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Circulating microRNAs as potential biomarkers in cutaneous and mucosal leishmaniasis

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Abstract

Tegumentary leishmaniasis (TL) caused by protozoan of the genus *Leishmania* encompasses a spectrum of clinical manifestations, including cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML). Disease outcomes are strongly influenced by host immune responses, which are regulated in part by microRNAs (miRNAs). Circulating miRNAs are promising non-invasive biomarkers, but their value for distinguishing cutaneous and mucosal forms of leishmaniasis and for monitoring treatment response is poorly understood. We profiled circulating miRNAs in plasma from patients with CL, patients with ML and healthy controls using a PCR array of 84 inflammation- and autoimmunity-related miRNAs. Patients with CL were further stratified by therapeutic response, and paired plasma samples collected before and 90 days after treatment were analyzed. An exploratory *in silico* analysis was performed to identify predicted target genes and biological pathways of candidate miRNAs. Compared with healthy controls, patients with CL showed significant downregulation ($P < 0.05$) of miR-19b-3p, let-7b-5p, miR-16-5p, miR-424-5p, miR-19a-3p, miR-101-3p, miR-15a-5p, miR-9-5p and miR-144-3p, whereas let-7g-5p was significantly downregulated in ML. Direct comparison between ML and CL showed significant upregulation of miR-1324, miR-524-5p, miR-302b-3p, let-7b-5p and miR-520d-3p in ML. Before treatment, responders had higher levels of miR-1324, miR-34c-5p and miR-373-3p than non-responders, and longitudinal analyses revealed treatment-associated changes in miR-372-3p in responders and in let-7b-5p and miR-301b-3p in non-responders. *In silico* analyses linked candidate miRNAs to the adherens junction pathway. Circulating miRNA profiles therefore differ between the clinical forms investigated and according to therapeutic response, supporting their potential as non-invasive biomarkers for disease stratification and treatment monitoring.

Keywords: *Leishmania braziliensis*, microRNA, biomarkers, cutaneous leishmaniasis, mucosal leishmaniasis, circulating miRNA, inflammation

1. Introduction

Tegumentary Leishmaniasis (TL) is a parasitic disease caused by diverse species of protozoan parasites of the genus *Leishmania* and represents a major public health problem in tropical and subtropical regions including Latin America **[1–2]**. In Brazil, *Leishmania (Viannia) braziliensis* is the predominant causative species and is associated with a broad clinical spectrum that includes cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and disseminated forms **[3–4]**. CL, the most common clinical manifestation, typically presents as ulcerative skin lesions that may heal spontaneously or progress to chronic forms **[5]**. ML, which affects 2-5% of CL patients caused by *L. (V.) braziliensis*, represents a severe and disfiguring form characterized by progressive destruction of nasal and oropharyngeal mucosa **[4].**

The clinical heterogeneity observed in TL reflects complex interactions between parasite virulence factors, vector characteristics, and host immune responses **[6]**. While a Th1-dominated immune response characterized by IFN-γ production and macrophage activation is essential for parasite control, an exacerbated inflammatory response has been associated with tissue damage, particularly in ML **[7–8]**. Conversely, regulatory mechanisms mediated by IL-10 and TGF-β can limit immunopathology but may also favor parasite persistence **[9–10]**. This delicate balance between protective immunity and immunopathology represents a central determinant of clinical outcome in TL, yet the molecular mechanisms governing these processes remain incompletely understood.

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 22 nucleotides that regulate gene expression post-transcriptionally by binding to complementary sequences in the 3' untranslated region (3'UTR) of target mRNAs, leading to translational repression or mRNA degradation **[11]**. It is estimated that more than 60% of protein-coding genes in the human genome

are regulated by at least one miRNA, positioning these molecules as fundamental components of gene regulatory networks **[11–12]**. miRNAs play central roles in regulating immune processes, including hematopoietic cell development and differentiation, macrophage activation, cytokine production, and modulation of inflammatory signaling pathways **[13–14]**.

Circulating miRNAs can be detected in body fluids such as plasma, serum, saliva, and urine, where they are protected from ribonuclease degradation through encapsulation in extracellular vesicles (exosomes and microvesicles), association with proteins such as Argonaute 2 (AGO2), or incorporation into high-density lipoproteins **[15–17]**. This stability in the extracellular environment and the possibility of quantification by RT-qPCR with high sensitivity make circulating miRNAs promising candidates as non-invasive biomarkers for diagnosis, prognosis, and therapeutic monitoring in various diseases **[15,18]**.

In the context of infectious diseases, studies have demonstrated that circulating miRNA profiles are altered in response to viral, bacterial, and parasitic infections, reflecting both the host's immunological status and the intensity of the inflammatory response **[13]**. In leishmaniasis, several studies have identified miRNAs differentially expressed in tissues and cells infected with *Leishmania* **[19–21]**. However, studies evaluating circulating miRNAs as biomarkers in TL remain limited, and their potential to distinguish between clinical forms and monitor therapeutic response has not been systematically explored.

This study aimed to characterize the expression profile of circulating miRNAs in plasma samples from patients with CL, ML, and healthy individuals, as well as in CL patients before and after treatment, using a PCR Array targeting inflammation- and autoimmunity-related miRNAs. Our findings identify candidate miRNAs associated with distinct clinical manifestations of TL and with treatment response, providing insights into the molecular mechanisms underlying disease pathogenesis and highlighting potential biomarkers for clinical application.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Brazilian National Research Ethics Committee (CONEP) under protocol number CAAE: 71602417.0.0000.0065. All participants provided written informed consent prior to sample collection, in accordance with the Brazilian national ethical guidelines for research involving human subjects.

2.2. Study population and sample collection

Participants were recruited at the Ambulatório de Dermatopatias Infecciosas do Instituto de Infectologia Emílio Ribas, São Paulo, Brazil, and were enrolled based on clinical, epidemiological, and laboratory criteria consistent with TL. The study population included individuals diagnosed with CL and ML before treatment initiation. Diagnosis was confirmed based on clinical and epidemiological criteria and laboratory methods, including parasitological, histopathological, or immunohistochemical confirmation, as well as anti-*Leishmania* serology, when applicable **(Table 1)**. In addition, healthy individuals with no previous history of leishmaniasis were included as a control group. The inclusion criteria comprised individuals aged 18 years or older with a confirmed diagnosis of TL and active lesions. Pregnant individuals, patients with diabetes mellitus, HIV coinfection, immunosuppression, debilitating clinical conditions, a previous history of chemotherapeutic treatment for leishmaniasis, or lesions located in areas unsuitable for biopsy or local treatment were excluded from the study.

Plasma samples from patients with CL were divided into four groups according to therapeutic response and time of sample collection: (a) responders before treatment (n = 6), (b) responders 90 days after treatment (n = 6), (c) non-responders before treatment (n = 6), and (d) non-responders 90 days after treatment (n = 6). Therapeutic response was defined clinically: patients with complete

healing of the lesion within 3 months after treatment were classified as responders, and patients without complete healing within this period were classified as non-responders (Table 1). In addition, samples from untreated patients with ML (n = 5) and healthy individuals (n = 5), who served as the control group, were included. After confirmation of clinical status, 10 mL of peripheral venous blood was collected from each participant into vacuum tubes. Samples were centrifuged at 2,000 × *gLeishmania (V.) braziliensis* for 10 min to separate the plasma, which was carefully aliquoted and stored at −80 °C until processing. Plasma samples were used to analyze the expression of circulating microRNAs associated with inflammatory and immune responses. In addition, molecular analyses performed at an independent laboratory confirmed as the infecting species in the study participants.

Table 1. Clinical and epidemiological characteristics of the study participants

(A) Patients with cutaneous leishmaniasis

	Sex	Age	Occupation	Probable place of infection	Lesion site	Lesion size (mm)	Disease duration (months)	PCR	IFI	ELISA	Direct exam	Treatment After First Collection	Treatment Duration	Lesion Healing Within 3 Months
Responders	M	35	Police Officer	Minas Gerais	Waist	60 × 25	5	+	-	+	ND	Gluc. System.	20 days	Yes
	M	40	Plasterer	Pará	Right Finger	15 × 15	3	+	-	+	ND	Anfo. Lipos.	10 days	Yes
	M	30	Shopkeeper	Embu/SP	Penis	30 × 25	3	+	-	+	ND	Gluc. System.	20 days	Yes
	F	59	Housewife	Minas Gerais	Abdomen	30 × 25	8	+	-	+	ND	Gluc. Intrales	4 days	Yes
	M	37	Not Reported	Minas Gerais	Left Leg	85 × 65	6	+	+	+	ND	Gluc. System.	20 days	Yes
	F	49	Saleswoman	Minas Gerais	Left Arm	45 × 35	9	+	-	+	ND	Anfo. Lipos.	10 days	Yes
Non-responders	F	59	Housewife	Embu/SP	Left Ankle	60 × 40	3	+	-	+	ND	Anfo. Lipos.	10 days	No
	F	47	Saleswoman	Paraná	Right Ankle	40 × 25	2	+	-	+	ND	Gluc. Intrales	4 weeks	No
	M	39	Bricklayer	Mairiporã/SP	Left Arm	35 × 35	3	+	+	+	ND	Gluc. System.	21 days	No
	M	40	General Services	Mairiporã/SP	Trunk	40 × 30	6	+	-	+	ND	Gluc. System.	20 days	No
	F	36	Not Reported	Pernambuco	Left Arm	25 × 20	2	+	ND	ND	ND	Gluc. Intrales	4 weeks	No
	M	65	Engineer	Mato Grosso	Right Leg	25 × 30	6	+	-	+	ND	Gluc. Intrales	4 weeks	No

(B) Patients with mucosal leishmaniasis

	Sex	Age	Occupation	Probable Place of Infection	Lesion site	PCR	IFI	ELISA	Immunohistochemistry	Direct Examination
Patients with Mucosal Leishmaniasis	F	33	Housewife	Comodoro/MT	Nasal	-	+	+	ND	+
	M	38	Bricklayer	Itambé/BA	Nasal, soft palate, uvula, hypopharynx	+	+	+	+	-
	F	73	Retired	Suzano/SP	Nasal	+	-	-	-	-
	M	85	Retired	Salinas/MG	Nasal, mouth, tongue, hard palate	ND	-	+	+	-
	M	52	Street Vendor	Ilheus/BA	Nasal, larynx, hard palate	ND	+	+	ND	+

M, male; F, female; PCR, polymerase chain reaction; IFI, indirect immunofluorescence; ELISA, enzyme-linked immunosorbent assay; +, positive; -, negative; ND, not done; Gluc. System., systemic meglumine antimoniate (Glucantime); Gluc. Intrales, intralesional meglumine antimoniate; Anfo. Lipos., liposomal amphotericin B; MT, Mato Grosso; BA, Bahia; MG, Minas Gerais; SP, São Paulo (Brazilian states). Satisfactory response, complete healing of the lesion within 3 months after treatment; unsatisfactory response, no complete healing within this period.

2.3. Total RNA extraction from plasma

Total RNA, including the microRNA fraction, was extracted from plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen, Cat. No. 217184), according to the manufacturer's instructions. To monitor RNA extraction efficiency and control for technical variation among samples, 3.5 µL of the miRNeasy Serum/Plasma Spike-In Control (cel-miR-39-3p) (Qiagen, Cat. No. 219610) was added to each sample prior to RNA extraction.

Briefly, 200 µL of plasma was mixed with 1,000 µL of QIAzol Lysis Reagent (Qiagen, Cat. No. 79306), homogenized, and incubated at room temperature for 5 min. Phase separation was then performed by adding 200 µL of chloroform, followed by centrifugation at 12,000 × g for 15 min at 4 °C. The upper aqueous phase was carefully transferred to a new tube, and absolute ethanol was added to precipitate the RNA. The samples were then loaded onto RNeasy MinElute spin columns, washed according to the manufacturer's protocol, and eluted in 14 µL of RNase-free water provided with the kit. The purified RNA was stored at −80 °C until cDNA synthesis.

2.4. miRNA-specific cDNA synthesis

miRNA-specific cDNA synthesis was performed using the miScript II RT Kit (Qiagen, Cat. No. 218161) according to the manufacturer's instructions. This system enables the addition of a poly(A) tail to mature miRNAs, followed by reverse transcription using oligo(dT) primers containing a universal tag sequence. Reverse transcription reactions were carried out in a thermal cycler under the following conditions: 37 °C for 60 min, followed by 95 °C for 5 min.

The resulting cDNA was stored at −20 °C until further use.

2.5. cDNA preamplification

Due to the low abundance of circulating microRNAs in plasma samples, a cDNA preamplification step was performed using the RT² PreAMP Kit (Qiagen, Cat. No.331452), according to the manufacturer's instructions. For each reaction, 2.5 µL of previously diluted cDNA was used. The diluted cDNA was prepared by mixing 10 µL of cDNA with 40 µL of nuclease-free water. Preamplification reactions were performed in a thermal cycler under the following conditions: an initial incubation at 95 °C for 15 min, followed by 12 cycles of 94 °C for 30 s and 60 °C for 3 min. The preamplified products were stored at −20 °C until further analysis.

2.6. miRNA expression profiling by PCR Array

The expression profile of circulating microRNAs was evaluated using the miScript miRNA PCR Array Human Inflammatory Response & Autoimmunity (MIHS-105Z; Qiagen, Cat. No. 331221) in combination with the miScript SYBR Green PCR Kit (Qiagen, Cat. No. 218076). This panel simultaneously analyzes the expression of 84 microRNAs associated with inflammatory and immune responses. PCR reactions were prepared according to the manufacturer's instructions using previously preamplified cDNA. Amplification was performed under the following cycling conditions: an initial activation step at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. At the end of the amplification, a melting curve analysis was performed to verify the specificity of the amplified products.

2.7. Relative miRNA expression analysis

MiRNA expression data were normalized using the endogenous housekeeping controls included in the miScript miRNA PCR Array. For each miRNA, ΔCt values were calculated by subtracting the

mean Ct of the selected reference genes from the Ct of the miRNA of interest. Relative expression was calculated using the 2^−ΔΔCt method, and results were expressed as fold regulation.

2.8. Data analysis

Raw cycle threshold (Ct) values were analyzed using the QIAGEN GeneGlobe Data Analysis Center. MiRNAs with Ct values ≥ 35 were considered undetectable and excluded from subsequent analyses according to the predefined detection criterion.

Expression profiles were compared between patients with cutaneous leishmaniasis (CL), patients with mucosal leishmaniasis (ML), and healthy controls. In addition, miRNA expression was evaluated in paired plasma samples collected from patients with CL before treatment and 90 days after treatment. The reference group was defined separately for each comparison.

2.9. Statistical analysis

Statistical analyses of miRNA expression were performed using the QIAGEN GeneGlobe Data Analysis Center. According to the software-generated analysis report, p-values were calculated using Student's t-test applied to replicate 2^−ΔCt values from the groups being compared. A nominal p-value < 0.05 was considered statistically significant. This analytical approach has previously been used in a study employing the same platform (Souza et al., 2021).

2.10. Target prediction and pathway enrichment analysis

To explore potential biological functions and pathways associated with selected miRNAs, target prediction and pathway enrichment analyses were performed using DIANA-miRPath v3.0 [22].

Predicted miRNA–target interactions were obtained from DIANA-microT-CDS [23], and pathway enrichment was assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Pathways with nominal p-values < 0.05 were considered statistically significant.

3. Results

3.1. Differential expression of circulating miRNAs in plasma samples

The differential expression of circulating miRNAs was evaluated in plasma samples from patients with cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and healthy controls using a miRNA PCR Array. The results are presented as volcano plots **(Fig. 1A–C)**. Comparison between patients with CL and healthy controls **(Fig. 1A)** identified several significantly downregulated miRNAs in CL, including miR-19b-3p (*fold regulationp* = −15.0709; = 0.000127), let-7b-5p (*fold regulationp* = −213.9766; = 0.001140), miR-16-5p (*fold regulationp* = −18.6758; = 0.003652), miR-424-5p (*fold regulationp* = −98.3844; = 0.004983), miR-19a-3p (*fold regulationp* = −19.0006; = 0.005649), miR-101-3p (*fold regulationp* = −25.8722; = 0.006092), miR-15a-5p (*fold regulationp* = −8.8502; = 0.013796), miR-9-5p (*fold regulationp* = −40.2344; = 0.022573), and miR-144-3p (*fold regulationp* = −42.9871; = 0.043305).

In the comparison between patients with ML and healthy controls **(Fig. 1B)**, only one miRNA, let-7g-5p, was significantly downregulated (*fold regulationp* = −45.5459; = 0.032234). No significantly upregulated miRNAs were identified in this comparison. Direct comparison between patients with ML and CL **(Fig. 1C)** revealed a set of miRNAs significantly upregulated in ML, including miR-1324 (*fold regulation* = 6.0968; $p = 0.003342$), miR-524-5p (*fold regulation* = 2.0621; $p = 0.016225$), miR-302b-3p (*fold regulation* = 3.3771; $p = 0.019201$), let-7b-5p (*fold regulation* = 11.0105; $p = 0.023137$), and miR-520d-3p (*fold regulation* = 2.0175; $p = 0.038337$).

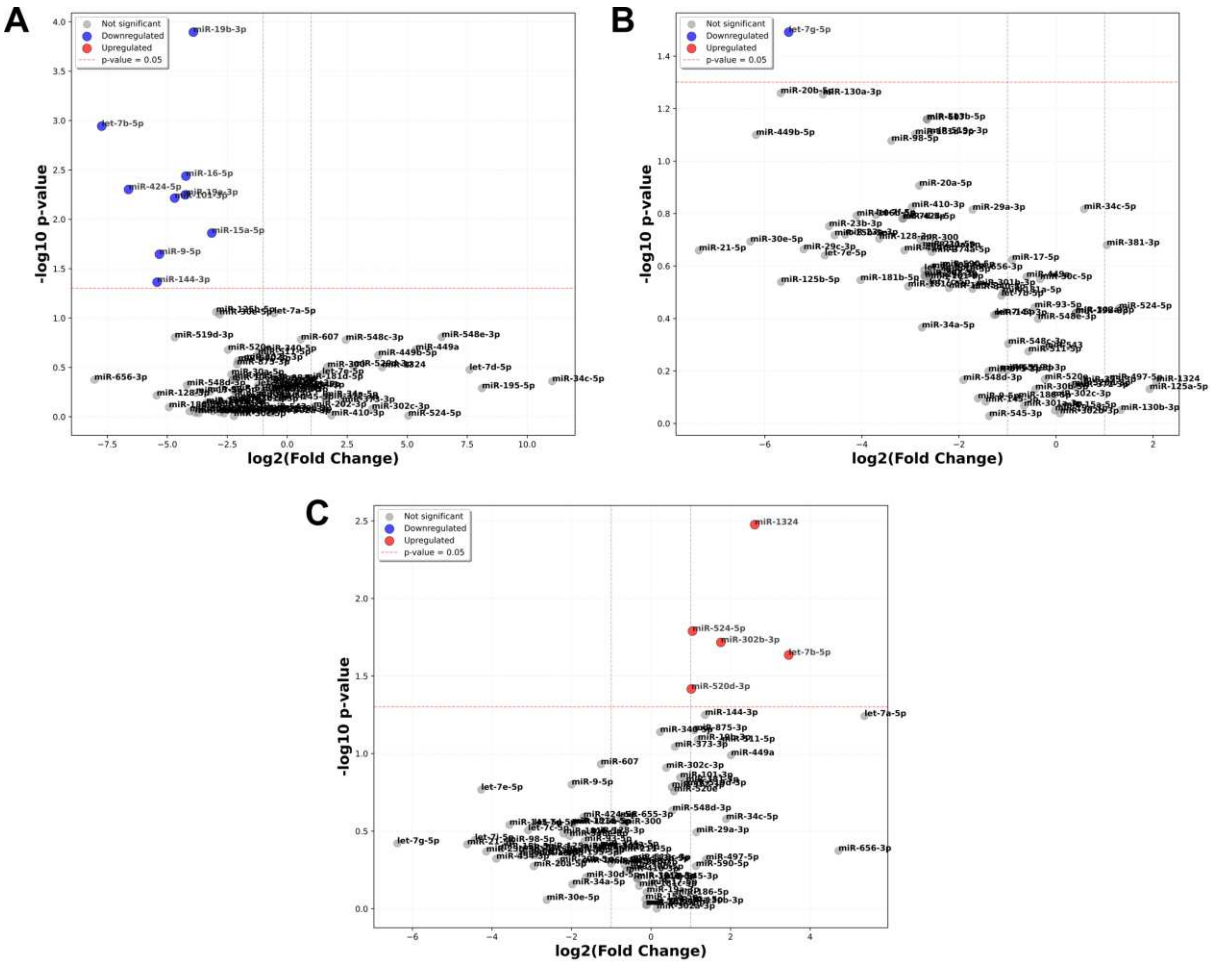


Fig. 1. Differential expression of circulating microRNAs in plasma samples from patients with cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and healthy controls. Volcano plots showing the differential expression of miRNAs based on $\log_2$ fold change and $-\log_{10}$ (p-value) thresholds. (**A**) Cutaneous leishmaniasis (CL) vs. healthy controls; (**B**) mucosal leishmaniasis (ML) vs. healthy controls; and (**C**) ML vs. CL. Each dot represents a single miRNA. Significantly upregulated miRNAs are shown in red on the right side of each plot, whereas significantly downregulated miRNAs are shown in blue on the left side ($p < 0.05$). Statistically significant miRNAs are located in the upper regions of the plots. The horizontal dashed line indicates the statistical significance threshold ($-\log_{10}(p) = 1.301$, corresponding to $p = 0.05$), with miRNAs located above this line considered statistically significant ($p < 0.05$), while the vertical dashed lines represent the fold-change cutoffs ($\log_2$ fold change ≥ 1 or ≤ -1).

238 **Table 2. Cutaneous leishmaniasis (CL) versus healthy controls**

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
miR-19b-3p	Downregulated	-15.0709	0.000127	TGF-β; fibroblasts; extracellular matrix/fibrosis	Gantier et al. [24]; Zou et al. [25]
let-7b-5p	Downregulated	-213.9766	0.001140	Skin repair; fibroblasts; migration and extracellular matrix	Zhou et al. [26]
miR-16-5p	Downregulated	-18.6758	0.003652	M1/Th1 polarization; NF-κB/JAK-STAT	Zheng et al. [27]
miR-424-5p	Downregulated	-98.3844	0.004983	Tissue repair response; TGF-β/WNT; α-SMA	Min et al. [28]
miR-19a-3p	Downregulated	-19.0006	0.005649	TGF-β/SMAD2/3; fibroblast activation; collagen/fibrosis	Zou et al. [25]; Shi et al. [29]
miR-101-3p	Downregulated	-25.8722	0.006092	TGFBR1/SMAD2; fibroblasts; remodeling/fibrosis	Huang et al. [30]; Eun et al. [31]
miR-15a-5p	Downregulated	-8.8502	0.013796	Inflammation/fibrosis; VEGFA	Shang et al. [32]
miR-9-5p	Downregulated	-40.2344	0.022573	TLR/NF-κB; innate immunity	Bazzoni et al. [33]
miR-144-3p	Downregulated	-42.9871	0.043305	Fibroblasts; collagen/extracellular matrix; fibrosis	Yuan et al. [34]; Bahudhanapati et al. [35]

239 **Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

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241 **Table 3. Mucosal leishmaniasis (ML) versus healthy controls**

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
let-7g-5p	Downregulated	-45.5459	0.032234	NF-κB/NLRP3 signaling; regulation of inflammatory responses	Huang et al. [36]

242 **Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

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246 **Table 4. Mucosal leishmaniasis (ML) versus cutaneous leishmaniasis (CL)**

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
miR-1324	Upregulated	6.0968	0.003342	Cell proliferation/invasion; limited direct immune evidence	NCBI Gene [37]

miR-524-5p	Upregulated	2.0621	0.016225	T-cell activation; IFN-γ production/Notch	Lu et al. [38]
miR-302b-3p	Upregulated	3.3771	0.019201	Fibroblast senescence; skin aging; JNK2/SIRT1	Ma et al. [39]; Tan et al. [40]
let-7b-5p	Upregulated	11.0105	0.023137	Skin repair; fibroblasts; migration and extracellular matrix	Zhou et al. [26]
miR-520d-3p	Upregulated	2.0175	0.038337	Proliferation, migration and EMT; limited direct immune evidence	Zhang et al. [41]

**Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

3.2. Comparison of circulating miRNA expression in patients with cutaneous leishmaniasis before and after treatment

To investigate the potential association between circulating miRNA expression and therapeutic response in CL, comparisons were performed between patients classified according to their clinical outcome after treatment, as well as longitudinal analyses using samples collected before treatment and 90 days after therapy **(Fig. 2A–D)**.

Comparison between responders and non-responders before treatment identified differentially expressed miRNAs between the two groups **(Fig. 2A)**. Responders exhibited significantly higher expression of miR-1324 (*fold regulation* = 16.32; $p = 0.0099$), miR-34c-5p (*fold regulation* = 23.78; $p = 0.0435$), and miR-373-3p (*fold regulation* = 5.00; $p = 0.0459$) compared with non-responders. Although miR-30e-5p (*fold regulation* = 2.01; $p = 0.4990$) and miR-15b-5p (*fold regulationp* = −4.27; = 0.3445) exhibited differences in expression, neither reached statistical significance. These findings suggest that differences in the baseline circulating miRNA profile may be associated with treatment outcome.

Comparison between responders and non-responders after treatment did not identify any significantly differentially expressed miRNAs **(Fig. 2B)**.

Among responders, longitudinal comparison of plasma samples collected after and before treatment revealed a significant decrease in miR-372-3p expression (*fold regulationp* = −7.43; = 0.0310) **(Fig. 2C)**.

Among non-responders, longitudinal comparison of samples collected after and before treatment revealed a significant decrease in let-7b-5p expression (*fold regulationp* = −14.50; = 0.0237) and a significant increase in miR-301b-3p expression (*fold regulation* = 3.53; $p = 0.0272$). This difference was not statistically significant **(Fig. 2D)**.

Taken together, these findings indicate that the circulating miRNA expression profile may be associated with both baseline therapeutic response and treatment-associated changes in patients with cutaneous leishmaniasis, supporting further investigation of these regulatory molecules as potential prognostic biomarkers and candidates for functional studies.

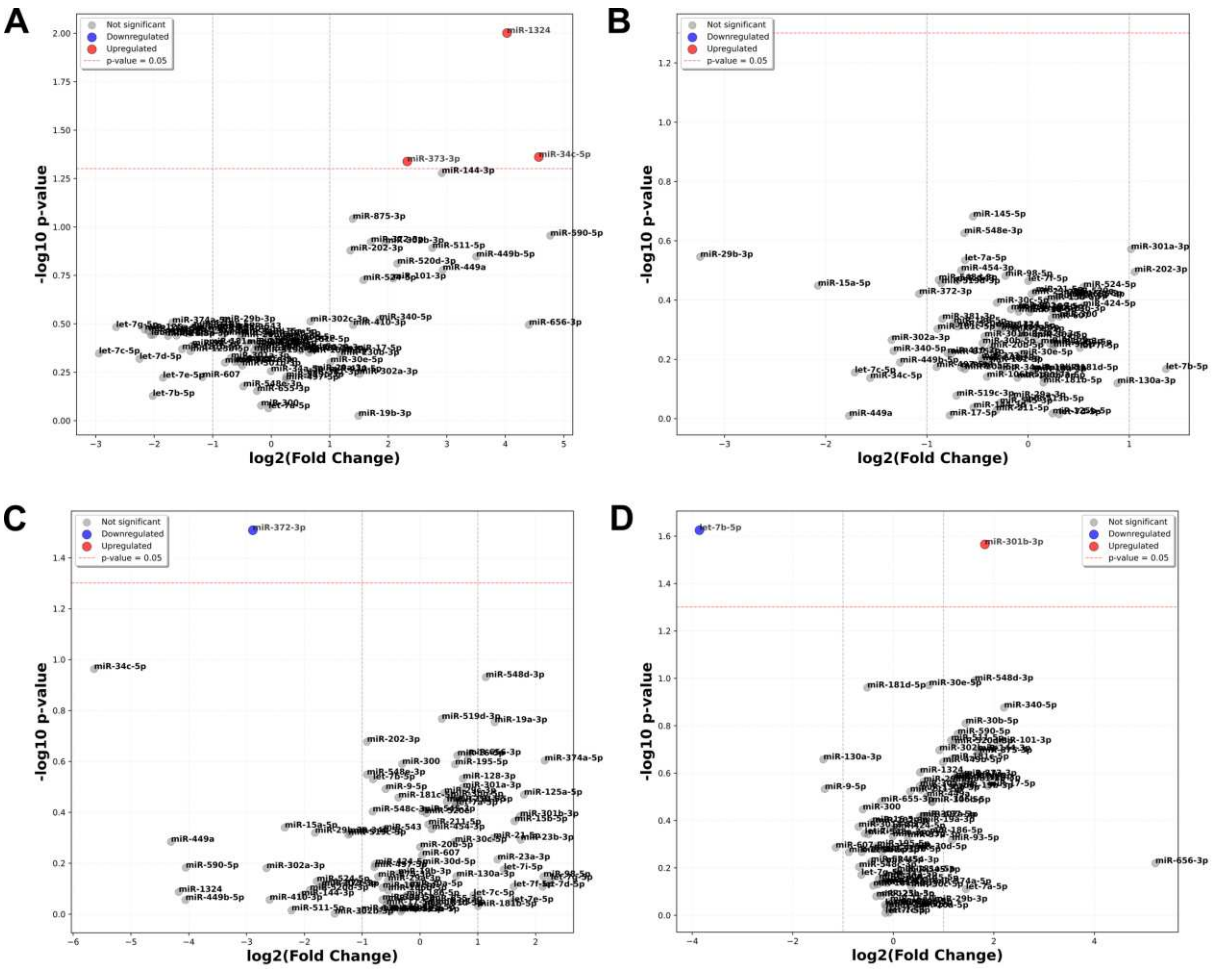


Fig. 2. Differential expression of circulating miRNAs in plasma samples from patients with cutaneous leishmaniasis classified according to therapeutic response. Volcano plots showing the differential expression of miRNAs based on $\log_2$ fold change and $-\log_{10}$ (*p*-value) thresholds. (A) Responders versus non-responders before treatment; (B) Responders versus non-responders after treatment; (C) Responders after treatment versus before treatment; and (**D**) Non-responders after treatment versus before treatment. Each dot represents a single miRNA. Significantly upregulated miRNAs are shown in red on the right side of each plot, whereas significantly downregulated miRNAs are shown in blue on the left side ($p < 0.05$). The horizontal dashed line indicates the statistical significance threshold ($-\log_{10}(p) = 1.301$, corresponding to $p = 0.05$), with miRNAs located above this line considered statistically significant ($p < 0.05$), whereas the vertical dashed lines represent the fold-change cutoffs ($\log_2$ fold change ≥ 1 or ≤ -1).

Table 5. Comparison of circulating miRNA expression between responders and non-responders before treatment.

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
miR-1324	Upregulated	16.32	0.0099	Cell proliferation/invasion; limited direct immune evidence	NCBI Gene [37]
miR-34c-5p	Upregulated	23.78	0.0435	Fibroblasts; extracellular matrix; fibrosis/remodeling	Wei et al. [42] ; Hu et al. [43]
miR-373-3p	Upregulated	5.00	0.0459	Cell migration/invasion; ECM remodeling (MMP-9); fibrosis	Lee et al. [44]; Xuan et al. [45]

**Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

Table 6. Comparison of circulating miRNA expression in responders before and after treatment.

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
miR-372-3p	Downregulated	-7.43	0.0310	Endothelial function; apoptosis; FGF16	Meng et al. [46]

**Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

Table 7. Comparison of circulating miRNA expression in non-responders before and after treatment.

miRNA	Regulation	Fold regulation	p-value	Associated processes*	References
let-7b-5p	Downregulated	-14.50	0.0237	Skin repair; fibroblasts; migration and extracellular matrix	Zhou et al. [26]
miR-301b-3p	Upregulated	3.53	0.0272	Infection-associated inflammation; TLR4/MyD88/NF-κB; c-Myb	Li et al. [47]

**Associated processes have been reported in other experimental or clinical contexts and do not establish causality in tegumentary leishmaniasis.*

3.3. Candidate miRNA selection and exploratory in silico analysis

To identify candidate miRNAs potentially involved in mechanisms associated with the pathogenesis of TL, an exploratory *in silico* reanalysis was performed using data previously generated in our laboratory from clinical samples and *in vitro* infection experiments using THP-1 cells. These previous datasets demonstrated differential expression of several miRNAs in patients with active cutaneous leishmaniasis, patients with spontaneous healing, and healthy controls, as well as in THP-1 cells infected with *Leishmania (V.) braziliensis* at different time points after infection. Considering the importance of ulcerative lesion formation in CL and previous evidence

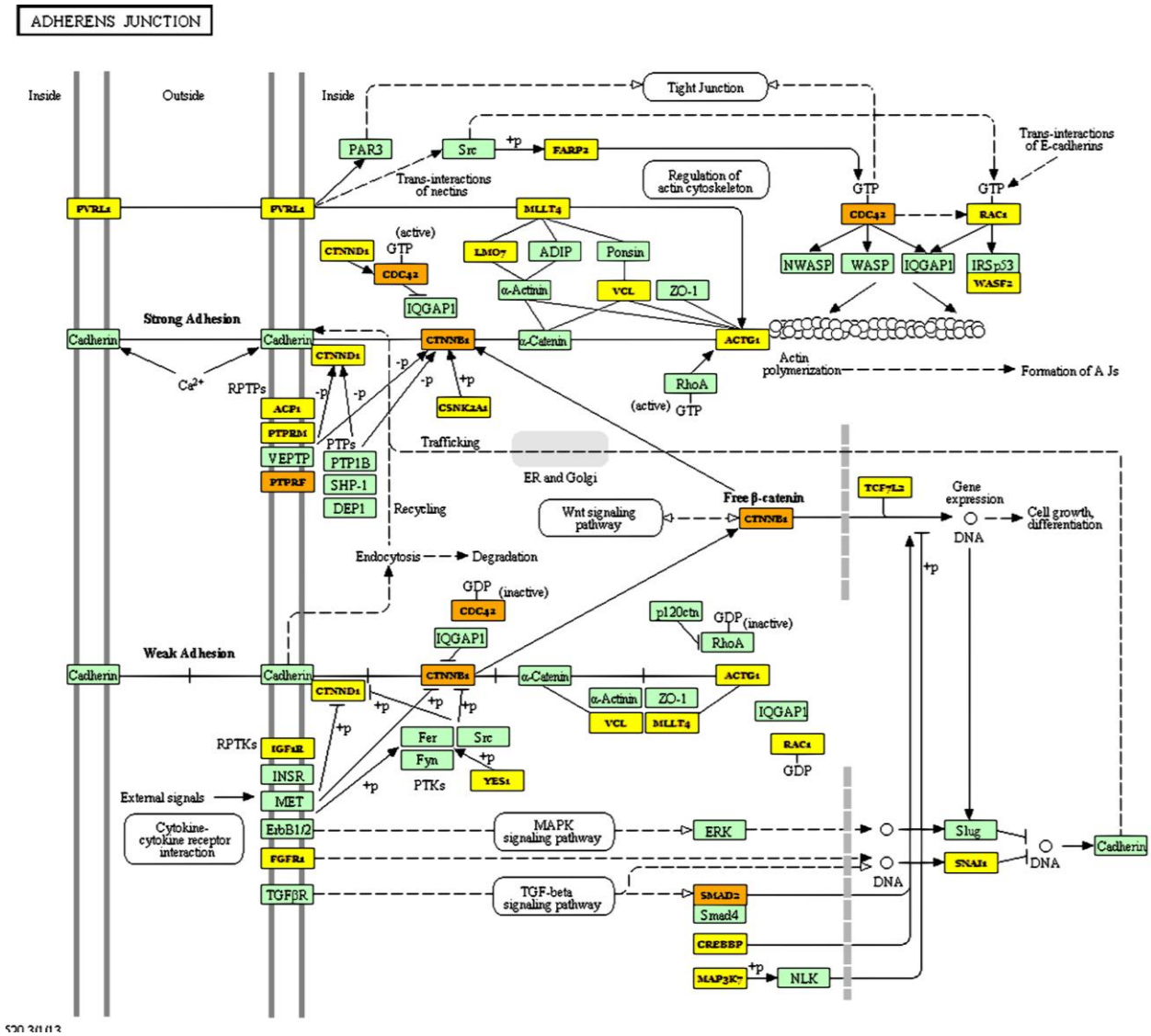
implicating cell adhesion, cell junction organization, and extracellular matrix remodeling in lesion healing **[48]**, an exploratory analysis focusing on pathways related to cell adhesion was performed.

For this reanalysis, miRNAs previously identified as differentially expressed in *L. (V.) braziliensis*-infected THP-1 cells and in plasma samples from patients with spontaneous healing compared with patients with active disease were considered, regardless of the direction of regulation. Bioinformatic analysis was performed using DIANA-miRPath v3.0, a platform that predicts miRNA target genes and identifies biological pathways potentially regulated by miRNAs. The reanalysis identified several miRNAs potentially involved in the adherens junction pathway in the THP-1-derived and patient plasma datasets **(Table 8; Fig. 3)**. Among the identified candidates, hsa-miR-15b-5p and hsa-miR-181a-5p were highlighted, with 20 and 18 predicted target genes, respectively, in plasma samples. In addition, hsa-miR-29b-3p was identified in both datasets, exhibiting downregulation in infected THP-1 cells and upregulation in patient plasma, with 14 predicted target genes **(Table 8)**. These findings contributed to the selection of biologically relevant candidate miRNAs for subsequent investigations.

Table 8. Predicted target genes identified using DIANA-miRPath v3.0 for miRNAs differentially expressed in plasma samples from patients with spontaneous healing compared with patients with active cutaneous leishmaniasis and in *Leishmania (Viannia) braziliensis*-infected THP-1 cells. The miRNAs listed in the table were predicted to be involved in the adherens junction pathway by modulating the expression of different target genes. The only miRNA, hsa-miR-

29b-3p, predicted to be regulated in both infected THP-1 cells (downregulated) and patient plasma samples (upregulated), is highlighted in red.

miRNAs	Target genes
hsa-miR-15b-5p	*ACTB, SMAD2, ACTG1, LMO7, IGF1R, PTPRF, ACP1, MLLT4, CDH1, CTNNB1, CSNK2A1, PRKAC1, CDC42, EP300, PVRL3, YES1, FGFR1, MAP3K7, CREBBP, PVRL1*
hsa-miR-29b-3p	*ACTB, CTNND1, SMAD2, TCF7L2, PTPRM, VCL, PTPRF, SNAI1, WASF3, CDH1, CTNNB1, WASF2, FARP2, CDC42*
hsa-miR-181a-5p	*ACTB, MET, WASF1, WASL, TCF7L2, SNAI2, IGF1R, EGFR, VCL, TJP1, CTNNB1, FARP2, MAPK3, SSX2IP, EP300, YES1, MAPK1, TGFBR2*



329

330 **Fig. 3. Signaling pathways associated with the predicted target genes of miRNAs differentially expressed in**
331 ***Leishmania (Viannia) braziliensis*-infected THP-1 cells and in plasma samples from patients with spontaneous**
332 **healing compared with patients with active cutaneous leishmaniasis.** Genes predicted to be targeted by more than
333 one miRNA are highlighted in orange, whereas genes predicted to be targeted by a single miRNA are highlighted in
334 yellow. Genes shown in green were not predicted to be targeted by these miRNAs.

335

336

337 **4. Discussion**

In the present study, we identified distinct circulating miRNA expression profiles in patients with cutaneous and mucosal leishmaniasis, as well as differences associated with therapeutic response. These findings suggest that circulating miRNAs may reflect molecular alterations associated with the clinical heterogeneity of TL. Considering the role of miRNAs as post-transcriptional regulators of immune and inflammatory responses, their differential expression may provide insights into mechanisms involved in disease progression and clinical outcome.

The clinical heterogeneity of TL is closely associated with differences in the host immune response. Mucosal leishmaniasis, in particular, is characterized by exacerbated inflammation, increased production of pro-inflammatory cytokines, and $CD8^+$ T cell-mediated cytotoxic activity, which contribute to progressive tissue damage **[8,49]**. Previous studies have also demonstrated alterations in immunoregulatory mechanisms, including reduced responsiveness to IL-10 and dysregulation of IL-10- and TGF-β-mediated pathways **[9,50]**. In this context, the distinct circulating miRNA profiles observed in our study may reflect differences in the regulation of inflammatory responses between clinical forms, although their functional involvement remains to be established.

miRNAs have been recognized as important post-transcriptional regulators of gene expression, capable of simultaneously controlling multiple cellular pathways involved in immunity, inflammation, and tissue remodeling **[11,13–14]**. Therefore, alterations in their expression levels may reflect profound changes in the regulation of the host response to infection.

In the present study, a predominance of differentially downregulated miRNAs was observed in the plasma of patients with cutaneous leishmaniasis (CL) compared with healthy controls, including miR-19b-3p, let-7b-5p, miR-16-5p, miR-424-5p, and miR-19a-3p. The reduced expression of these miRNAs suggests a coordinated dysregulation of pathways controlling the magnitude of the inflammatory response. Indeed, the miR-19 subfamily acts as a critical molecular

362 regulator of NF-κB signaling, playing an essential role in fine-tuning inflammatory responses **[24]**.
363 Furthermore, miR-424 has been shown to participate in human monocyte/macrophage
364 differentiation through a regulatory mechanism involving the transcription factor PU.1 and the
365 repression of NFI-A **[51]**. miR-424-5p has also been implicated in the regulation of cellular
366 responses to oxidative stress **[52]**. More recently, Toro and colleagues [53] demonstrated in human
367 cell models that experimental suppression of miR-16-5p exerts a cytoprotective effect by restoring
368 antioxidant defenses (such as catalase and superoxide dismutase) and mitigating cellular
369 inflammation. From this mechanistic perspective, the reduced expression of miR-16-5p and miR-
370 424-5p observed in patients with CL may reflect a homeostatic attempt by the host to mimic a
371 protective profile by preventing the repression of antioxidant and immune regulatory genes, thereby
372 counterbalancing the oxidative stress and exacerbated tissue damage characteristic of lesions
373 induced by infection. Among the identified miRNAs, members of the let-7 family deserve particular
374 attention. Schulte et al. [54] demonstrated that reduced let-7 expression in macrophages infected
375 with *Salmonella Typhimurium* releases IL-6 and IL-10 from post-transcriptional control, thereby
376 amplifying the inflammatory response. Similarly, Kumar et al. [55] demonstrated that let-7
377 participates in the antimicrobial response during *Mycobacterium tuberculosis* infection through
378 regulation of the NF-κB pathway. Beyond immune regulation, let-7b has also been implicated in
379 cellular processes relevant to tissue repair. Experimental studies have demonstrated that let-7b
380 regulates keratinocyte migration and cutaneous re-epithelialization through the modulation of
381 IGF2BP2 **[56].** Additionally, exosome-associated let-7b has been implicated in macrophage
382 polarization, inflammatory resolution, and improved cutaneous wound healing in experimental
383 models [57]. These processes are particularly relevant to TL, as persistent inflammation and
384 extracellular matrix degradation may compromise tissue regeneration and wound healing **[58]**.
385 Thus, the reduced circulating expression of let-7b-5p observed in patients with CL and let-7g-5p in
386 patients with ML may reflect alterations in inflammatory regulatory mechanisms, while the

387 differential expression of let-7b-5p between CL and ML may also be associated with differences in
388 tissue repair and remodeling. However, these possibilities remain speculative because circulating
389 miRNA levels do not necessarily reflect their expression or functional activity within lesions.

390 In addition, miR-16-5p, miR-19a-3p, and miR-19b-3p have been associated with the
391 regulation of cell proliferation, apoptosis, and immune cell activation. miR-19a/b belongs to the
392 miR-17~92 cluster, which is essential for immune homeostasis and for the regulation of pro-
393 apoptotic proteins such as Bim **[59]**, whereas miR-16-5p is involved in the regulation of pathways
394 associated with cellular stress and programmed cell death **[60]**. Collectively, the reduced expression
395 of these miRNAs suggests a scenario of impaired post-transcriptional regulation that may contribute
396 to the maintenance of the inflammatory process and the persistence of infection.

397 In contrast to the predominance of downregulated miRNAs observed in CL compared with
398 healthy controls, direct comparison between CL and ML identified a distinct subset of upregulated
399 miRNAs, including miR-1324, miR-524-5p, miR-302b-3p, let-7b-5p, and miR-520d-3p. Among
400 these, miR-302b-3p deserves particular attention because, although it has not previously been
401 investigated in leishmaniasis, experimental evidence indicates that it participates in biological
402 processes related to tissue homeostasis. **Tan et al. [40]** demonstrated that miR-302b-3p promotes
403 skin fibroblast senescence by directly targeting JNK2, resulting in reduced SIRT1 expression and
404 accelerated cellular aging. Since fibroblast function is essential for extracellular matrix remodeling
405 and wound repair, these findings suggest that miR-302b-3p may influence mechanisms associated
406 with lesion evolution in TL. Interestingly, ulcer formation, chronic inflammation, tissue destruction,
407 and subsequent healing are hallmarks of cutaneous leishmaniasis. Therefore, the differential
408 expression of miR-302b-3p observed between CL and ML raises the possibility that this miRNA
409 contributes to molecular pathways involved in tissue remodeling rather than solely inflammatory

regulation. However, because no previous studies have evaluated miR-302b-3p in leishmaniasis, this interpretation should be considered hypothesis-generating and requires functional validation.

More broadly, our findings are consistent with previous observations demonstrating that miRNA expression profiles are closely associated with host–parasite interactions. In this regard, Lago et al. [61] demonstrated that macrophages infected with different clinical isolates of *Leishmania braziliensis* exhibit distinct miRNA expression profiles that correlate with parasite burden and inflammatory parameters. Together, these observations support the concept that altered miRNA expression is closely associated with the host response to *Leishmania* infection and may participate in the regulatory networks that influence disease progression and clinical outcome. In addition to their biological relevance, circulating miRNAs have emerged as promising minimally invasive biomarkers because of their remarkable stability in biological fluids, resulting from their association with extracellular vesicles, Argonaute proteins, and lipoproteins, as well as their resistance to degradation during sample processing **[62–64]**. In leishmaniasis, emerging evidence suggests that non-coding RNAs may serve as candidate biomarkers for disease diagnosis and clinical characterization, although their potential applications in predicting clinical outcomes and monitoring treatment responses require further validation **[65]**.

Analysis of therapeutic response revealed differences in circulating miRNA expression profiles even before treatment initiation. Responders showed significantly higher expression of miR-1324, miR-34c-5p, and miR-373-3p compared with non-responders. These findings suggest that baseline circulating miRNA profiles may be associated with treatment outcome, highlighting their potential prognostic value. Although the biological functions of these miRNAs remain poorly understood in the context of leishmaniasis, members of the miR-34 family, including miR-34c, have been identified as components of the p53 regulatory network involved in cell cycle control and cellular stress responses **[66–67]**. Furthermore, He et al. [68] demonstrated that miR-34c-5p carried

by bone marrow stromal cell-derived extracellular vesicles attenuated inflammation and lung injury in experimental models of bronchopulmonary dysplasia through regulation of the OTUD3/PTEN axis. Although these findings were obtained in a different pathological context, they suggest that miR-34c-5p may participate in inflammatory regulation and tissue repair-related processes, warranting further investigation of its potential relevance to therapeutic response in cutaneous leishmaniasis.

In addition, miR-373-3p has been shown to regulate cell migration, invasion, and extracellular matrix-associated processes through the modulation of CD44, radixin, and MMP-9 in experimental models **[44]**. Collectively, these observations suggest that miRNAs associated with favorable treatment outcomes may participate in biological pathways relevant to cellular stress responses, inflammatory regulation, and tissue remodeling, although their functional contribution to lesion healing in cutaneous leishmaniasis remains to be established.

Longitudinal analyses further revealed treatment-associated changes in circulating miRNA expression. A significant reduction in miR-372-3p expression was observed in responders, whereas non-responders exhibited significantly decreased let-7b-5p expression and increased miR-301b-3p expression after treatment. These distinct patterns suggest that circulating miRNAs may reflect biological changes associated with clinical evolution and therapeutic response. Together, these findings support the potential use of circulating miRNAs not only as candidate prognostic biomarkers but also as molecular indicators for monitoring treatment response, although further validation in larger and independent cohorts is required.

Consistent with these observations, the distinct circulating miRNA signatures identified in the present study discriminated not only patients from healthy controls but also different clinical manifestations of TL, reinforcing the potential of these molecules as biomarkers for disease

457 characterization. Nevertheless, validation in larger and independent patient cohorts will be essential
458 before their clinical application can be established.

459 This study has limitations. First, the number of patients per group was small (five or six),
460 which limits statistical power and the generalizability of the findings; the differentially expressed
461 miRNAs identified here should therefore be regarded as candidates. Second, patients received
462 different therapeutic regimens (systemic or intralesional meglumine antimoniate, or liposomal
463 amphotericin B) of variable duration, and these regimens were not evenly distributed between
464 responders and non-responders, so treatment-specific effects on circulating miRNA levels cannot be
465 excluded. Third, miRNA levels were measured with a PCR array after cDNA preamplification and
466 were not validated by individual RT-qPCR assays. Validation of the candidate miRNAs by
467 individual assays in larger and independent cohorts is required before their use as biomarkers can be
468 considered.

469

470 **5. Conclusions**

471 This study demonstrates that circulating miRNAs are differentially expressed in plasma
472 samples from patients with CL and ML compared to healthy controls, and that specific miRNA
473 signatures can distinguish between these clinical forms of TL. Furthermore, we identified miRNAs
474 whose expression changes significantly following treatment, suggesting potential utility for
475 monitoring therapeutic response. These findings support the hypothesis that circulating miRNAs
476 represent promising non-invasive biomarkers for TL and provide insights into the molecular
477 mechanisms underlying disease pathogenesis. Future validation studies with larger cohorts and
478 functional investigations of candidate miRNAs and their target genes will be essential to translate
479 these findings into clinical applications.

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Competing interests

DECLARATION OF INTERESTS: Pegine Walrad is a Subject Editor for Parasites & Vectors and an Editor for the “Translational leishmaniasis” Special Issue, and was not involved in the peer review of this article. The authors declare no other conflicts of interest.

Abbreviations

AGO2: Argonaute 2

TL: Tegumentary Leishmaniasis

cDNA: Complementary DNA

CL: Cutaneous Leishmaniasis

IFN-γ: Interferon gamma

IL: Interleukin

KEGG: Kyoto Encyclopedia of Genes and Genomes

miRNA: MicroRNA

ML: Mucosal Leishmaniasis

mRNA: Messenger RNA

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

TGF-β: Transforming growth factor beta

Th1: T helper 1

UTR: Untranslated region

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