


Plasma extracellular microRNAs are related to AIDS/cerebral toxoplasmosis co-infection

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Abstract

This study investigated the potential of five miRNA candidates for cerebral toxoplasmosis/HIV co-infection (CT/HIV) biomarkers. miR-155-5p, miR-146a-5p, miR-21-5p, miR-125b-5p and miR-29c-3p were tested in 79 plasma divided into groups: 32 CT/HIV patients; 27 individuals with asymptomatic toxoplasmosis (AT); and 20 individuals seronegative for toxoplasmosis (NC). From each was collected peripheral blood/EDTA for laboratory diagnosis. Blood cells for DNA extractions (molecular diagnosis), plasma for RNA extractions (gene expression) and ELISA (serological diagnosis). miRNA expression was performed by qPCR, and values were expressed in Relative Quantification (RQ). Among the five miRNAs, miR-21-5p and miR-146a-5p were up-expressed in CT/HIV group when compared with AT and NC groups. RQ means for miR-21-5p and miR-146a-5p in CT/HIV group were 3.829 and 2.500, while in AT group, were 1.815 and 1.661, respectively. Differences between 3 groups were statistically significant (Kruskal-Wallis ANOVA test), as well as CT/HIV and AT groups (Mann-Whitney test). Plasma of CT/HIV and AT groups expressed similar levels of miR-29c-3p, miR-155-5p and miR-125b-5p. As NC group was different of CT/HIV and AT groups, differences between three groups were statistically significant (Kruskal-Wallis ANOVA test). No difference was shown between CT/HIV and AT groups (Mann-Whitney test). These results suggest the host miRNAs modulation by *Toxoplasma gondii*.

KEYWORDS

cerebral toxoplasmosis, cytokine regulation, extracellular miRNA, gene expression

1 | INTRODUCTION

Toxoplasmosis is a worldwide zoonosis with important repercussions on public health. *Toxoplasma gondii* infects around 80% people in some regions of the world. Generally, infection is benign as immune system keeps the parasite in check and parasitaemia is self-limited resulting in an asymptomatic clinical form in most cases. However,

around 10%-20% of the infected individuals may occur rupture of tissue cysts in eyes, brain or muscles causing local necrosis, inflammation and tachyzoite multiplication.^{1,2} Ocular alterations may acquire congenitally, post-natal acquired infection, or result of disease reactivation in immunocompromised individuals.³⁻⁵

Immunocompromised hosts may reactivate the latent toxoplastic infection, converting bradyzoites to tachyzoites. In these

settings, toxoplasmosis can result in severe disability or to be life-threatening.⁶

Cerebral toxoplasmosis is the most common HIV-related opportunistic infection of the central nervous system (CNS), as well as the main cause of focal brain lesions in human immunodeficiency virus (HIV)-infected patients.^{2,7,8} Cerebral toxoplasmosis is fatal if untreated and a major cause of death in HIV-infected patients from low- and middle-income countries.⁷⁻⁹

Cellular response is totally altered in HIV-infected patients. Th1/Th2 imbalance due to HIV and the decrease in CD4 T cells (CD4 T-lymphocyte counts below 100 cells/ μ L) lead to reactivation of latent toxoplasmosis due to a fail of antiparasitic CD4 T-cell response.^{10,11} The induction of TH1 inflammatory cytokine response occurs early in immune response against *T gondii*. Thus, the role of interferon gamma (IFN- γ) is significant because it is the principal mediator inducing host resistance for parasite.

Nevertheless, other cytokines have capacity to stimulate or decrease IFN- γ levels. One of them is tumour necrosis factor alpha (TNF- α), which have been related to increased permeability of the blood-brain barrier, and the interleukin 10 (IL-10) due to its anti-inflammatory action and suppression of TH 1 response.^{10,12-14}

As imaging diagnosis is unable to distinguish *T gondii* infection of other neurological opportunistic infections, early laboratorial diagnosis and prompt treatment of cerebral toxoplasmosis patients/HIV co-infection reduce risk of neurological sequelae and mortality. Thus, more effective strategies are required for differential diagnosis.^{7,8} For this propose, studies have been shown that microRNAs (miRNA, miR) play an important role in regulating gene expression in eukaryotic cells.

At least 100 different miRNAs have been shown to be expressed by cells of the immune system, viral replication, neurotransmitter synthesis, circadian rhythm and insulin secretion as others. miRNAs are detected in biological fluids as serum, saliva and others, and exhibit a good potential as noninvasive biomarkers.¹⁵⁻¹⁷

miRNAs participate in molecular pathways, controlling the development and function of innate and adaptive immune cells.¹⁸ In addition, miRNAs regulate T-cell activation and differentiation and act on subsets of effector T cells and regulatory T (Treg) cells that are defined by cytokine profiles.¹⁹ Some miRNAs are essential for immune cell function changing the host immune response against parasite infections contributing to parasite survival.^{20,21}

The purpose of this study was to investigate gene expression of five miRNAs, in plasma from patients with cerebral toxoplasmosis/HIV co-infection (CT/HIV). The miRNAs, miR-155-5p, miR-146a-5p, miR-21-5p, miR-125b-5p and miR-29c-3p, which were correlated with modulation of some cytokines, could be potential biomarker candidates for CT/HIV. These enquiries were based in our previous studies, that showed the importance of immune modulation in CT/HIV patients.^{13,14}

miR-155-5p and miR-146a-5p participate in modulation of immune cell activity in *T gondii* infection in mouse model.²² miR-155-5p acts on signalling pathways for differentiation of Th1 response. Its deficiency was involved in decrease of cellular and humoral T

cell-dependent immune response.²³⁻²⁶ miR-155-5p is highly expressed in Th17-cell subpopulations in humans and mice. In murine *T gondii* infection, miR-155-5p is required for cytokine expression by Treg.²⁷

Furthermore, miR-146a-5p not only was related as suppressor of Th17-cell differentiation,²⁸ but also is highly expressed in Treg cells and control IFN- γ -mediated Th1 response. In human T cells, miR-146a-5p is induced in regulator of toll-like receptor (TLR) coupling and is abundant in memory cells.²⁹

miR-21-5p is abundantly expressed in mammalian cells and evolutionarily conserved in a wide range of vertebrate species.³⁰ miR-21-5p, also, acts as a down-regulator of TLR-4 and inhibits the expression of pro-inflammatory cytokines.³¹ Studies have shown an overexpression of miR-21-5p in several cancer tissues, including rectal, gastric and pulmonary tissues,^{30,32} and murine livers infected by *T gondii*.³³

miR-125b-5p (from miR-125 family) was related in the development of inflammatory diseases, as well as T-cell differentiation.³⁴ In addition, miR-125b-5p was down-regulated in macrophages infected with *T gondii* RH strain.³⁵

miR-29 family includes the miR-29a, miR-29b and miR-29c. They are distributed in the chromosomes 1q32 and 7q32.³⁶ miR-29c-3p has been described as one of the regulators of retinal neurogenesis, since its targets are retinal regulatory genes, an integral part of CNS. Thus, miR-29c-3p may play a similar role during neurogenesis in other parts of the brain.^{26,37}

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

The Ethic Committees of Instituto Adolfo Lutz (CONEP-IAL/SES number: 2922263) and Instituto Emilio Ribas (CONEP-IIER number: 1133380) approved this study, which was performed according to the recommendations of Plataforma Brasil (from Brazilian Ministry).

2.2 | Patients and clinical samples

This study evaluated 79 plasma samples divided into three groups. The first group was composed of 32 plasma samples from CT/HIV patients. They were admitted and treated at Instituto de Infectologia Emilio Ribas, São Paulo, Brazil. Diagnosis was established by clinical and radiological features, ELISA, and real-time PCR (qPCR).^{2,7,8,38} Clinical and radiological diagnosis of cerebral toxoplasmosis in HIV-infected patients was based on (a) progressive neurological deficits; (b) contrast-enhancing mass lesion(s) on computed tomography and/or magnetic resonance imaging; and (c) successful clinical and radiological response to antiparasitic treatment within 10-14 days.^{7,8} The second group consisted of 27 plasma samples from individuals with asymptomatic toxoplasmosis

(AT), seropositive for toxoplasmosis in ELISA. For control, the third group was formed of 20 plasma samples from healthy individuals (NC), seronegative for toxoplasmosis in ELISA. For each patient/individual was collected peripheral blood with EDTA (5 mL) for laboratory diagnosis. Blood cells were used for DNA extractions (molecular diagnosis). Plasma samples were used for total RNA containing miRNA extractions (gene expression) and ELISA (serological diagnosis). Blood samples were sent to the laboratory within 48 hours after collection and immediately processed. In CT/HIV patients, blood samples were collected before the antiparasitic therapy for toxoplasmosis.

2.3 | Laboratorial diagnosis

Protocols for molecular diagnosis were performed as described before for blood samples.³⁸ DNA molecules were extracted from peripheral blood and tachyzoites (positive control) by QIAamp DNA Mini Kit. The protocols for DNA purification were done according to the manufacturer's instructions in a Robotic workstation for automated purification of DNA (QIAcube, Qiagen). qPCR was performed using the REP-529 molecular marker, which amplifies a highly repetitive 112 bp sequence in *T gondii* genome. The design included the forward (5'AGAGACACCGGAATGCGATCT3') and reverse (5'TTCGTCCAAGCCTCCGACT3') primers, and the hybridization probe (5'TCGTGGTGATGGCGGAGAGAATTGA3') labelled with FAM and BHQ1.

To verify the absence of PCR inhibitors, samples were also assayed using a housekeeping gene, the eukaryotic 18S rRNA gene (GenBank accession code X03205.1) (Applied Biosystems). Amplifications were run in the following thermal profile: 2 minutes, 50°C and 95°C for 10 minutes. Next, 40 cycles were performed at 95°C for 15 seconds and 60°C for 1 minute in an AriaMx Real-Time PCR System (Agilent Technologies).

Serological diagnosis was performed by ELISA using a *T gondii* lysate antigen (TLA).^{39,40} Tachyzoites (RH strain) released from Vero cell cultures were purified by centrifugation. Then, parasites were washed, suspended in PBS, and lysed using glass beads by vortex for 8 cycles for 4 minutes with 2-minute intervals. Parasite extract was centrifuged (3000 g) and dissolved in 0.3 mol/L NaCl. Protein concentration was determined at 280 nm by spectrometry in a NanoDrop ND100 (Thermo Scientific).

2.4 | RNA isolation and cDNA synthesis

To minimize the RNA degradation by RNases during the experimental process, all materials and working surfaces were cleaned using RNaseZap® RNase Decontamination Solution (Ambion™) prior to handling the samples. The total RNA, including miRNA, was extracted from plasma using the miRNeasy Serum/Plasma Kit (Qiagen). Plasma (250 µL) was mixed with denaturing buffer in the volumes described in manufacturer's protocol. The homogenate was incubated at room temperature for 5 minutes. Then, 5 µL (25 fmol) of the synthetic *Caenorhabditis elegans* miRNA (Cel-miR-39, Ambion) was spiked to each sample as the external control.⁴⁰ Subsequently, the protocols for RNA extraction were performed as manufacturer's instructions. miRNAs (and total RNA) were eluted into 30 µL of nuclease-free water.

Next, 2 µL of total RNA (including miRNAs) was reverse-transcribed (RT) using TaqMan® Advanced miRNA cDNA Synthesis Kit (Applied Biosystems). RT was performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems) according to the manufacturer's instructions, in four steps and under the following thermal conditions: 45 minutes at 37°C, 10 minutes at 65°C for poly (A) tailing reaction; 60 minutes at 16°C for ligation reaction; 15 minutes at 42°C, 5 minutes at 85°C for reverse transcription reaction; 5 minutes at 95°C, followed by 14 cycles of 95°C for 3 seconds and 60°C for 30 seconds;

TABLE 1 Description of genes investigated in this study

Assay name	Assay ID ^a	miR base accession number	Location chromosome	Mature miRNA sequence
hsa-miR-155-5p	477927_miR	MIMAT0000646	21	UUA AUGCUAAUCGUGAUAGGGGU
hsa-miR-146a-5p	478399_miR	MIMAT0000449	5	UGAGAACUGAAUCCAUGGGUU
hsa-miR-21-5p	477975_miR	MIMAT0000076	17	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-125b-5p	477885_miR	MIMAT0000423	11	UCCUGAGACCCUAAUUGUGA
hsa-miR-29c-3p	479229_miR	MIMAT0000681	1	UAGCACCAUUUGAAUCGGUUA
hsa-miR-26b-5p ^b	478418_miR	MIMAT0000083	2	UUCAAGUAAUUCAGGAUAGGU
hsa-miR-423-3p ^b	478327_miR	MIMAT0001340	17	AGCUCGGUCUGAGGCCCCUCAGU
hsa-miR-484 ^b	478308_miR	MIMAT0002174	16	UCAGGCUCAGUCCCUCCCGAU
cel-miR-39 ^c	478293_miR	MIMAT0000010	ND	UCACCGGGUGUAAUACAGCUUG

Abbreviations: cel, *Caenorhabditis elegans*; hsa, *Homo sapiens*; ND, nondetermined.

^aPurchased from Applied Biosystems.

^bControl endogenous sequence.

^cExternal control sequence.

TABLE 2 Clinical and laboratory diagnoses of patient groups

	Groups		
	CT/HIV (n = 32)	AT (n = 27)	NC (n = 20)
Clinical diagnosis ^a	Cerebral toxoplasmosis/HIV co-infection	Chronic toxoplasmosis (asymptomatic)	Healthy
Anti- <i>Toxoplasma gondii</i> IgG (ELISA)	Reagent (32/32)	Reagent (27/27)	Nonreagent (20/20)
PCR in blood (for <i>T gondii</i> DNA)	Positive (17/32)	Negative (26/26)	ND
Gender	15M - 17F	12M - 15F	2M - 18F

Abbreviation: AT, asymptomatic toxoplasmosis; CT/HIV, cerebral toxoplasmosis/HIV co-infection; NC, negative control; ND, nondetermined.

^aDiagnosis was defined by clinical, images and laboratory data as described in Materials and Methods section.

stop reaction at 99°C for 10 minutes for miR-Amp reaction. All cDNA samples were stored at -70°C until use in quantitative qPCR.

2.5 | Quantitative qPCR

qPCR amplification mixture contained 5 µL of 2× TaqMan Fast Advanced Master Mix and 0.5 µL of each TaqMan® Advanced miRNA Assays (miR-155-5p, miR-146a-5p, miR-21-5p, miR-29c-3p, miR-125b-5p, miR-26b-5p and cel-miR-39) (both Applied Biosystems). Description and assay IDs of each miRNA are shown in Table 1. Next, each template cDNA, diluted 1:10 in RNase-free water (2.5 µL), in triplicate were added to the mixture in a total volume of 10 µL. Amplifications were performed in a StepOne™ Real-Time PCR System (Applied Biosystems) using the following thermal profile: 95°C for 20 seconds, followed by 40 cycles performed at 95°C for 1 second and 60°C for 20 seconds. Results without amplification in qPCR were repeated three times.

2.6 | Data analysis

The values of miRNA expression were shown as 'Relative Quantification' (RQ) and calculated by the comparative CT method ($2^{-\Delta\Delta CT}$) as described before.⁴¹ The amplification plot, reflecting the fluorescent signal at each cycle, was determined based on the threshold cycle (CT) values for each sample. The average of CT values was calculated after values, in triplicate, for each sample.

The calibrators for calculations in miRNA expression experiments were constituted of 20 plasma samples from NC group (negative controls).

Normalization of gene expression assay was conducted testing the endogenous genes miR-484, miR-423-3p and miR-26b-5p, in which description and assay IDs are shown in Table 1.

Statistical analyses were performed using GraphPad Prism software version 6.0 (San Diego, CA, USA). Differences between the three groups (CT/HIV, AT and NC) for each miRNA were analysed by using

the nonparametric test Kruskal-Wallis ANOVA. Differences between CT/HIV and AT groups; or female and male for each miRNA were analysed by Mann-Whitney (nonparametric t test). In all cases, differences or similarities were considered statistically significant at $P \leq .05$.

3 | RESULTS

3.1 | Clinical and laboratorial diagnosis

Clinical and laboratory diagnoses from the 79 patients/individuals are shown in Table 2. The clinical diagnosis for the cerebral toxoplasmosis/HIV co-infection was defined in the 32 CT/HIV patients. All of them were seropositive in ELISA and 17 of them had positive qPCR. The 27 asymptomatic individuals (AT) were seropositive for toxoplasmosis. The 20 individuals of NC group were seronegative for toxoplasmosis. The gender of each group was 15 men and 17 women (CT/HIV), 12 men and 15 women (AT) and 2 men and 18 women (NC).

3.2 | Selecting the ideal endogenous gene

cel-miR-39 synthetic molecule was added after RNA extraction in all plasma samples tested in this study. Thus, all of them had positive result by qPCR, ensuring a good extraction quality.

In first step, gene expression analysis was devoted for methodology standardizations. After testing the miR-484, miR-423-5p and miR-26b-5p in 10 plasma samples from CT/HIV patients and C_T results analysed by geNorm software, M values were 1.354 for miR-484, 1.463 for miR-423-3p and 1.927 for miR-26b-5p. miR-423-3p and miR-484 genes were appropriate since RQ values were below 1.5 (considered as cut-off value).⁴¹ Nevertheless, according to geNorm classification, miR-484 was chosen as the endogenous control because of its uniform expression throughout plasma samples. miR-26b-5p was highly variable and an inadequate candidate for normalizer.⁴¹

3.3 | Up-expression of miR-21-5p and miR-146a-5p in CT/HIV patients

In the next step, RQ results of each miRNA level and comparison between groups were performed. Among five miRNAs, miR-21-5p and miR-146a-5p were up-expressed in plasma samples of CT/HIV patients when compared with AT and NC individuals. Mean RQ for miR-21-5p was 3.829 ± 0.5787 and for miR-146a-5p, 2.500 ± 0.3243 . Plasma samples of AT individuals had lower RQ mean, for miR-21-5p was 1.815 ± 0.1514 and for miR-146a-5p, 1.661 ± 0.2131 (mean \pm SEM). Differences between three groups (CT/HIV, AT and NC) were statistically significant at $P < .0001$ (miR-21-5p and miR-146a-5p) in Kruskal-Wallis ANOVA test (Figure 1).

Differences between CT/HIV and AT groups were, also, statistically significant at $P = .0302$ (miR-21-5p) and $P = .0107$ (miR-146a-5p) in Mann-Whitney test (Figure 2).

Differences were also observed between three groups at $P < .0001$ for miR-29c-3p, miR-155-5p and miR-125b-5p in Kruskal-Wallis ANOVA test (Figure 1). However, CT/HIV patients and AT individuals expressed almost similar levels of miR-29c-3p (1.486 ± 0.1164 and 1.315 ± 0.0964 , respectively); miR-155-5p (1.243 ± 0.0341 and 1.651 ± 0.2536 , respectively); and miR-125b-5p

(2.041 ± 0.2451 and 2.026 ± 0.2150 , respectively). These analyses were done using Mann-Whitney test (Figure 2).

The literature reports that some miRNAs can be differently expressed in samples from males or females.^{42,43} Thus, the comparison between miRNA levels in plasma from women and men was analysed. Only miR-21-5p, miR-146a-5p and miR-125b-5p were more expressed in plasma men than plasma women from CT/HIV group. In AT and NC groups, nondifference related to gender was observed in all miRNAs studied (Figure 3).

4 | DISCUSSION

miRNAs regulate activation and differentiation of T-effector and Treg cells.¹⁹ In the adaptive immune system, T cells play an important role in preventing pathogen invasion. The start and resolution of these responses by cells must be tightly regulated to avoid inflammatory processes and possible pathologies.¹⁹ T-cell activation is triggered by the T-cell receptor (TCR), which determines cell specificity.⁴⁴ TCR coupling induces a signalling cascade leading to activation of transcription factors, including nuclear factor kappa B (NF- κ B) which is involved in T-cell proliferation, survival and effector functions of T cells.⁴⁵ miRNAs can be involved in this

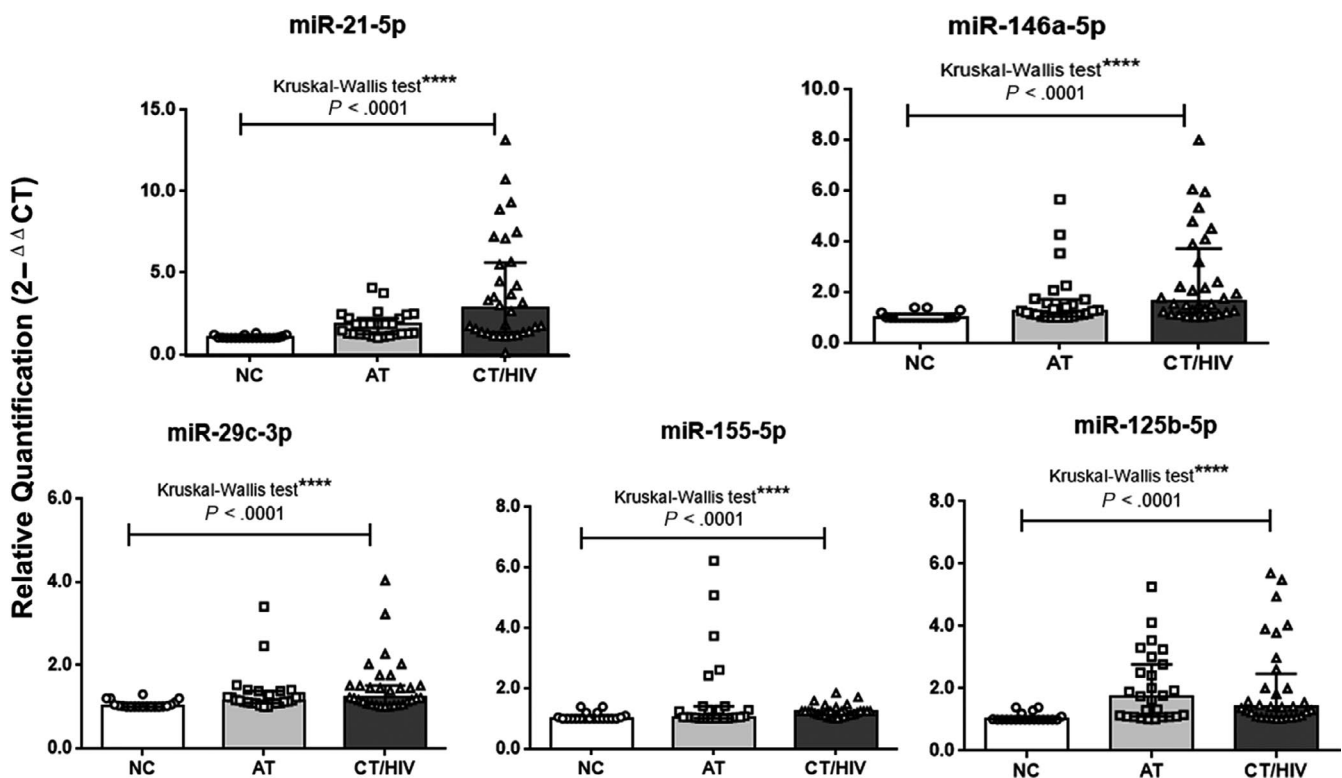


FIGURE 1 Expression levels of miR-21-5p, miR-146a-5p, miR-29c-3p, miR-155-5p and miR-125b-5p in plasma from 20 seronegative individuals for toxoplasmosis (NC), 27 asymptomatic toxoplasmosis individuals (AT) and 32 patients with cerebral toxoplasmosis/HIV co-infection (CT/HIV). Gene expression of each miRNA was determined by qPCR, after miRNA isolation and cDNA synthesis. Results are expressed as 'Relative Quantification' that was determined by $2^{-\Delta\Delta CT}$ method. All miRNA expression values were normalized to the miR-484 endogenous control. Scatter dot plots show medians, and comparison of expression levels between the three groups was performed by Kruskal-Wallis ANOVA test. Differences were statistically significant at * $P < .05$, ** $P < .005$, *** $P < .0005$, **** $P < .00005$

regulation system, especially at transcriptional and processing levels. miRNAs can regulate cytokine production, and cytokines can modulate miRNA levels.²⁶

miR-21-5p was up-expressed in CT/HIV patients when compared with AT individuals. Considering that miR-21-5p can down-regulate IL-10 and TNF- α , these findings are in agreement with our previous studies, in which CT/HIV patients had low IL-10 levels.^{26,30,31} miR-21-5p is correlated with resolution of inflammation and pro-inflammatory response induced by many stimuli that trigger miR-21-5p induction. In particular, miR-21-5p is a key mediator for anti-inflammatory process,

since interfere on levels of the anti-inflammatory cytokine IL-10 TNF- α production.^{31,32} The regulation between those cytokines acts a key signal for the balance and transition between both states of the infection, symptomatic and asymptomatic. Thus, miR21-5p presence is not solely a feature of pro-inflammatory or immunosuppressive state. miR-21-5p can regulate the inflammatory switch.⁴⁶

CD4 and CD8 T cells revealed that TCR stimulation activates NF- κ B, which induces miR-146a-5p expression. On the other hand, miR-146a-5p down-regulates NF- κ B activity by acting as a negative feedback regulatory network.²⁸ miR-146a-5p, also, contributes to the continuous suppression of the Th1-like features in Treg cells controlling IFN- γ -mediated Th1 responses.^{22,29} Down-expression of miR-146a-5p in mouse Treg results in immunopathology mediated by CD4 + T-lymphocyte (Th1), IFN- γ -dependent, causing an interruption in tolerance.⁴⁵ In addition, *T gondii*-infected mice with miR-146a-5p deficiency had better control of parasite infection resulting in the long-term survival.²⁵ In this study, CT/HIV patients had an up-expression of miR-146a-5p. These results suggest that miR-146a-5p may represent an alternative regulatory mechanism for adjusting the IFN- γ -mediated inflammatory response, helping the excessive CNS inflammation after NF- κ B stimulation.^{47,48} Here, CT/HIV patients up-expressed miR-146a-5p and miR-21-5p. These are a few possible explanations for this finding by previous studies, that miR-146a-5p and miR-21-5p target different components of the TLR and NF- κ B pathways. However, they act together to suppress the activation of these pathways.^{32,47,48}

Among miRNAs investigated in this study, miR-125b-5p, miR-155-5p and miR-29c-3p were equally expressed in CT/HIV patients

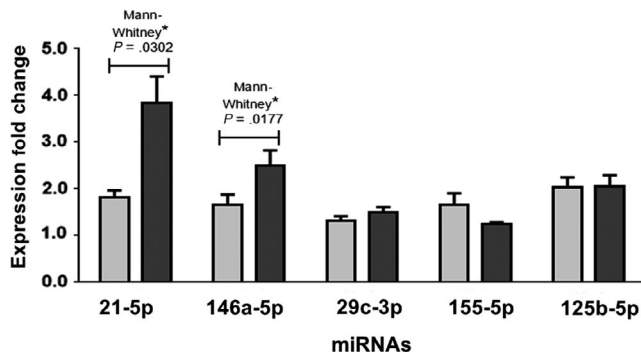


FIGURE 2 Mean of expression levels of miR-21-5p, miR-146a-5p, miR-29c-3p, miR-155-5p and miR-125b-5p in plasma of asymptomatic toxoplasmosis individuals (light grey bars) and CT/HIV patients (dark grey bars). Comparison of expression levels between two groups was performed by Mann-Whitney test. Differences were statistically significant at * $P < .05$, ** $P < .005$, *** $P < .0005$, **** $P < .00005$

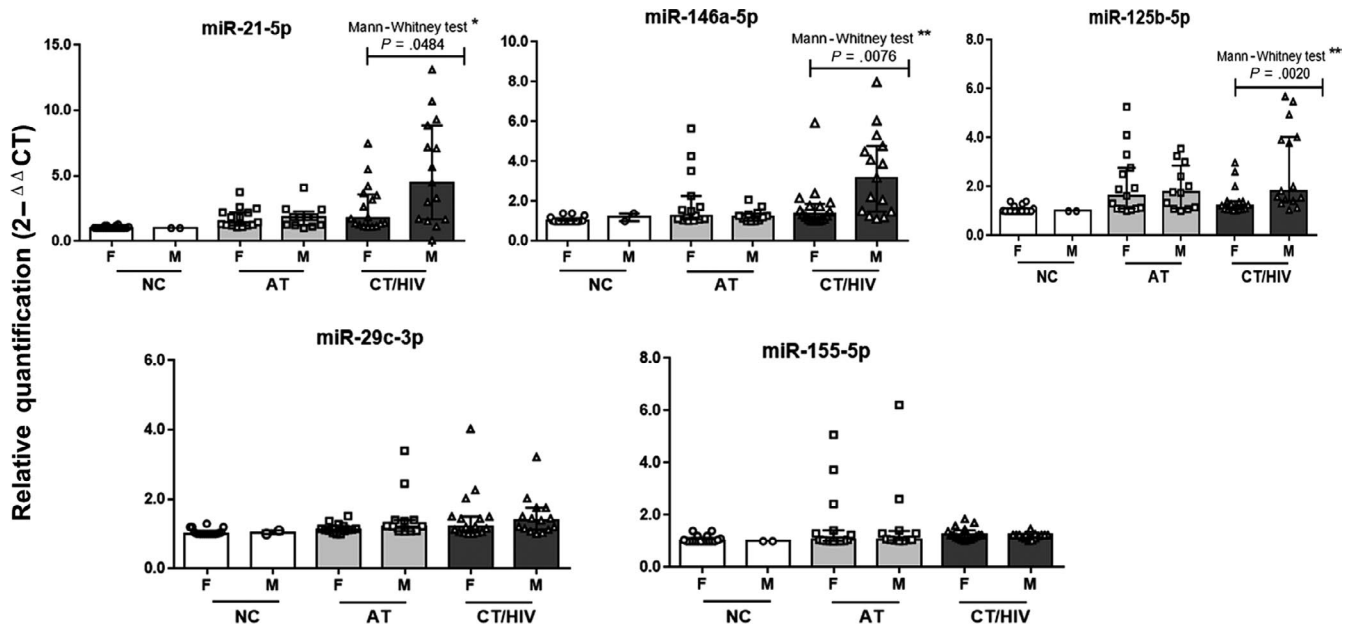


FIGURE 3 Expression levels of miR-21-5p, miR-146a-5p, miR-29c-3p, miR-155-5p and miR-125b-5p in plasma. Gene expression of each miRNA was determined by qPCR, after miRNA isolation and cDNA synthesis. Results are expressed as 'Relative Quantification' that was determined by $2^{-\Delta\Delta CT}$ method. All miRNA expression values were normalized to miR-484 endogenous control. Scatter dot plots show medians. Comparison of expression levels was performed by Mann-Whitney test, between 18 women (F) and 2 men (M) seronegative for toxoplasmosis (NC); 15 F and 22 M with asymptomatic toxoplasmosis (AT); and 17 F and 15 M with cerebral toxoplasmosis/HIV co-infection (CT/HIV). Differences were statistically significant at * $P < .05$, ** $P < .005$, *** $P < .0005$, **** $P < .00005$

and AT individuals. These results can suggest that these miRNAs were not directly correlated with this co-infection. However, in a previous study, we showed that patients with ocular toxoplasmosis up-expressed miR-155-5p and miR-29c-3p.⁴⁹ In agreement, these patients had elevated pro-inflammatory cytokines (IL-6, TNF- α , and TGF- β and IL-1B) production.^{13,50}

Although some miRNAs could be differently expressed in samples from males and females,^{42,43} the miRNAs analysed in this study were equally expressed in plasma collected from male and female from CN or AT groups. However, miR-21-5p, miR-146a-5p and miR-125b-5p were more expressed in plasma collected from men than those collected from women (both with CT/HIV). This divergence could indicate that gender may be an important factor of disease-associated miRNAs. Therefore, miRNA expression and gender difference may be more complex than previously supposed.

Despite the significant increase in miRNA studies, few of them have evaluated the miRNAs/toxoplasmosis relationship, principally during toxoplasmosis/HIV co-infection. The data showed in this study can contribute for identify specific patterns of miRNAs as biomarkers⁵¹ for diagnosis and prognosis in CT/HIV patients. Anyhow, further research on this topic should be done before the association between toxoplasmosis and miRNAs is more clearly understood.

CONFLICT OF INTEREST

The authors have no other relevant affiliations or financial involvement with any organization or entire with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION

VLP-C and CSM-S coordinated the experiments and designed the study. VLP-C and ISP wrote the manuscript. ISP and VLP-C performed the selection of clinical samples. ISP, MMM, ABC and CSM-S performed all experiments including miRNA purifications, gene expression and serological diagnosis for toxoplasmosis. ISP and CSM-S analysed the gene expression results. JEV and JPMT performed the clinical diagnosis of patients. RG performed the molecular diagnosis for toxoplasmosis. All authors contributed substantially to interpretation of the data and to the manuscript. In addition, all authors revised the manuscript, approved the final version submitted, published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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