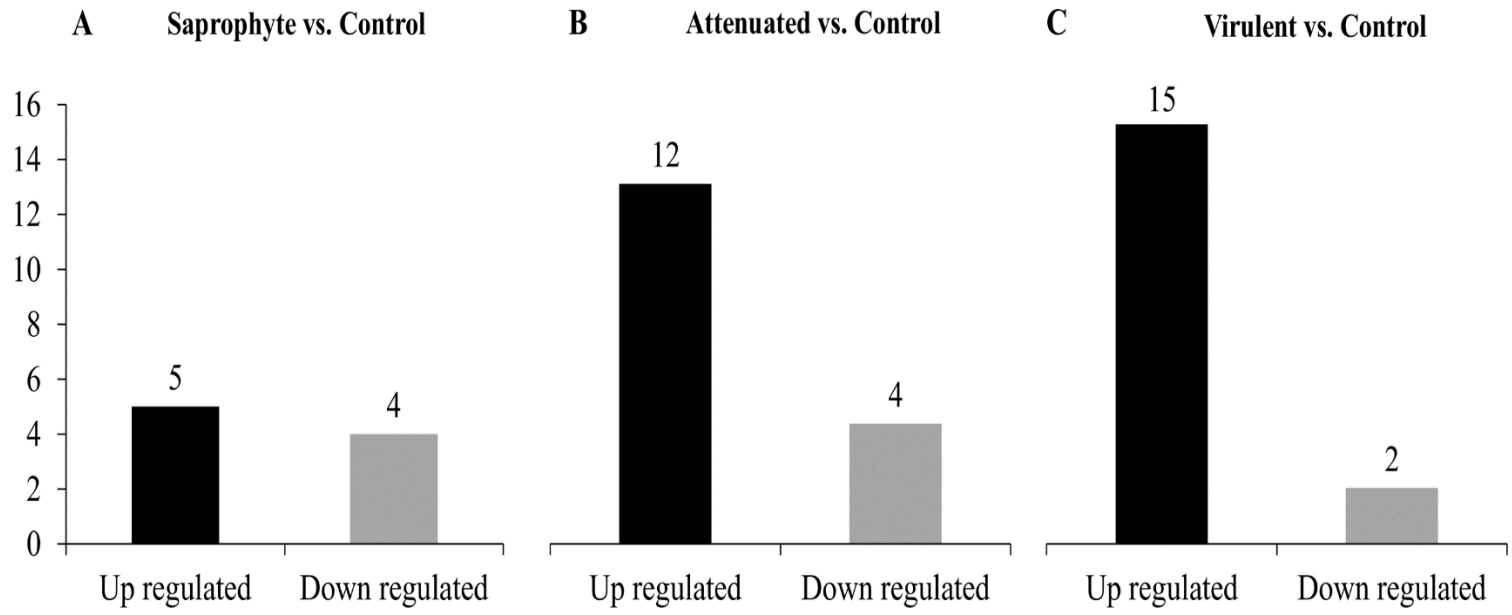


**Fig 1.** Quantity of miRNAs modulated by macrophages following 8h of infection by different strains of *Leptospira* spp. Total number of miRNAs/treatment (n = 3/treatment; p-value<0.01; linear fold change ± 1.5) in the contrasts A) Saprophyte; B) Attenuated and C) Virulent vs. Non-infected Control.

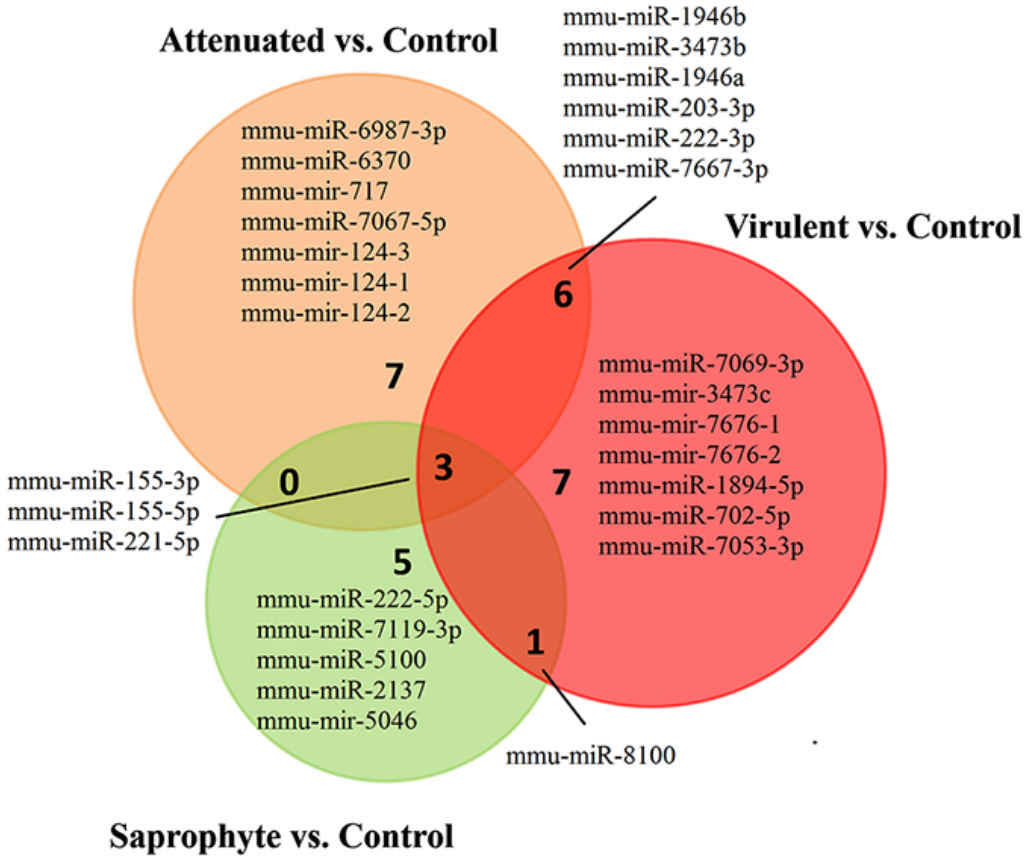


**Fig 2.** Venn diagram of miRNAs modulated by macrophages at 8h of infection by different strains of Leptospira spp. Total number of miRNAs/treatment (n = 3/treatment; p-value <0.01; linear fold change ±1.5) in the contrasts Infected (Saprophyte; Attenuated and Virulent) vs. Non-infected Control.

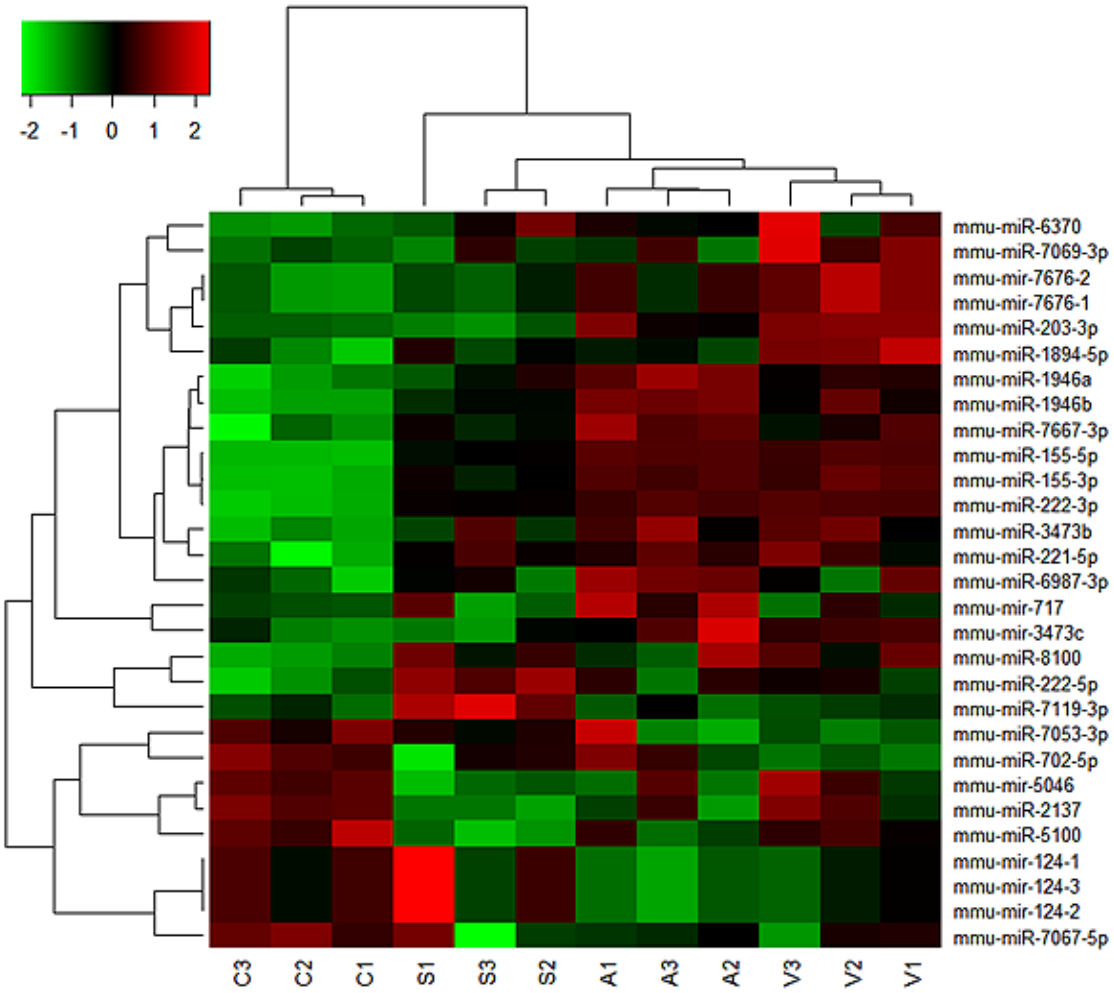


**Fig 3.** Heatmap of miRNAs modulated by macrophages at 8h of infection by different strains of Leptospira spp. Heatmap shows the average signal of 29 miRNAs/treatment (n = 3/treatment; p-value <0.01; linear fold change ±1.5).

## 2.2 miRNA target prediction and functional enrichment

For prediction of target genes to the differentially expressed miRNAs in all treatments, we used the tool miRNA Target Filter present in the IPA software. We utilized databases from miRecords, Tarbase, TargetScan, and the Ingenuity Knowledge Base, and filtered for targets with high prediction and that have been experimentally observed only. In Table 1, we demonstrate that 10 out of the 29 differentially expressed miRNAs have predicted mRNA targets. To identify the relationships, mechanisms, functions, and pathways relevant to the list of target genes, we employed the feature Core Analysis available in the IPA software package with values of significance–log (BH corrected p-value)>1.3. From our list of predicted genes for each treatment, we report 21 and 5 canonical pathways for virulent and attenuated treatment, respectively (Tables 2 and 3). For the saprophyte treatment, specific miRNAs did not have predicted targets. Further, only the virulent treatment had specific pathways identified. Five biological processes, Molecular Mechanisms of Cancer, Fcγ Receptormediated Phagocytosis in Macrophages, PI3K/AKT Signaling, PTEN Signaling and Role of Macrophages in Rheumatoid Arthritis are potentially regulated by miRNAs in response to *L*. *interrogans* infection, regardless of virulence. These five pathways were therefore classified as common to *L*. *interrogans*. In regards to specific processes, 16 pathways were identified only in response to the virulent strain compared to noninfected controls.

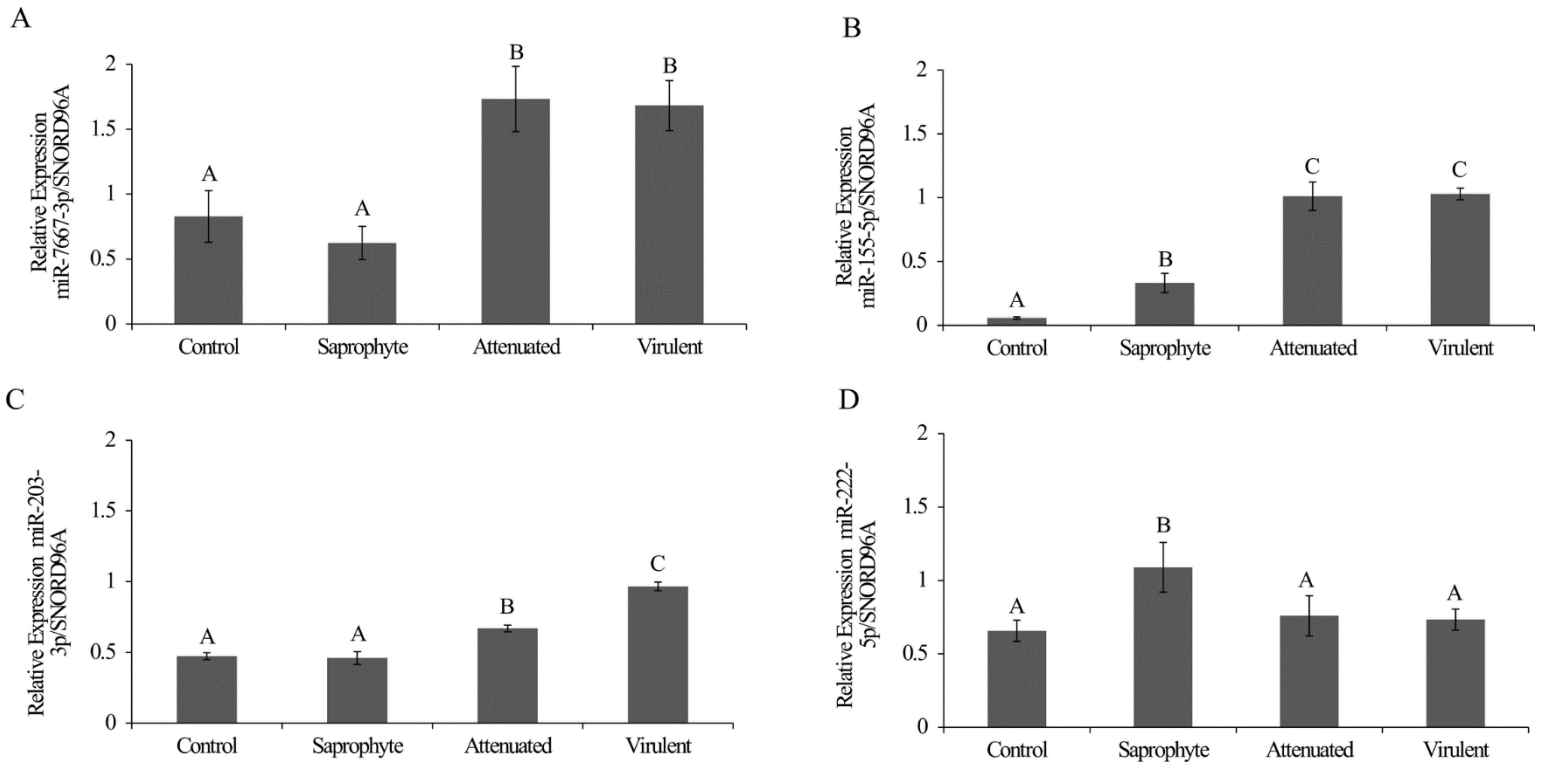


**Fig 4.** qRT-PCR of miRNA expression levels in macrophages infected with different strains of Leptospira compared to non-infected controls. A) Relative expression of miR-7667-3p, B) Relative expression of miR-155-5p, C) Relative expression of miR-203-3p, D) Relative expression of miR-222-5p. Different superscript letters differ significantly ($p<0.05$).

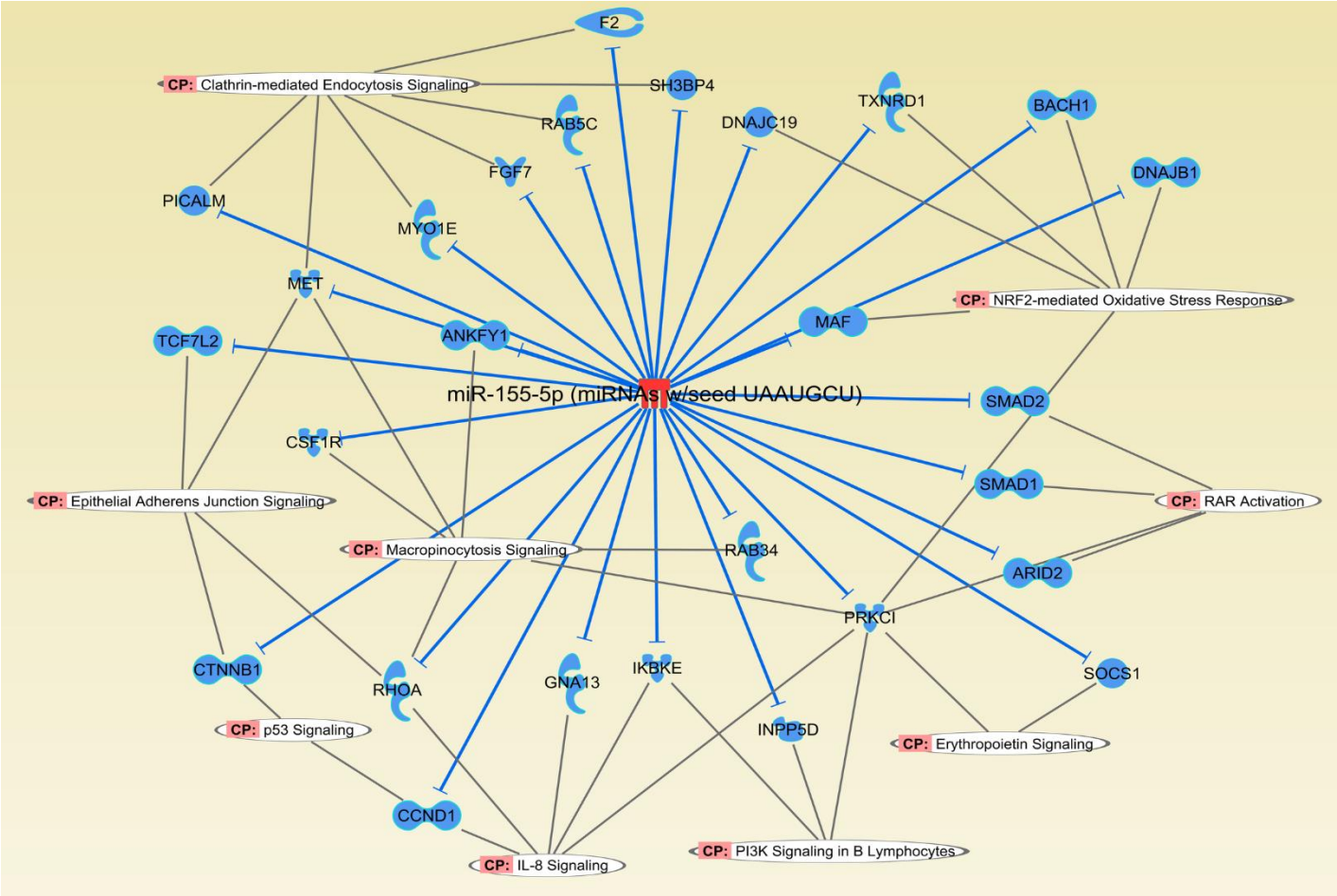


**Fig 5.** Canonical pathways of miR-155-5p targets identified in virulent treatment compared to non-infected control. Network shows potential downregulated targets of mmu-miR-155-5p participating in biological pathways identified only in virulent treatment when compared to control. In red, miR-155 was upregulated and blue indicates the potentially downregulated targets of miR-155-5p in macrophages infected with virulent L. interrogans following 8h of infection in vitro. (CP = Canonical pathways).

**Table 1.** Number of miRNA targets to the differentially expressed miRNAs following infection with virulent, attenuated or saprophyte Leptospira. (CT = control; FC = fold change).

| miRNAs | Virulent x CT | | Attenuated X CT | | Saprophyte X CT | | Number of Targets |
|---|---|---|---|---|---|---|---|
| | FC | p-value | FC | p-value | FC | p-value | |
| **miR-155-3p** | **14.3** | **0.000066** | **14.4** | **0.000006** | **6.3** | **0.000286** | 2 |
| **miR-155-5p** | **12.1** | **0.000004** | **12.4** | **2.46E-07** | **5.9** | **0.000007** | 224 |
| **miR-221-5p** | **1.8** | **0.012387** | **1.7** | **0.005452** | **1.6** | **0.009546** | 298 |
| **miR-1946b** | **1.5** | **0.002698** | **2.0** | **0.000014** | 1.4 | 0.000770 | - |
| **miR-3473b** | **1.6** | **0.003419** | **1.5** | **0.006212** | 1.2 | 0.038841 | - |
| **miR-1946a** | **1.5** | **0.00278** | **1.8** | **0.001035** | 1.1 | 0.490372 | - |
| **miR-203-3p** | **2.3** | **0.000001** | **1.5** | **0.019451** | -1.1 | 0.312029 | 5 |
| **miR-222-3p** | **1.5** | **0.000009** | **1.5** | **0.00003** | 1.3 | 0.000024 | 177 |
| **miR-7667-3p** | **3.2** | **0.021577** | **4.7** | **0.005985** | 2.4* | 0.031972 | 219 |
| **miR-7069-3p** | **1.6** | **0.010383** | 1 | 0.350463 | 1 | 0.488653 | 189 |
| **mir-3473c** | **1.5** | **0.009386** | 1.65 | 0.051234 | 1 | 0.868143 | - |
| **mir-7676-1** | **1.5** | **0.001562** | 1.3 | 0.018375 | 1.1 | 0.092322 | - |
| **mir-7676-2** | **1.5** | **0.001562** | 1.3 | 0.018375 | 1.1 | 0.092322 | - |
| **miR-1894-5p** | **1.5** | **0.00436** | 1 | 0.096391 | 1.2 | 0.082984 | - |
| **miR-702-5p** | **-1.5** | **0.001429** | -1 | 0.416406 | -1.1 | 0.162260 | 64 |
| **miR-7053-3p** | **-1.5** | **0.006674** | -1.7 | 0.411229 | -1.1 | 0.162706 | - |
| **miR-6987-3p** | 1.3 | 0.204396 | **1.8** | **0.006432** | 1.3 | 0.242025 | 78 |
| **miR-6370** | 2 | 0.076150 | **1.6** | **0.0016** | 1.71 | 0.068848 | - |
| **mir-717** | 1 | 0.441789 | **1.5** | **0.010607** | -1 | 0.799834 | - |
| **miR-7067-5p** | -1.3 | 0.127036 | **-1.8** | **0.010926** | -1.9 | 0.218096 | 730 |
| **mir-124-3** | -1.4 | 0.107360 | **-1.9** | **0.010232** | 1 | 0.697852 | - |
| **mir-124-1** | 1 | 0.580372 | **-1.9** | **0.010232** | 1 | 0.697852 | - |
| **mir-124-2** | -1.4 | 0.107370 | **-1.9** | **0.010232** | -1 | 0.909340 | - |
| **miR-222-5p** | 1.5* | 0.041164 | 1.5 | 0.111593 | **1.6** | **0.004998** | - |
| **miR-7119-3p** | 1 | 0.589778 | - 1 | 0.867308 | **-1.5** | **0.005767** | - |
| **miR-5100** | -1 | 0.183061 | 1.2 | 0.067205 | **-1.5** | **0.000297** | - |
| **miR-2137** | - 1 | 0.453161 | -1.3 | 0.073805 | **-1.6** | **0.0031** | - |

**Table 2.** Common canonical pathways identified using the targets of miRNAs expressed by macrophages infected with different strains of *Leptospira* spp. Core analysis from IPA (Ingenuity Pathway Analysis) software was used to identify significant pathways trough B-H Multiple testing correction -log(B-H p-value)>1.3.

| Ingenuity Canonical Pathways | Contrast | -log(B-H p-value) | Genes in Pathway |
|---|---|---|---|
| **Molecular Mechanisms of Cancer** | Virulent x Control | 3.13 | 41/376 |
| | Attenuated x Control | 1.96 | 48/376 |
| **Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes** | Virulent x Control | 1.98 | 15/93 |
| | Attenuated x Control | 2.01 | 19/93 |
| **PI3K/AKT Signaling** | Virulent x Control | 1.91 | 17/125 |
| | Attenuated x Control | 1.96 | 22/125 |
| **PTEN Signaling** | Virulent x Control | 1.80 | 16/119 |
| | Attenuated x Control | 2.01 | 22/119 |
| **Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis** | Virulent x Control | 1.56 | 29/311 |
| | Attenuated x Control | 1.55 | 40/311 |

**Table 3.** Specific canonical pathways identified using the targets of miRNAs modulated by macrophages infected with virulent strain (L. interrogans serovar Copenhageni L1-130). Core analysis from IPA (Ingenuity Pathway Analysis) software was used to identify significant pathways trough B-H Multiple testing correction log(B-H p-value) >1.3.

| Ingenuity Canonical Pathways | -log(B-H p-value) | Genes in Pathway |
|---|---|---|
| **PI3K Signaling in B Lymphocytes** | 1.98 | 18/130 |
| **NRF2-mediated Oxidative Stress Response** | 1.62 | 21/193 |
| **IL-8 Signaling** | 1.91 | 23/197 |
| **Clathrin-mediated Endocytosis Signaling** | 1.56 | 21/199 |
| **p53 Signaling** | 1.70 | 15/111 |
| **Ovarian Cancer Signaling** | 1.36 | 16/144 |
| **Osteoarthritis Pathway** | 1.36 | 21/210 |
| **Small Cell Lung Cancer Signaling** | 1.56 | 12/85 |
| **Tec Kinase Signaling** | 1.36 | 18/170 |
| **Erythropoietin Signaling** | 1.62 | 12/81 |
| **Macropinocytosis Signaling** | 1.62 | 12/81 |
| **Epithelial Adherens Junction Signaling** | 1.56 | 17/146 |
| **Gα12/13 Signaling** | 1.36 | 15/131 |
| **Regulation of IL-2 in Activated and Anergic T Lymphocytes** | 1.36 | 29/526 |
| **Pancreatic Adenocarcinoma Signaling** | 1.36 | 14/118 |
| **RAR Activation** | 1.88 | 22/190 |

## 3 Discussion

Despite its prevalence, morbidity and mortality rates, pathogenicity of leptospirosis is still largely unknown. To better understand the processes involved in the host-pathogen interplay of Leptospira infection, we carried out, for the first time, a global evaluation of microRNA modulation of murine macrophages infected with different strains of Leptospira spp at 8h post-infection in vitro. Here, we compared miRNA profiles to address whether Leptospira affect macrophageal miRNA expression, if this modulation is species specific and if bacterial virulence plays a role in this modulation.

In Fig 3, we show that Leptospira spp, regardless of species or virulence, modulate the miRNAs mmu-miR-155-5p, mmu-miR-155-3p and mmu-miR-221-5p. Notwithstanding, specific miRNAs signatures were also obtained for each strain, and they differed within species based on virulence. Differential host response has been previously suggested to be associated to protein differences between the strains. In fact, Haake et al., [34] reported that attenuated *L. interrogans* cultures expressed different proteins and LPS profiles when compared to virulent cultures. In addition, Picardeau et al. [35], compared the genomic sequence of saprophyte L. biflexa with that of the pathogenic strain, L. interrogans, identifying differences such as a larger number of genes encoding proteins containing leucine-rich repeat (LRR) domains in the pathogenic strain, shown to be involved in attachment and invasion of host cells in other bacteria. Therefore, it is not surprising that differing bacterial genomic and protein profiles elicit different responses in their host cells. Further, Xu et al. [36] demonstrated significant differences in gene expression, particularly in genes related to antigen processing and presentation, regulation of membrane potential, cell migration, cytoskeleton organization and biogenesis are mostly up-regulated in murine macrophage cells, following infection by different species of Leptospira. Our current results indicate that, beyond the previously reported genomic and transcriptional differences, control of gene expression by means of post-

transcriptional modifications may be dependent on species and virulence in leptospiral infection.

Among the miRNAs identified in our study as commonly regulated by the genus *Leptospira* sp, independent of virulence, is mmu-miR-155-5p. This miRNA is known to be upregulated in inflammatory processes such as rheumatoid arthritis, cancer, cardiovascular disease, as well as in other bacterial infections in macrophages like *Listeria*, *Salmonella*, *Helicobacter* and *Mycobacteria* [37] reviewed by [31,38]. MiR155 has a considerable number of mRNA targets, and all canonical pathways identified here, in virulent and attenuated treatments, have highly predicted and experimentally observed targets of mmu-miR-155, suggesting an important role for miR-155 as a post-transcriptional regulator in leptospiral induced host response.

Macrophages are fundamental against leptospiral infection. They have different receptors that activate a plethora of responses, such as phagocytosis, cytokine/chemokine production and antigen presentation [39–41]. The cross-linking of IgGs (immunoglobulin-g) with Fc receptors in macrophages initiates crucial cellular events for host immune response. The pathway Fc-gamma receptors in macrophages, play an important role in recognizing IgG-coated pathogen targets during the phagocytosis process in the host [42]. Our target analysis identified previously reported genes in this pathway, significantly identified following virulent and attenuated infection, such as SHIP-1, VAV3 and VAMP3 suggested to be downregulated by mmumiR-155-5p, and PTEN, downregulated by mmu-miR-222-3p, involved in phagosome formation and recycling of cell membrane, respectively. Phagocytosis is vital for internalization of leptospires, and previous work has indicated that one of the resistance mechanism of pathogenic Leptospira in the host, is evasion of the alternative and classical complement system pathways [43]. VAV3, is a potential target to miR-155-5p, and downregulation of this gene can cause inhibition in B- and T-cell development and activation, given its involvement in activating pathways that lead to actin cytoskeletal rearrangements and greater cellular movement [44]. More studies are needed to correlate *Leptospira* evasion and VAV3

function. Inositol polyphosphate-5-phosphatase D (INPPD5 or SHIP-1) is a well established target for mmu-miR-155-5p [37] and this correlation is associated with cell proliferation. Increased expression of mmu-miR-1555p, with a resulting decrease of INPPD5, promotes transcription of major proinflammatory cytokines in macrophages [45–46]. Restoration of INPPD5 levels is related to an inhibition in PI3K-AKT signaling and anti-inflammatory response in raw264.7 cells and primary bone marrow-derived macrophages (BMDMs), as reported in bowel disease [46]. It is tempting to hypothesize that, at early infection (8h in this study), INPPD5 is downregulated by mmu-miR-155-5p, leading to the production of major pro-inflammatory cytokines, such as 1L-1α and TNF-α, previously observed in macrophages [36], as a first response to infection by Leptospira.

Another common pathway identified in this study, between attenuated and virulent strains, was PI3K/AKT signaling with several target genes for mmu-miR-155-5p involved in pro-inflammatory response. This pathway is responsible for B cell development trough activation of several genes. Inhibited PI3K signaling leads to immunodeficiency, autoimmunity activation and leukemia [47–48]. Cheung et al., [49] have shown that IL-10 inhibits miR-155, but this process is dependent on the presence of INPPD5 (a target to miR-155), and also that the activation of PI3K/AKT signaling abolished IL-10-inhibition of miR-155, resulting in an increase of miR-155 [49]. It is plausible to infer that miR-155 can be dependent of PI3K/AKT signaling to promote inflammation in *L. interrogans* infection, and that mmu-miR-155 could be a master regulator of pro-inflammatory process in leptospiral infection.

Another goal in the present study was to identify differences in macrophageal response to strains, varying only in virulence. We have identified 16 specific canonical pathways potentially regulated by miRNAs modulated following infection with virulent L. interrogans. Cellular movement and cell-to-cell signaling interactions are functions related with the canonical pathways IL-8 (CXCL8) signaling, Tec kinase signaling, Epithelial adherens junction signaling and Gα12/13 signaling. It is well know that Leptospira causes changes

in adherens junctions and endothelial cells increasing vascular permeability, potentially leading to severe illness [50]. The small GTPase RhoA (ras homolog family member A), a protein that regulates actin cytoskeleton and the remodeling of cell junctions, appears in most of the pathways mentioned above, as a direct target of miR-155-5p. In a recent study, Sato & Coburn [50] found a slight elevation of RhoA protein levels in endothelial cells following infection of both virulent and saprophyte Leptospira strains. In our macrophage cells, we observed an increase of miR-155-5p, which could lead to a decrease in RhoA, contrary to what was observed by Sato & Coburn in the endothelial cell line. This could be due to a cell type difference, to a difference in time of infection (8h in our study versus 24h in the aforementioned study), or to a difference in regulation of RhoA between the cell types.

A very common occurrence in patients with leptospirosis is a coagulation disorder causing lung hemorrhage [4]. The mechanism behind this Leptospira-induced hemorrhage is not fully understood. Fernandes et al, [51] provide evidence that lower serum levels of prothrombin and antithrombin III in patients with the disease is related to the observed hemorrhage. Liver-produced prothrombin remains inactive in circulation until being proteolytically cleaved to form thrombin to start clot formation by converting fibrinogen to fibrin. Here, we can suggest that Prothrombin (Coagulation Factor II) is potentially downregulated by miR-155-5p, suggesting a role for epigenetically mediated post-transcriptional control of clot formation in leptospirosis patients. In fact, recent studies support the idea that miR-155-5p can be secreted to act as modulators elsewhere. Wang et al. [52] have just shown that macrophages secrete miR-155-5p that can act as paracrine regulators of inflammation during cardiac injury. Alexander et al. [53] have also demonstrated that miR-155 present in exosomes can pass between immune cells in vivo and promote endotoxin-induced inflammation in mice.

Pathogen invasion into host cells is crucial for pathogenicity. Through phagocytosis, macrophages can kill the invading bacteria early in the process of infection. Both macropinocytosis signaling and clathrin-mediated endocytosis

signaling are pathways significantly identified following infection with the virulent strain. In the macropinocytosis process, two genes appear to be down-regulated, Colony Stimulating Factor 1 Receptor (CSF1R), a target of miR-155-5p and SRC (Proto-oOncogene, nNonReceptor Tyrosine Kinase (SRC), targeted by mmu-miR-203-3p. CSF1R is a type III tyrosine kinase receptor, involved in cell proliferation and survival, and when phosphorylated, this receptor activates SRC kinases to initiate the signal cascade required for macropinocytosis process [54–55]. Therefore these miRNAs have potential to control initial signaling cascades of macropinocitosis.

During phagocytosis, one of the most effective weapons used by macrophages to kill invading leptospires is nitric oxide (NO) and reactive oxygen species (ROS), which induce an antimicrobial response [56–57]. On the other hand, an excessive production of $O_2$ by macrophages can affect homeostasis [56]. This burden of intracellular oxygen demand by macrophages to kill pathogens has important collateral effects that can contribute to the inflammatory process through hypoxia in tissues [58] and DNA damage [59].

Further, Luo et al., [56] reported that Erythropoietin signaling, significantly identified in our study as a response to virulent infection, has a vital function in regulation of acute inflammatory conditions in hypoxia. This process of inflammation regulation appears to be inhibited in our acute infection, which could ultimately be associated with the exacerbated inflammation commonly seen in leptospirosis.

Hu and colleagues [59] report that leptospiral infection in macrophages induces cell cycle arrest dependent of p53/p21. Here we identified that the target pathway p53 signaling is regulated, following virulent infection, by modulation of miRNAs. This pathway can be activated by DNA damage, hypoxia, cytokines, metabolic changes, viral infection or oncogenes [60–61], and our study adds bacterial infection by Leptospira sp as another activator. This pathway triggers three important processes in the host cell, cell cycle arrest for DNA repair, apoptosis and cell survival. Here we found that BCL2 (anti-apoptotic gene) is potentially downregulated by mmu-miR-7667-3p following infection with L.

interrogans, leading us to suggest that cell survival could be at risk following L. interrogans infection of macrophages.

Lastly, another virulent specific pathway identified in our study, the retinoic acid receptor (RAR) activation, has not been previously described in leptospirosis. This canonical pathway is related to development, differentiation, apoptosis and homeostasis, mostly participating in phosphorylation of several signaling pathways, as reviewed by [62], and also has fundamental importance in acquired and adaptative immune responses, with an important role in clonal expansion, differentiation, survival of pathogen-specific CD8 T cells, and bacterial clearance, as confirmed by a knockout model of RAR in mice [63]. These nuclear receptors act on recruitment of the transcriptional machinery to DNA response elements, regulating other complexes like nuclear factor kappa B (NF-κB complex) [62], SMAD complex [63], and also interact with other signaling pathways like PI3K/Akt and PTEN [62], which are vital to immune response. Here we found genes like Mothers Against Decapentaplegic Homolog (SMAD1/2) as a predicted target to mmu-miR-155-5p, SMAD7/9 targeted by miR- 7667-3p, TGF-β, that indirectly increases activation of SMAD complexes and is a target of mmu-miR-7069-3p and, finally, nuclear factor kappa B (NF-κB complex), a predicted target of mmu-miR-155-5p [64]. We suggest that upregulation of these miRNAs following macrophageal infection by L. interrogans can negatively regulate immune response to virulent leptospires through modulation of RAR activation. Interestingly, it has been demonstrated that vitamin A, a ligand of RAR, has antimicrobial activity against monocytes infected with Mycobacterium tuberculosis, in a mechanism dependent of intracellular cholesterol transporter 2 (NPC2) [65], raising the question as to whether this effect extends to other bacterial infections.

Our data provides the first evidence that host miRNAs are regulated by Leptospira infection in macrophages in a virulence- and species-specific manner in vitro. A large number of the identified miRNA targets participate in key processes involved in the immune response. Characterization of this regulatory network may help to understand the pathogenesis of leptospirosis

and to identify miRNAs as biomarkers of infection or as targets for therapy. In conclusion, we suggest that post-transcriptional regulation by miRNAs play a role in the host's response to leptospirosis infection.

## 4 Methods

### 4.1 Bacterial strains

Three types of bacterial samples were utilized in this study, *Leptospira interrogans* serovar Copenhageni strain FIOCRUZ-L1-130, as a virulent strain; the pathogenic culture-attenuated *L. interrogans* serovar Copenhageni strain M-20; and *Leptospira biflexa* serovar Patoc strain FIOCRUZ-Patoc I as a saprophyte strain. Bacteria were maintained in Fletcher semi solid culture medium, and incubated at 30°C. To restore bacterial virulence in strain L1-130, 1mL of cultured bacteria was inoculated intraperitoneally in hamsters (Mesocricetus auratus) and later recovered from kidneys. Attenuated strain did not undergo intraperitoneal inoculation in hamsters [66]. The inoculum was quantified using the camera of Petroff-Hausser.

### 4.2 Ethics statement

Bacterial samples were provided by Laboratory of Preventive Veterinary Medicine of University of São Paulo (USP). Production of these samples were in accordance with Ethics Committee for Animal Use (FOA-FMVA UNESP), under protocol number 2015–00895. Following bacterial arrival, no animal experimentation was performed in the experiments described herein.

### 4.3 Macrophage culture

Murine monocyte-macrophage cells (Mus musculus monocyte-macrophage cell line J774A.1), provided by the Paul Ehrlich cell bank, Rio de Janeiro, Brazil, was maintained in RPMI1640 media (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100ug/mL streptomycin (Sigma Chemical Co St. Louis, MO), 0.03% Lglutamine solution (Sigma) and 100 UI/mL of penicillin. Cells were incubated at 37° C, 5% CO2 until formation of a confluent monolayer in 6-well cell culture plates (3cm/well).

### 4.4 Infection of macrophages

Cultured cells were washed three times with sterile phosphate buffer solution (pH 7,2) for removal of antibiotics and non-adherent cells. L. interrogans and L. biflexa were harvested by centrifugation and the pellet was resuspended in RPMI-1640 media (Sigma), and 100:1 bacteria:cell were added to macrophages at confluency (MOI of 100), as previously described [24]. Treatments, performed in three biological replicates, were carried as follows: infection of macrophages with a virulent strain (L. interrogans), infection with attenuated strain (L. interrogans), saprophyte strain (L. biflexa) and non-infected macrophages (control). All treatments were incubated in fresh RPMI medium, without antibiotics, for 8h at 37° C, 5% C02. Rate of infection did not differ between strains (78, 85 e 80% for saprophyte, attenuated and virulent,

respectively). Following this period, RNA extraction was immediately performed as described below.

### 4.5 RNA extraction and quantification

Total RNA was extracted from macrophages with a miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA samples were immediately stored at -80°C. Quantification was performed using NanoDrop (ND-2000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA) and quality of samples was assessed using capillary electrophoresis (Bioanalyzer 2100 Agilent, Santa Clara, CA, USA). All samples used for microarray analysis had a RIN of 10.

### 4.6 Microtranscriptome array

MicroRNA profiles were obtained from 250ng/sample of total RNA (RIN 10) using the FlashTag Biotin HSR RNA Labeling Kit, and the Affymetrix miRNA 4.1 Array strip (Affymetrix, Santa Clara, California, EUA), containing 3195 murine specific probes of miRNA, according to the manufacturer's instructions. A recommended ELOSA quality control assay was run for all samples, and hybridization of samples to the strips was carried at 48°C for 20h. Following this period, strips were processed and scanned using the GeneAtlas System (Affymetrix). Raw intensity values were background corrected, log2 transformed and then quantile normalized by the software Expression Console (Affymetrix) using the Robust Multi-array Average (RMA) algorithm. Data files were deposited at Gene Expression Omnibus (GSE105104). Statistical analysis was performed in the TAC software (Affymetrix) by ANOVA (fold change ± 1.5, $p < 0.01$).

### 4.7 Identification of microRNA targets and functional enrichment

For identification of target genes we employed the miRNA Target Filter Analysis from the Ingenuity Pathway Analysis (IPA) software (Qiagen). For selection, we opted to use conservative filters, allowing only experimentally observed and highly predicted targets to be selected (Supplementary table). For the identification of canonical pathways potentially regulated by the differentially expressed miRNAs, we employed the Benjamini-Hochberg (BH) correction for multiple testing (BH corrected $p<0.05$).

### 4.8 Validation of microarray results by qRT-PCR

For validation of miRNA expression in infected macrophages (saprophyte, attenuated and virulent strains) and non-infected control macrophages, we employed the miScript miRNA PCR System (Qiagen-Valencia, CA, USA) for preparation of cDNA and realtime PCR, according to manufacturer´s instructions. Validated inventoried primers employed were purchased from Qiagen. PCR was performed using a Stratagene QPCR System Mx3005P (Agilent Technologies, Santa Clara, CA, USA), following instructions on the miScript miRNA PCR System´s manual. Expression levels were determined using standard curves for all miRNAs at each individual run, and the expression of candidate miRNAs is presented as a ratio to the control miRNA SNORD96A.

### 4.9 Statistical analysis

Differential expression of each miRNA was determined by ANOVA with two criteria, a fold change of ±1.5 comparing all infected groups to the non-infected control and p-value<0.01. Real time PCR data was analyzed using least-squares analysis of variance and the general linear model procedures of SAS (SAS Institute, Cary, NC, USA; $p<0.01$). Comparison of means was done using Duncan's multiple range test, and significance was set at $p<0.05$.

### 4.10 Accession numbers

mmu-miR-155-3p (MIMAT0016993); mmu-miR-155-5p (MIMAT0000165); mmu-miR-221-5p (MIMAT0016070); mmu-miR-1946b (MIMAT0009443); mmu-miR-3473b (MIMAT0020367); mmu-miR-1946a (MIMAT0009412); mmu-miR-203-3p (MIMAT0000236); mmu-miR-222-3p (MIMAT0000670); mmu-miR-7667-3p (MIMAT0029841); mmu-miR-7069-3p (MIMAT0028045); mmu-mir-3473c (MI0018015); mmu-mir-7676-1 (MI0025017); mmu-mir-7676-2 (MI0025018); mmu-miR-1894-5p (MIMAT0007877); mmu-miR-702-5p (MIMAT0022931); mmu-miR-7053-3p (MIMAT0028011); mmu-miR-6987-3p (MIMAT0027877); mmu-miR-6370 (MIMAT0025114); mmu-mir-717 (MI0004704); mmu-miR-7067-5p (MIMAT0028038); mmu-mir-124-3 (MI0000150); mmu-mir-124-1 (MI0000716); mmu-mir-124-2 (MI0000717); mmu-miR-222-5p (MIMAT0017061); mmu-miR-7119-3p (MIMAT0028136); mmu-miR-5100 (MIMAT0020607); mmu-miR-2137 (MIMAT0011213); mmu-miR-5046 (MIMAT0020540); mmu-miR-8100 (MIMAT0031403).

## 4.12 Supporting information

S1. **Common pathways with respective miRNAs and targets obtained from IPA software.**

| Pathway | ID | Confidence | Symbol |
|---|---|---|---|
| **Fc-gammaR-mediated phagocytosis in macrophages** | mmu-miR-155-5p | High (predicted) | ACTA1 |
| | mmu-miR-155-5p | Experimentally Observed | SHIP-1 |
| | mmu-miR-155-5p | Experimentally Observed,High (predicted) | PRKCI |
| | mmu-miR-155-5p | High (predicted) | RPS6KB1 |
| | mmu-miR-155-5p | Experimentally Observed | VAMP3 |
| | mmu-miR-155-5p | High (predicted) | VAV3 |
| | mmu-miR-7069-3p | High (predicted) | GAB2 |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | High (predicted) | ACTR3 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | PIK3R1 |
| | mmu-miR-222-3p | Experimentally Observed | PTEN |
| | mmu-miR-221-5p | High (predicted) | PRKCZ |
| | mmu-miR-7667-3p | High (predicted) | CBL |
| | mmu-miR-7667-3p | High (predicted) | FYB |
| | mmu-miR-7667-3p | High (predicted) | GAB2 |
| | mmu-miR-7667-3p | High (predicted) | RAC2 |
| | mmu-miR-7067-5p | High (predicted) | PLA2G6 |
| | mmu-miR-7067-5p | High (predicted) | PXN |
| | mmu-miR-7067-5p | High (predicted) | VASP |
| | mmu-miR-6987-3p | High (predicted) | DGKB |
| **PI3K/AKT Signaling** | mmu-miR-155-5p | Experimentally Observed | CCND1, CTNNB1, INPP5D |
| | mmu-miR-155-5p | Experimentally Observed,Moderate (predicted) | IKBKE, RHEB |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-222-3p | Experimentally Observed | FOXO3, PTEN |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | CDKN1B, PPP2R2A, PIK3R1 |
| | mmu-miR-155-5p | High (predicted) | RPS6KB1 |
| | mmu-miR-7069-3p | High (predicted) | GAB2 |
| | mmu-miR-221-5p | High (predicted) | CCND1, PRKCZ |
| | mmu-miR-7667-3p | High (predicted) | BCL2, GRB2, EIF4E, GAB2 |
| | mmu-miR-7067-5p | High (predicted) | EIF4EBP1, SFN, PPP2R5D, PPP2R5B, |

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|---|---|---|---|
| | | | ITGA3, FOXO3 |
| | mmu-miR-6987-3p | High (predicted) | YWHAH |
| **Molecular mechanism of Cancer** | mmu-miR-155-5p | Experimentally Observed | CCND1, FADD, CTNNB1, GNA13, PMAIP1, RHOA, SMAD2 |
| | mmu-miR-155-5p | Experimentally Observed,Moderate (predicted) | SMAD1, TAB2 |
| | mmu-miR-155-5p | Experimentally Observed,High (predicted) | PRKCI |
| | mmu-miR-155-5p | High (predicted) | APAF1, FOS, TCF4 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-7069-3p | High (predicted) | BCL2L11, WNT8B, CASP7, CDKN2D, GAB2, RND2, TGFB3 |
| | mmu-miR-203-3p | Experimentally Observed | ABL1, SRC |
| | mmu-miR-222-3p | Experimentally Observed | BCL2L11 |
| | mmu-miR-222-3p | Experimentally Observed,Moderate (predicted) | APAF1, BBC3 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | DIRAS3, CDKN1B, FOS, PIK3R1 |
| | mmu-miR-222-3p | High (predicted) | CDKN2B |
| | mmu-miR-7667-3p | High (predicted) | ARHGEF7, SMAD9, SMAD7, RAC2, BCL2, CBL, GAB2, GRB2 |
| | mmu-miR-7067-5p | High (predicted) | BAK1, WNT5B, TLR9, TGFBR2, SMAD7, PRKAR1B, PAK4, ITGA3, GNAI2, FZD9, FADD, CAMK2G, BCL2L11, WNT7B |
| | mmu-miR-221-5p | High (predicted) | CAMK2A, SUV39H1, CCND1, |

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|---|---|---|---|
| | | | FADD, GNA14, NOTCH1, PRKCZ, RHOD |
| | mmu-miR-6987-3p | High (predicted) | HIF1A. WNT7A |
| **PTEN Signaling** | mmu-miR-155-5p | Experimentally Observed | CCND1, INPP5D |
| | mmu-miR-155-5p | Experimentally Observed,Moderate (predicted) | IKBKE |
| | mmu-miR-155-5p | High (predicted) | RPS6KB1 |
| | mmu-miR-7069-3p | High (predicted) | BCL2L11 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-222-3p | Experimentally Observed | BCL2L11, PTEN, FOXO3 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | CDKN1B, PIK3R1 |
| | mmu-miR-222-3p | High (predicted) | KDR |
| | mmu-miR-221-5p | High (predicted) | CCND1, PRKCZ |
| | mmu-miR-7667-3p | High (predicted) | BCL2, RAC2, CBL, GRB2 |
| | mmu-miR-7067-5p | High (predicted) | BCL2L11, DDR1, FOXO3, FOXO4, ITGA3, NGFR, TGFBR2, TNFRSF11A |
| | mmu-miR-6987-3p | High (predicted) | YWHAH |
| **Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis** | mmu-miR-155-5p | Experimentally Observed | CCND1, RHOA, RIPK1, CTNNB1, MYD88 |
| | mmu-miR-155-5p | Experimentally Observed,High (predicted) | CEBPB, PRKCI |
| | mmu-miR-155-5p | Experimentally Observed,Moderate (predicted) | IKBKE, SOCS1, TCF7L2 |
| | mmu-miR-155-5p | High (predicted) | CSNK1A1, TCF4, FOS, IL36G |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-7069-3p | High (predicted) | PDGFB, WNT8B, PROK1 |
| | mmu-miR-203-3p | Experimentally Observed | SOCS3, SRC |
| | mmu-miR-222-3p | Experimentally Observed | ICAM1 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS, PIK3R1 |

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|---|---|---|---|
| | mmu-miR-222-3p | Experimentally Observed,Moderate (predicted) | MMP1 |
| | mmu-miR-222-3p | High (predicted) | PLCL2, SOCS3, PPP3R1 |
| | mmu-miR-221-5p | High (predicted) | CAMK2A, PRKCZ, CCND1, IRAK4 |
| | mmu-miR-7667-3p | High (predicted) | GRB2, PROK1, BCL2, CBL, GRB2, SMAD9 |
| | mmu-miR-7067-5p | High (predicted) | CALM1 (includes others), FZD9, IL1RN, ITGA3, MAP2K7, NGFR, TLR9, TNFRSF11A, WNT5B, WNT7B |
| | mmu-miR-6987-3p | High (predicted) | CALM1 (includes others), WNT7A |

S2.Specific pathways with respective miRNAs and targets obtained from IPA software.

| Pathway | ID | Confidence | Gene Symbol |
|---|---|---|---|
| **PI3K Signaling in B Lymphocytes** | mmu-miR-155-5p | High (predicted) | FOS, VAV3 |
| | | Experimentally Observed,Moderate (predicted) | IKBKE |
| | | Experimentally Observed | INPP5D |
| | | Experimentally Observed,High (predicted) | PRKCI |
| | mmu-miR-7069-3p | High (predicted) | IRS4 |
| | | Experimentally Observed | KRAS |
| | mmu-miR-203-3p | Experimentally Observed | ABL1 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS, PIK3R1 |
| | | Experimentally Observed | FOXO3, PTEN |
| | | High (predicted) | PLCL2, PLEKHA2, PPP3R1 |
| | mmu-miR-221-5p | High (predicted) | CAMK2A, PRKCZ, CD180 |
| | mmu-miR-7667-3p | High (predicted) | CBL, CD19, CD180 |
| **IL-8 Signaling** | mmu-miR-155-5p | Experimentally Observed | CCND1, GNA13, RHOA |
| | | High (predicted) | FOS, RPS6KB1 |
| | | Experimentally Observed,Moderate (predicted) | IKBKE |
| | | Experimentally Observed,High (predicted) | PRKCI |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | | High (predicted) | PROK1, RND2 |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | DIRAS3, FOS, PIK3R1 |
| | | Experimentally Observed | ICAM1 |
| | | High (predicted) | KDR |
| | mmu-miR-221-5p | High (predicted) | CCND1, IRAK4, PRKCZ, RHOD |
| | mmu-miR-7667-3p | High (predicted) | BCL2, GNG13, GRB2, MYL9, PROK1, RAC2 |
| | mmu-miR-702-5p | High (predicted) | GNG13 |

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| **RAR Activation** | mmu-miR-155-5p | Experimentally Observed,High (predicted) | ARID2, PRKCI |
| | | High (predicted) | FOS, RBP2 |
| | | Experimentally Observed,Moderate (predicted) | SMAD1 |
| | | Experimentally Observed | SMAD2 |
| | mmu-miR-7069-3p | High (predicted) | CITED2, RXRB, SDR9C7, TGFB3 |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS, PIK3R1 |
| | | Experimentally Observed,Moderate (predicted) | MMP1 |
| | | Experimentally Observed | PTEN |
| | | High (predicted) | RBP2 |
| | mmu-miR-221-5p | High (predicted) | ALDH1A2, IL3RA, NRIP2, PRKCZ |
| | mmu-miR-7667-3p | High (predicted) | SMAD7, SMAD9, ZBTB16 |
| | mmu-miR-702-5p | High (predicted) | GTF2H5 |
| **p53 Signaling** | mmu-miR-155-5p | High (predicted) | APAF1 |
| | | Experimentally Observed | CCND1, CTNNB1, PMAIP1 |
| | | Experimentally Observed,Moderate (predicted) | TNFRSF10A |
| | | Experimentally Observed,High (predicted) | TP53INP1 |
| | mmu-miR-7069-3p | High (predicted) | CSNK1D, DRAM1 |
| | mmu-miR-203-3p | Experimentally Observed | TP63 |
| | mmu-miR-222-3p | Experimentally Observed,Moderate (predicted) | APAF1, BBC3 |
| | | Experimentally Observed,High (predicted) | PIK3R1 |
| | | Experimentally Observed | PTEN |
| | | High (predicted) | TP53BP2 |
| | mmu-miR-221-5p | High (predicted) | CCND1, TP63 |
| | mmu-miR-7667-3p | High (predicted) | BCL2, GRB2 |
| | mmu-miR-155-3p | Experimentally Observed | DNAJA2, DNAJB1 |

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| **NRF2-mediated Oxidative Stress Response** | mmu-miR-155-5p | High (predicted) | ACTA1, FOS |
| | | Experimentally Observed,High (predicted) | BACH1, PRKCI |
| | | Experimentally Observed | DNAJB1, MAF, DNAJC19, TXNRD1 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS, PIK3R1 |
| | | Experimentally Observed,Moderate (predicted) | SOD2 |
| | mmu-miR-221-5p | High (predicted) | DNAJB6, PRKCZ, GSTM4, GSTK1 |
| | mmu-miR-7667-3p | High (predicted) | DNAJC5G, GPX2, GRB2, MAFF |
| | mmu-miR-702-5p | High (predicted) | DNAJB5 |
| **Erythropoietin Signaling** | mmu-miR-155-5p | High (predicted) | FOS, RPS6KB1 |
| | | Experimentally Observed,High (predicted) | PRKCI |
| | | Experimentally Observed,Moderate (predicted) | SOCS1 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-203-3p | Experimentally Observed | SOCS3, SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS, PIK3R1 |
| | mmu-miR-222-3p | High (predicted) | SOCS3 |
| | mmu-miR-221-5p | High (predicted) | EPO, PRKCZ |
| | mmu-miR-7667-3p | High (predicted) | CBL, GRB2 |
| **Macropinocytosis Signaling** | mmu-miR-155-5p | Experimentally Observed | ANKFY1, MET, RHOA |
| | | Experimentally Observed,Moderate (predicted) | CSF1R, RAB34 |
| | | Experimentally Observed,High (predicted) | PRKCI |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | | High (predicted) | PDGFB |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | PIK3R1 |
| | mmu-miR-221-5p | High (predicted) | PRKCZ |

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| | mmu-miR-7667-3p | High (predicted) | GRB2 |
| **Clathrin-mediated Endocytosis Signaling** | mmu-miR-155-5p | High (predicted) | ACTA1 |
| | | Experimentally Observed | F2, SH3BP4, MYO1E, MET |
| | | Experimentally Observed,High (predicted) | FGF7, PICALM |
| | | Experimentally Observed,Moderate (predicted) | RAB5C |
| | mmu-miR-7069-3p | High (predicted) | PDGFB, PROK1 |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | High (predicted) | ACTR3, PPP3R1, HSPA8 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | PIK3R1 |
| | mmu-miR-221-5p | High (predicted) | APOL1, LDLR |
| | mmu-miR-7667-3p | High (predicted) | APOL1, PROK1, PIP5K1C, CBL, GRB2 |
| | mmu-miR-702-5p | High (predicted) | APOM |
| **Small Cell Lung Cancer Signaling** | mmu-miR-155-5p | High (predicted) | APAF1 |
| | | Experimentally Observed | CCND1 |
| | | Experimentally Observed,Moderate (predicted) | IKBKE |
| | mmu-miR-7069-3p | High (predicted) | RXRB |
| | mmu-miR-203-3p | Experimentally Observed | ABL1 |
| | mmu-miR-222-3p | Experimentally Observed,Moderate (predicted) | APAF1, PTEN |
| | | Experimentally Observed,High (predicted) | CDKN1B, PIK3R1 |
| | | High (predicted) | CDKN2B |
| | mmu-miR-221-5p | High (predicted) | CCND1, SUV39H1 |
| | mmu-miR-7667-3p | High (predicted) | BCL2, GRB2 |
| **Epithelial Adherens Junction Signaling** | mmu-miR-155-5p | High (predicted) | ACTA1, TCF4 |
| | | Experimentally Observed | CTNNB1, RHOA, MET |
| | | Experimentally Observed,Moderate (predicted) | TCF7L2 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS, NOTCH2 |

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| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | High (predicted) | ACTR3, NECTIN1, TUBA1A |
| | | Experimentally Observed | PTEN |
| | | Experimentally Observed,Moderate (predicted) | PTPRM |
| | mmu-miR-221-5p | High (predicted) | NOTCH1 |
| | mmu-miR-7667-3p | High (predicted) | EPN2, MYL9 |
| **Ovarian Cancer Signaling** | mmu-miR-155-5p | Experimentally Observed | CCND1, CTNNB1 |
| | | High (predicted) | RPS6KB1, TCF4 |
| | | Experimentally Observed,Moderate (predicted) | TCF7L2 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-7069-3p | High (predicted) | PROK1, WNT8B |
| | mmu-miR-203-3p | Experimentally Observed | ABL1, SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | PIK3R1 |
| | mmu-miR-222-3p | Experimentally Observed | PTEN |
| | mmu-miR-221-5p | High (predicted) | CCND1, SUV39H1, RAD51 |
| | mmu-miR-7667-3p | High (predicted) | BCL2, GRB2, PROK1 |
| **Osteoarthritis Pathway** | mmu-miR-155-5p | Experimentally Observed,High (predicted) | CEBPB |
| | | Experimentally Observed | CTNNB1, SMAD2, FADD |
| | | Experimentally Observed,Moderate (predicted) | SMAD1, TCF7L2 |
| | | High (predicted) | TCF4 |
| | mmu-miR-7069-3p | High (predicted) | CASP7, WNT8B, LEP, PROK1 |
| | mmu-miR-203-3p | Experimentally Observed | RUNX2 |
| | mmu-miR-222-3p | Experimentally Observed,Moderate (predicted) | DDIT4, MMP1 |
| | | Experimentally Observed | FOXO3, TIMP3 |
| | mmu-miR-221-5p | High (predicted) | CASP2, SDC4, NOTCH1, FADD |

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|---|---|---|---|
| | mmu-miR-7667-3p | High (predicted) | PROK1, SMAD7, SMAD9 |
| **Tec Kinase Signaling** | mmu-miR-155-5p | High (predicted) | ACTA1, VAV3, FOS |
| | | Experimentally Observed | FADD, RHOA, GNA13 |
| | | Experimentally Observed,High (predicted) | PRKCI |
| | | Experimentally Observed,Moderate (predicted) | TNFRSF10A |
| | mmu-miR-7069-3p | High (predicted) | RND2 |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | DIRAS3, FOS, PIK3R1 |
| | mmu-miR-221-5p | High (predicted) | FADD, TNFRSF21, RHOD, PRKCZ, GNA14 |
| | mmu-miR-7667-3p | High (predicted) | GNG13, GRB2 |
| | mmu-miR-702-5p | High (predicted) | GNG13 |
| **Gα12/13 Signaling** | mmu-miR-155-5p | Experimentally Observed | CTNNB1, RHOA, GNA13, F2 |
| | | Experimentally Observed,Moderate (predicted) | IKBKE |
| | | High (predicted) | LPAR6, VAV3 |
| | mmu-miR-7069-3p | High (predicted) | CDH20, LPAR5 |
| | | Experimentally Observed | KRAS |
| | mmu-miR-203-3p | Experimentally Observed | SRC |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | PIK3R1 |
| | mmu-miR-221-5p | High (predicted) | F2RL3 |
| | mmu-miR-7667-3p | High (predicted) | GRB2, MYL9 |
| **Regulation of IL-2 in Activated and Anergic T Lymphocytes** | mmu-miR-155-5p | High (predicted) | FOS, VAV3 |
| | | Experimentally Observed,Moderate (predicted) | IKBKE |
| | | Experimentally Observed | SMAD2 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | | High (predicted) | TGFB3 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | FOS |

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|---|---|---|---|
| | | High (predicted) | PPP3R1 |
| | mmu-miR-221-5p | High (predicted) | CD3E |
| | mmu-miR-7667-3p | High (predicted) | CD28, GRB2 |
| | mmu-miR-702-5p | High (predicted) | CD247 |
| **Pancreatic Adenocarcinoma Signaling** | mmu-miR-155-5p | Experimentally Observed | CCND1, SMAD2 |
| | mmu-miR-7069-3p | Experimentally Observed | KRAS |
| | mmu-miR-7069-3p | High (predicted) | PROK1, TGFB3 |
| | mmu-miR-203-3p | Experimentally Observed | ABL1 |
| | mmu-miR-222-3p | Experimentally Observed,High (predicted) | CDKN1B, PIK3R1 |
| | | High (predicted) | CDKN2B |
| | mmu-miR-221-5p | High (predicted) | CCND1, SUV39H1, RAD51, NOTCH1 |
| | mmu-miR-7667-3p | High (predicted) | BCL2, PROK1, GRB2 |

## 4.11 Acknowledgments

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