RESEARCH ARTICLE

Variants of MIRNA146A rs2910164 and MIRNA499 rs3746444 are associated with the development of cutaneous leishmaniasis caused by Leishmania guyanensis and with plasma chemokine IL-8

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Abstract

Leishmania are intracellular protozoan parasites that cause a wide spectrum of clinical manifestations in genetically susceptible individuals with an unsatisfactory or balanced Th1 immune response to eliminate the parasite. MiRNAs play important regulatory roles in numerous biological processes including essential cellular functions. miR146-a acts as an inhibitor of interleukin 1 receptor associated kinase 1 (IRAK1) and tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) present in the toll-like receptors pathway while miR499a modulates TGF-β and TNF signalling pathways. Here, we investigated whether MIRNA146A rs2910164 and MIRNA499 rs3746444 variants are associated with the development of L. guyanensis (Lg)-cutaneous leishmaniasis (CL). The variants MIRNA146A rs2910164 and MIRNA499 rs3746444 were assessed in 850 patients with Lg-CL and 891 healthy controls by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Plasma cytokines were measured using the BioPlex assay. Carriers of rs2910164 GC genotype have 30% higher odds of developing CL (OR_adj.age/sex = 1.3 [95% CI 0.9–1.8]; Padj.age/sex 0.14) compared to individuals with the genotype GG (OR_adj.age/sex = 0.77 [95% CI 0.56–1.0]; Padj.age/sex 0.14) if exposed to Lg-infection. Heterozygous GC individuals also showed lower odds of developing CL (OR_adj.age/sex = 0.77 [95% CI 0.5–1.1]; Padj.age/sex 0.09). Homozygosity for the allele C is suggestive of an association with the development of Lg-CL among exposed individuals to Lg-infection. However, the odds of developing CL associated with the CC genotype was evident only in male individuals.
Individuals homozygous for the G allele tend to have higher plasma IL-8 and CCL5. Similarly, for the MIR499A rs3746444, an association with the G allele was only observed among male individuals (OR = 1.4 [1.0–1.9]; P = 0.009). In a dominant model, individuals with the G allele (GG-GA) when compared to the AA genotype reveals that carriers of the G allele have 40% elevated odds of developing Lg-CL (ORadjage = 1.4 [1.1–1.9]). Individuals with the GG genotype have higher odds of developing Lg-CL (ORadjage/sex = 2.0 [95%CI 0.83–5.0]; Padjage = 0.01). Individuals homozygous for the G allele have higher plasma IL-8. Genetic combinations of both variants revealed that male individuals exposed to Lg bearing three or four susceptible alleles have higher odds of developing Lg-CL (OR = 2.3 [95% CI 1.0–4.7]; p = 0.017). Both MIR146A and MIR499A variants are risk factors to developing cutaneous leishmaniasis caused by L. guyanensis in Amazonas state of Brazil. Individuals with these variants are susceptible to the development of CL.

Introduction

Leishmaniasis, a vector-borne disease caused by protozoan parasites from the genus Leishmania, is endemic in the tropical and subtropical regions, including more than 98 countries. Nearly, one billion of people are at risk of infection [1]. Leishmania species cause a spectrum of clinical forms of the disease: visceral (VL), cutaneous (CL), diffuse cutaneous, disseminated cutaneous and mucocutaneous leishmaniasis (ML) [2].

CL is considered the most common form of Leishmania-infection. Approximately 0.7 million to 1.2 million human beings are affected by this disease [1,3]. In Brazil, the main species involved in the etiology of CL are L. braziliensis (Lb), L. guyanensis (Lg), L. lainsoni, L. amazonensis, L. shawi, L. naiffi and L. lindbergi [4]. In the Amazonas state of Brazil, Lg is responsible for 95% of CL cases [5].

Resistance to Leishmania-infection or healing is associated with a Th1 cell immune response and production of pro-inflammatory cytokines (IL-12, IFN-γ and TNF-α) [6]. Susceptibility to infection and uncontrolled parasitic replication are associated with a Th2 anti-
inflammatory cytokines IL-4, IL-5 and IL-13 [7]. Th17 cells, producing IL-22 and IL-17, and T regulatory (Treg) cells producing IL-10 and TGF-β, also contribute to disease progression [6].

MicroRNAs (miRNAs) are small, single-stranded, untranslated endogenous RNAs, composed of 18–26 nucleotides that regulate gene expression. miRNAs bind to their target messenger RNA (mRNA) through base complementarity mechanism resulting in the regulation or degradation of protein translation [8,9]. More than 30% of human protein-coding genes are under post-transcriptional control of miRNAs [10]. miRNAs can regulate many physiological processes such as cell cycle, metabolism, and apoptosis. miRNAs play a crucial role in hematopoiesis, immune cells development and differentiation, inflammation, and immunomodulation [11,12].

MiRNAs can modulate macrophages polarization during Leishmania-infection, creating mix M1/M2 as shown in murine macrophages infected with L. amazonensis [13,14]. L. donovani glycoprotein can downregulate pre-miRNA-122 affecting post-transcriptional regulation of host mRNA/miRNA interactions leading to accumulation of the parasites in the liver [15]. L. amazonensis upregulates miR-294-30 and miR-721 that bind to Nos2 mRNA leading to low levels of NO and increased infectivity in BALB/c-BMDM [16]. miRNAs, let-7a, miR-25, miR-26a, miR-132, miR-140, miR-146a, and miR-155 are upregulated in L. major-infected human macrophages and negatively correlated with the expression of the corresponding chemokine targets, CCL2, CCL5, CXCL10, CXCL11, and CXCL12 [17]. let-7a, let-7b, and miR-103 are upregulated in L. donovani-infected DCs and macrophages but downregulated in L. major infections [9]. Recently, it was shown that a super-enhancer mediates the transcription of MIR146A and drives the polarization of macrophages into M2 suppressing immune responses in L. donovani-infection [18].

miR146-a is a key modulator of innate immune response and acts as an inhibitor of interleukin 1 receptor associated kinase 1 (IRAK1) and tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) [19]. Both IRAK1 and TRAF6 are signalling transducers of the nuclear factor kappa B (NF-κB) in the Toll-like receptors (TLR) pathways. Inhibitions the NF-κB transcriptional activity leads to the impairment of the biosynthesis of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α [19]. miR499-a modulates different biological functions, including immune cells development and maturation, TGF-β and TNF signalling pathways [20]. miR499-a is correlated to the expression of IL-17RB, IL-23a, IL-2R, IL-6, IL-2, and IL-18R [21].

Single nucleotide polymorphisms (SNPs) in miRNA precursors may affect the miRNA biogenesis, causing a reduction of mature miRNA expression levels [22]. SNPs in mature miRNA may affect miRNA target specificity and leads to altered cellular protein levels [23,24]. These SNPs or variants in miRNA may by their actions alter the course of various diseases.

MIR499A and MIR146A genes are located on 20q11.22 and 5q33.3 chromosomes, respectively. The variant rs3746444 located in the seed region of mature miR499-a leads to the disruption of miRNA-mRNA interactions and creation of new gene targets [20]. The variant rs2910164 present in the seed sequence of miR146-a precursor results in low production of mature miR146-a and consequently to a decrease inhibition of TRAF6 and IRAK1, leading to a higher production of pro-inflammatory cytokines upon TLRs activation [25]. miRNA146-a and miR499-a have been associated with susceptibility to multiple types of cancer, psoriasis and rheumatoid arthritis [26–29].

Taken into account the potential role of miR146-a and miR499-a in modulating the T helper cell immune response, we investigated whether the variants MIR146A rs2910164 and MIR499A rs3746444 are associated with susceptibility or protection to the development of Lg-CL in the Amazonas. The influence of the MIR146A and MIR499A genotypes on plasma cytokine levels were also investigated.
Methods

Area of study and population

The study was conducted at the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD), the referral regional center for treatment of leishmaniasis. The study population and the endemic area of recruitment of the participant of the study are described elsewhere [30]. Briefly, all the participants are from the perirural areas of Manaus, the capital city of the Amazonas State where *L. guyanensis* is endemic. Patients with active CL were followed at the FMT-HVD. The healthy controls with no history of CL and devoid of any scar suggestive of CL are from the same endemic area of the patients with CL.

Ethical statement

This study was conducted according to the Declaration of Helsinki and approved by the Research Ethics Committee of the FMT-HVD granted under the file number CAAE:09995212.0.0000.0005 on 31 May 2013. All the participants or their responsible party for individuals less than 18 years of age provided written informed consent for the collection of samples and subsequent analysis.

Sample size calculation

Sample size calculation is described elsewhere [31]. Briefly, using the Genetic Power calculator developed at Harvard University (http://pngu.mgh.harvard.edu/~purcell/gpe), we assumed a minor allele frequency of 5%, disease prevalence of 5%, complete linkage disequilibrium 1 between marker and case-control discrete trait, case-control ratio 1, and 5% type 1 error rates with an odds ratio of 1.5 and 2.0 for heterozygosity and homozygosity, respectively. For 80% power, the genetic allelic model provided a sample size of 789 individuals for cases and 789 for controls.

*Leishmania spp* identification from biopsy specimens and DNA extraction from whole blood for SNP typing

All the patients with CL provided a biopsy specimen from the cutaneous lesion for the isolation of parasite DNA. For the identification of the *Leishmania spp.*, the discrimination of the *Leishmania Viannia* subgenus specific PCR was in accordance with established protocols [32,33]. Identification of *Leishmania spp.* was performed by nucleotide sequencing of a fragment of *HSP 70* and *miexon* genes [34,35]. Venous blood was drawn from all participants and collected into EDTA-containing Vacutainers (Becton Dickinson, Brazil) for DNA extraction and cytokine assay. Genomic DNA was extracted by the salting out method [36].

SNP genotyping

The SNPs *MIR146A* rs2910164 and *MIR499A* rs3746444 were performed by PCR-RFLP with the restriction enzymes *HpyCH4 III* and *HpyCH4 IV* (New England Biolabs, Ipswich, MA, United States), respectively. The respective pairs of primers for the amplification the region flanking the SNPs and the fragments generated for alleles discrimination by the restriction enzymes as well as the cycling protocols for PCR are shown in S1 Table. The pair of primers for *MIR499A* was designed from the reference sequence NC 000020.11 from NCBI. The underline G nucleotide from the forward primer substitutes A from the reference sequence to eliminate a site of restriction for the HpyCH4IV. Primers for the *MIR146A*
were designed from the reference sequence NC 000005.10 and the underline A nucleotide substitutes C from the reference sequence to create restriction site when the G allele is present.

The PCR mix contains: 0.2 μM of each primer (Thermofisher, MA USA), 40 nM of dNTP (Thermofisher, MA USA), 1.0 mM of MgCl2 (Thermofisher, MA USA), 1 U of Taq DNA polymerase (Thermofisher, MA USA), 1X of 10X Taq polymerase buffer containing 500 mmol/L KCl and 100 mol/L of Tris-HCl (pH 8.3) and 50 ng of DNA in a final volume of 25 μL. A volume of 10 μL of the PCR products was digested with 1 unit of the respective restriction enzyme and buffer in a final volume of 20 μL and size separated in a 3% agarose (Ultrapure Agarose, Thermofisher, MA USA) gel electrophoresis.

Cytokine assay

Cytokine assay of IL-1β, TNF-α, IL-2, IL-6, IL-8, IL-17, IFN-γ, CCL2 and CCL5 in the plasma were measured using the multiplex cytokine commercial kit Human Cytokine Grp I Panel 27-Plex (Bio-Rad, USA) according to the instructions of the manufacturer in the Bio-plex 200 Protein Array System (Luminex Corporation, USA).

Statistical analysis

The genotype and allele frequencies were calculated by direct gene counting. For calculation of Hardy-Weinberg equilibrium (HWE), the website http://ihg.gsf.de/cgibin/hw/hwa1 was used, that also compared cases with the control groups by logistic regression analysis to determine associations to susceptibility or resistance for the different genotypes and alleles by χ2 test along with OR and 95% confidence interval. The correlation of the different genotypes of MIR146A rs2910164 and MIR499A rs3746444 to the concentration of circulating plasma cytokines was performed by the R software version 4.0.0 of SNPassoc package for quantitative traits analysis and ggplot2 package for visualizing. P values for the correlations of cytokines by genotypes were corrected for false discovery rate (FDR) of Benjamini-Hochberg.

Results

Study population

The study population is the same as described previously [30]. A total of 850 patients with Lg-CL and 891 healthy controls (HC) were included in the study. The HC are from the same endemic area of the patients with Lg-CL. Among the patients with Lg-CL, 639 (75%) patients were male (mean age 34.4 ± SD 13.7 years) and 211 (25%) were females (37.5 ± SD 15.7 years). In the controls group, 608 (68%) were male (42 ± SD 17.5 years) and 283 (32%) were female (40 ± SD 17.4 years). Overall, the mean age among the patients with Lg-CL and controls is 35.17 ± SD 14.25 and 41.4 ± SD 17.5 years, respectively. The HC is older than the cases (P<0.0001). Men were older among the HC group compared to group of male patients with Lg-CL (P<0.0001) while there was no age difference among females (P<0.077). All the participants of the study were devoid of HIV and the patients had fewer or equal to six lesions and treatment naïve at the time of enrolment. Pregnant women were excluded from the study. Of note, there are more females in the HC compared to the group of patients with CL (P<0.0013).

Association MIR146A rs2910164 and MIR499A rs3746444 with susceptibility to Cutaneous Leishmaniasis

MIR146A rs2910164 was assessed in 826 patients with Lg-CL and 886 controls. Genotype and allele frequencies for the two variants are demonstrated in Table 1. rs2910164 was in Hardy-
Weinberg equilibrium (HWE) among the patients with Lg-CL and HC. The MIR146A rs2910164 CC genotype was prevalent in patients with Lg-CL (12%) compared with HC (9%). Carriers of rs2910164 CC genotype have 30% higher odds of developing CL (ORadj\text{age/sex} = 1.3 [95%CI 0.9–1.8]; Padj\text{age/sex} = 0.14) compared to individuals with the genotype GG (ORadj\text{age/sex} = 0.77 [95%CI 0.56–1.0]; Padj\text{age/sex} = 0.14) if exposed to Lg-infection. Heterozygous GC individuals also have lower odds of developing CL compared with homozygous carriers of the C allele (ORadj\text{age/sex} = 0.77 [95%CI 0.55–1.1]; Padj\text{age/sex} = 0.09). In a recessive model, when homozygous individuals for the C allele are compared with individuals carrying a G allele (CC versus GC—GG), carrier of G allele have 23% lower odds of developing Lg-CL (ORadj\text{age/sex} = 0.77 [95%CI 0.56–1.0]; Padj\text{age/sex} = 0.098). Homozygous for the C allele have 30% higher odds of developing CL (ORadj\text{age/sex} = 1.3 [95%CI 0.9–1.8]; Padj\text{age/sex} = 0.098). The C allele is suggestive of an association with the development of Lg-CL.

### Table 1. Genotype and allele frequencies for the MIR146A rs2910164 and MIR499A rs3746444 in patients with Leishmania guyanensis-Cutaneous Leishmaniasis (Lg-CL) and healthy controls.

| Genotypes and Alleles Frequencies | Cases\(^a\) (%) | HC\(^b\) (%) |
|----------------------------------|-----------------|-------------|
| rs2910164                        |                 |             |
| GG                               | 375 (46)        | 405 (46)    |
| GC                               | 349 (42)        | 398 (45)    |
| CC                               | 102 (12)        | 83 (9)      |
| G                                | 1099 (67)       | 1209 (68)   |
| C                                | 553 (33)        | 563 (32)    |
| rs3746444                        |                 |             |
| AA                               | 649 (79)        | 706 (83)    |
| AG                               | 153 (19)        | 135 (16)    |
| GG                               | 16 (2)          | 10 (1)      |
| A                                | 1451 (89)       | 1547 (91)   |
| G                                | 185 (11)        | 155 (9)     |

| Genotypes and alleles comparisons | Comparisons | P value\(^c\) | OR [95% CI]\(^d\) | Padj\(^e\) | ORadj[95%CI]\(^f\) |
|----------------------------------|-------------|---------------|------------------|-----------|--------------------|
| rs2910164                        | GG vs CC    | 0.084         | 1.3 (0.96–1.8)   | 0.14      | 1.3 (0.9–1.8)      |
| CC vs GC                         | 0.040       | 1.4 (1.0–1.9) | 0.09             | 1.3 (0.9–1.8) |
| CC+GC vs GG                     | 0.087       | 1.0 (0.8–1.2) | 0.064            | 1.0 (0.8–1.2) |
| GG+GC vs CC                     | 0.047       | 1.4 (1.0–1.9) | 0.098            | 1.3 (0.9–1.8) |
| G vs C                          | 0.304       | 0.9 (0.8–1.0) |                 |           |                    |
| rs3746444                        | AA vs GG    | 0.167         | 1.7 (0.8–3.9)    | 0.15      | 2.0 (0.83–5.0)     |
| AA vs AG                        | 0.106       | 1.2 (0.9–1.6) | 0.13             | 1.3 (1.0–1.7) |
| AA+GA vs GG                     | 0.196       | 1.7 (0.8–3.7) | 0.170            | 1.8 (0.8–4.0) |
| AA vs GA+GG                     | 0.058       | 1.3 (1.0–1.6) | 0.067            | 1.3 (1.0–1.6) |
| A vs G                          | 0.035       | 1.3 (1.0–1.6) |                 |           |                    |

\(^a\)Cases: patients with Lg-CL  
\(^b\)HC: healthy controls  
\(^c\)P value: normal p-value  
\(^d\)OR: odds ratio with 95% confidence interval (CI)  
\(^e\)Padj: p adjusted by gender and age  
\(^f\)ORadj: odds ratio adjusted by gender and age; P value <0.05 is statistically significant.

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As male individuals were prevalent in both patients with Lg-CL and HC, we stratified according to sex as shown in Table 2. The odds of developing CL associated with the CC genotype was evident only in male individuals (OR_{adj/age} = 1.3 [95% CI = 0.9–2.0 \ P_{adj/age} = 0.06]) compared with GG genotype. Individuals with GG genotype had lower odds of developing Lg-CL (R_{adj} = 0.77 [95%CI 0.5–1.0]; p = 0.06). The genotype CC is suggestive of an association with the development of CL compared to GG genotype among Lg-infected individuals.

**Table 2. Distribution and Comparison of Genotypes and Alleles frequencies of the MIR146A rs2910164 between Patients with Leishmania guyanensis-Cutaneous Leishmaniasis (Lg-CL) and Controls stratified according to gender.**

|                  | Patients with Lg-CL, no. (%) | Controls, no. (%) |
|------------------|------------------------------|------------------|
|                  | Males | Females | Males | Females |
| Genotypes       |       |         |       |         |
| G/G              | 284 (46) | 91 (44) | 286 (47) | 120 (42) |
| G/C              | 254 (41) | 95 (47) | 261 (44) | 136 (48) |
| C/C              | 83 (13) | 19 (9) | 56 (9) | 27 (10) |
| Alleles         |       |         |       |         |
| G                | 822 (66) | 277 (67) | 833 (69) | 376 (66) |
| C                | 420 (34) | 133 (33) | 373 (31) | 190 (34) |

| Genotypes and alleles comparisons | Males | Females |
|-----------------------------------|-------|---------|
|                                   | P_v  | OR [95%CI] | P_{adj} | OR_{adj} [95%CI] | P_v  | OR [95%CI] | P_{adj} | OR_{adj} [95%CI] |
| GG vs CC                          | 0.036 | 1.5 [1.0–2.2] | 0.06 | 1.3 [0.9–2.0] | 0.82 | 0.9 [0.5–1.8] | 0.45 | 1.0 [0.5–1.8] |
| CC vs GC                          | 0.03  | 1.5 [1.0–2.2] | 0.04 | 1.4 [1.0–2.0] | 0.98 | 1.0 [0.5–1.9] | 0.45 | 1.0 [0.5–2.0] |
| GG+GC vs CC                      | 0.02  | 1.5 [1.0–2.2] | 0.04 | 1.4 [0.95–2.0] | 0.92 | 1.0 [0.5–1.9] | 0.38 | 1.0 [0.7–1.5] |
| G vs C                           | 0.12  | 1.1 [0.96–1.3] | 0.71 | 1.1 [0.8–1.4] | 1.1 |

_aP_v: normal p-value_  
bOR: odds ratio with 95% confidence interval (CI)  
cP_{adj}: p adjusted by age  
dOR_{adj}: odds ratio adjusted by age.  
P value < 0.05 is statistically significant.

As male individuals were prevalent in both patients with Lg-CL and HC, we stratified according to sex as shown in Table 2. The odds of developing CL associated with the CC genotype was evident only in male individuals (OR_{adj/age} = 1.3 [95% CI = 0.9–2.0 \ P_{adj/age} = 0.06]) compared with GG genotype. Individuals with GG genotype had lower odds of developing Lg-CL (R_{adj} = 0.77 [95%CI 0.5–1.0]; p = 0.06). The genotype CC is suggestive of an association with the development of CL compared to GG genotype among Lg-infected individuals.

MIR499A rs3746444 was genotyped in 818 patients with Lg-CL and 851 HC. The variant was in HWE in both groups of patients with Lg-CL and HC. The distribution of genotypes among patients with Lg-CL and HC was different as shown in Table 1, revealing a common odds ratio of 1.3 for the G allele compared with the A allele (p = 0.04). The rs3746444 AA genotype was more prevalent among the HC (83%) compared to 79% in the patients with Lg-CL group. Comparison of the genotype AA to GG revealed that individuals with the GG genotype have 100% higher odds of developing Lg-CL, with a 95% CI ranging from a decreased odds of 17% to an elevated odd of 400% (OR_{adj/age/sex} = 2.0 [95%CI 0.83–5.0]; P_{adj/age/sex} = 0.15). Similarly, the G allele confers 27% elevated odds of developing Lg-CL suggesting that the G allele contribute to susceptibility to the development of Lg-CL (OR = 1.27 [95% CI 1.0–1.6]; p = 0.035). In a dominant model, individuals with the G allele (GG-GA) when compared to the AA genotype reveals that carriers of the G allele have 30% elevated odds of developing Lg-CL (OR_{adj/sex} = 1.3 [95% CI 1.0–1.6]; P_{adj/sex} = 0.067).

We stratified into male and female individuals to look for the strength of the association. Like the MIR146A, the association was more evident among the male individuals as shown in Table 3. Male individuals with GG genotype compared with AA genotype revealed an elevated odds of 50% with a 95% confidence interval ranging from a decreased odds of 40% to an increased odds of 310% to the development of Lg-CL (OR_{adj} = 1.5 [0.6–4.1]; P_{adj} = 0.38).
Similarly, female individuals had higher odds of developing Lg-CL (OR\textsubscript{adj} = 2.3 [95%CI 0.5–10.0]; P\textsubscript{adj} = 0.26).

### Genetic combinations of both MIR146A rs2910164 and MIR499A rs3746444 genotypes

The frequencies of the combined genotypes are shown in Supplementary Table 3. Male individuals carrying at least one susceptibility allele (MIR146A rs2910164C or MIR499A rs3746444G) revealed an OR of 1.1 [95%CI 0.8–1.4] in comparison to individuals with two alleles (1.4 [95% CI 1.0–1.9]; p = 0.035) or three or four alleles (2.3 [95% CI 1.0–4.7]; p = 0.017). Notably, these associations were not observed among female individuals strengthening that the associations are mainly among male individuals.

### Comparison of circulating plasma cytokines concentrations (pg/mL) with respect to the MIR146A rs2910164 and MIR499A rs3746444 genotypes

miR-146a is suggested to suppress IRAK-1 expression leading to a decrease of IL-6 and IL-8 secretions that are key mediators of inflammation [37]. Bone-marrow derived macrophages from knockout MIR146A−/− mice treated with monosodium urate expressed higher levels of IL-1β, TNFα, NLRP3, IRAK-1 and TRAF-6 compared with BMDM from wild type mice [38]. Inhibitors of miR146-a stimulate the expression of IL-8 and CCL5 [39]. We analysed whether the different genotypes of MIR146A may correlate with circulating plasma levels of IL-6, IL-8, IL-1β, TNFα and CCL5 (S1 Fig).

Only IL-8 showed a tendency to correlate with the genotypes of rs2910164 in healthy individuals albeit no statistical significance is reached. In a dominant model, individuals homozygous for the G allele seem to have higher levels of circulating IL-8 compared with individuals.
bearing a C allele (GG 1.28±0.06 pg/mL vs CC +GC 1.10±0.05 pg/mL; p = 0.02; P corrected for false discovery rates (P_{FDR}) = 0.06) as shown in Fig 1. Similarly, these individuals seem to have increased levels of CCL5 (GG 73.01±30.14 pg/mL compared with individuals with a C allele, CC +GC 31.68±2.68 pg/mL; p = 0.19) (S2 Fig).

miRNA499-a modulates many inflammation-related signalling pathways such as TGFβ, TNFα and Toll-like receptors pathways. miR499-a has also been reported to regulate the expression of TNFα, IL-6, IL17RB, IL-18R and IL-23a [21]. We assayed IL-1β, IL-2, IL-6, IL-8, IL-17, IFNγ, CCL2 and TNFα to correlate with the different genotypes of MIR499A rs374644 as shown in S3 Fig. A tendency of plasma circulating levels of IL-8, IL-6 and IL-17 by MIR499A rs374644 genotypes is observed. Among the HC, the distribution of plasma circulating IL-8 according to the genotypes is statistically different in a codominant model (p = 0.01; P_{FDR} = 0.02) as shown in Fig 2. In a recessive model, individuals homozygous for the G allele seem to have higher levels of IL-8 (2.14±0.64 pg/mL) compared with individuals (AA +GA 1.18±0.04 pg/mL) bearing a A allele (p = 0.003; P_{FDR} = 0.01) (Fig 2).

GG homozygotes have higher levels of IL-8 (GG = 2.14±0.64 pg/mL versus AA = 1.18±0.04 pg/mL) (p = 0.005; P_{FDR} = 0.01) (Fig 3).

In a recessive model, bearers of the A allele among HC (AA+GA; 0.40±0.01 pg/mL) seem to have lower levels of IL-6 (p = 0.09) compared with GG homozygotes (0.64±0.18 pg/mL) (S4 Fig). Individuals homozygous for the G allele have higher levels of IL-6 compared with AA
homozygotes individuals (GG = 0.64±0.18 pg/mL versus AA = 0.41±0.01 pg/mL; p = 0.09) (Fig 3).

Similarly, MIR499A rs3746444 have a tendency on influencing the plasma circulating levels of IL-17 among the HC. GG homozygotes (5.48±1.97 pg/mL) have higher levels of IL-17

Fig 3. MIRNA499A rs3746444 effects on IL-6, IL-8, and IL-17 plasma levels between AA homozygous versus GG homozygous in healthy controls. Comparisons of circulating plasma levels of IL-6, IL-8 and IL-17 between the individuals AA and GG homozygous. The mean concentrations are displayed in picogram per millilitre (pg/mL) with standard error (SE) of mean. The means are represented by black bars, whereas SE are represented by error bars. *Corrected for Benjamini-Hochberg False Discovery Rate assuming three tests. p < 0.05 is statistically significant.
compared to heterozygotes GA (3.48±0.31 pg/mL) and homozygotes AA (3.98±0.16 pg/mL) (S4 Fig).

Genetic combinations of both variants revealed that individuals bearing three or four G alleles tend to correlate with higher plasma IL-8 (2.24 ± 0.48 pg/mL) and CCL5 (63.4± 29.2 pg/mL) as shown in S5 Fig.

Discussion

The manifestation of clinical symptoms in *Leishmania*-infected individuals depends on the capacity of the individual to have a fine regulation of the TH1 response to eliminate the parasite and avoid an exacerbation of expression of pro-inflammatory cytokines. Emerging evidence are showing that miRNAs regulate immune response [19,40,41].

In this study, we showed that male individuals homozygous for the C allele of *MIR146A* rs2910164 have 30% higher odds of developing *Lg*-CL compared with individuals bearing a G allele. The *MIR146A* rs2910164 C allele has been shown to be a risk factor for leprosy in Brazil and psoriasis in South African Indian [42,43]. Homozygous individuals for the C allele have increased risk of developing glioma and decreased survival [44]. A meta-analysis study on autoimmune diseases revealed that individuals bearing the C allele have increased risk of developing the disease [45].

Several studies have also reported that *MIR146A* rs2910164 is not a risk factor in several diseases. A meta-analysis on ischemic stroke showed that *MIR146A* rs2910164 is not associated with any risk occurrence of ischemic stroke [46]. A lack of association of *MIR146A* rs2910164 with the development of RA was also reported [47]. Another study cited that susceptibility to pulmonary tuberculosis is not influenced by the *MIR146A* rs2910164 [48]. However, a meta-analysis approach on psoriasis case-control studies with rs2910164 revealed that the CC genotype is correlated with decreased risk of psoriasis [28].

Conversely, other studies have reported that the G allele is a risk factor in several diseases. The G allele has been associated with pulmonary TB [49] and ankylosing spondylitis [50]. A meta-analysis study on cancer among Asian patients revealed that GG genotypes are associated with increased risk of cancer [51].

We also showed that in male individuals, the GG genotype of the *MIR499A* rs3746444 is associated with 50% increased odds of developing CL. The G allele confers 27% elevated odds of developing CL suggesting that the G allele may contribute to the development of *Lg*-CL.

A recent meta-analysis study showed that the *MIR499A* rs3746444 G allele is associated with high risk of developing breast cancer [52]. The allele G was also associated with hepatocellular carcinoma [53], and adenocarcinoma [54]. Other studies also showed that the G allele is associated with susceptibility to the development of bronchial asthma [20], RA [29,55], Behçet’s disease [56], ankylosing spondylitis [50], myocardial infarction [57] and coronary artery disease [58,59]. Immune response plays critical role in all these diseases. Of note, there is no study of both variants with protozoan infectious diseases to date.

The gender difference observed for both variants in this study may be due to either sexual hormonal interaction with the variant or the small sample size in the female group. Notably, we have 75% and 68% male individuals among patients with CL and HC, respectively. Interestingly, sex-based differences have been shown in *L. tropica* infected patients. *L. tropica*-infection manifests commonly in the form of CL. However, females present predominantly CL while male individuals are more inclined to develop viscerotropic leishmaniasis [60–62]. Furthermore, peripheral blood from patients with CL caused by *L. mexicana* stimulated or not with lipophosphoglycan demonstrated higher expression of IFNy and tumour necrosis factor alpha in females than in males [63]. An increased parasite growth has been observed when *L. tropica*.
mexicana promastigotes are treated with physiological doses of male dihydrotestosterone \[64,65\].

The \textit{MIR146A} is in the cytokine cluster 5q31 and the \textit{MIR146A} rs2910164 is situated in the stem region of the \textit{pre-miR-146a}. The change of guanine:uracil pair (G:U) to cytosine:uracil (C:U) in the stem structure affects the production of mature \textit{miR146-a} \[25,66\]. The G-allele is reported to be associated with high expression levels of mature \textit{miR146-a} while the C-allele with low expression \[25, 67\]. Other study cited that the C allele is correlated with higher \textit{miR146-a} and lower TNF\(\alpha\) expression from nerve biopsies of leprosy patients \[42\].

High expression of \textit{miR146-a} was also observed in specimens of healthy and tumour tissue from patients with gastric cancer bearing genotype CC compared to GG \[68\]. In Lupus, CC genotypes was correlated with high \textit{miR146-a} expression \[69\]. One study observed that the genotype CC is associated with increased expression of IRAK1 and TRAF6 \[70\]. \textit{miR146-a} regulates the IL-17 pathway in human keratinocytes, the first line of defense against pathogens in the skin, to restrain IL17-induced inflammation \[71\].

In this study, we did not observe any correlation of plasma levels of TNF\(\alpha\), IL-6, IL-1\(\beta\), IL-2, IL-17, IFN\(\gamma\) and CCL2 by \textit{MIR146A} rs2910164 genotypes neither among the patients with \textit{Lg}-CL nor the HC. However, individuals homozygous for the G allele seem to have higher levels of circulating IL-8 and CCL5 compared with individuals bearing a C allele.

Knockout \textit{MIR146A} \(-/-\) mice developed exaggerated pro-inflammatory response upon challenging with lipopolysaccharide due to chronic dysregulation of nuclear factor kappa-light-chain enhancer of activated B cells (NFkB) signalling \[74,75\]. \textit{miR146-a} is reported to target IRAK and TRAF-6 in the TLRs downstream pathway to downregulate the upregulation of NFkB leading to a decrease in the transcription of pro-inflammatory cytokines IL-6, IL-8, IL-1\(\beta\), and TNF\(\alpha\) \[74,76–80\]. A recent study showed that inhibition of \textit{miR146-a} resulted in a reduced secretion of IL-6 and IL-8 \[81\], suggesting that \textit{miR146-a} may downregulate an inflammatory reaction.

\textit{miRNA499-a} is involved in TLR-signalling \[82\]. Computational tool analysis suggests that \textit{miRNA499-a} may target IL-13 and IL-23 (microRNA.org). \textit{MIR}499A rs3746444 genotype GG has been suggested to correlate with high expression of \textit{miRNA499-a} \[83\]. Our data showed that individuals homozygous for the G allele have higher plasma IL-8 and a tendency to also have high IL-6, IL-17, and increased risk of developing \textit{Lg}-CL.

Indeed, in \textit{Lb}-infected patients with CL, high levels of IL-17 were observed \[84\] and peripheral blood from these patients released high IL-17 upon stimulation with soluble \textit{Leishmania} antigen \[85\]. \textit{L. major}-infected C57BL/6 mice developed larger lesion size with increased production of IL-17 and neutrophil infiltration at the site of lesion compared with mice treated with anti-IL-17 \[85\] while \textit{L. major}-infected BALB/c mice deficient for IL-17 develop smaller lesions \[86\], suggesting that IL-17 promotes lesions. High levels of IL-6 impaired the cytokine-enhanced antileishmanial activity of human macrophages by inhibiting IFN\(\gamma\) and TNF\(\alpha\) \[87\].

Our study has several limitations. Firstly, we considered that our healthy controls from the same endemic areas as the patients are exposed to the \textit{Lg}-vector and are infected but did not develop the disease. We did not perform DTH to ensure this despite most of the individuals included in the study are farming or agricultural workers. Secondly, the problem of multiple testing of many associations may result in spurious associations but may also discard a true association after correcting for multiple comparisons. Thirdly, our controls are slightly older
than our patients with CL and contain slightly more females. Stratification reduces the sample size.

Considering our data, we may hypothesize that MIR146A rs2910164 CC individuals are prone to develop Lg-CL due to an impairment neutrophil migration at the inoculation of the parasite by the sand fly bite. Additionally, these individuals have lower levels of CCL5. CCL5 has been shown to correlate with resistance to L. major infection in animal model [88]. Furthermore, our MIR499A rs3746444 data suggest that individuals with genotype GG are susceptible to develop Lg-CL by attracting too many neutrophils that may pass the parasite to macrophages leading to parasite persistence as they might be high producers of IL-8, IL-6 and IL-17.

 Altogether, this is the first study to date to investigate MIR146A rs2910164 and MIR499A rs3746444 in protozoan infectious diseases. Both variants are associated with the development of Lg-CL male individuals exposed to Lg-infection and correlate with plasma IL-8.

**Supporting information**

S1 Fig. Plasma levels of cytokines and chemokines according to the different genotypes of MIR146A rs2910164 in cases, controls and total individuals. (TIFF)

S2 Fig. MIR146A rs2910164 effects on circulating plasma CCL5 levels in healthy controls. (TIFF)

S3 Fig. Plasma levels of cytokines and chemokines according to the different genotypes of MIR499A rs3746444 in cases, controls and total individuals. (TIFF)

S4 Fig. MIR499A rs3746444 effects on circulating plasma IL-6 and IL-17 levels in healthy controls. (TIFF)

S5 Fig. Genetics combinations of genotypes by plasma cytokines. (TIFF)

S1 Table. Polymerase Chain Reactions protocols for the MIR146A rs2910164 and MIR499A rs3746444. (DOCX)

S2 Table. Mean values and standard error (SE) of the mean of plasma cytokines by the MIR146A rs2910164 and MIR499A rs3746444 genotypes according to inheritance models among the total individuals. (DOCX)

S3 Table. Genetics combinations of genotypes in patients with Cutaneous Leishmaniasis and Healthy Controls. (DOCX)

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References

1. World Health Organization. Available online: https://www.who.int/publications/i/item/who-who9525 (accessed on 20 October 2020).

2. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018; 392 (10151 ): 951–970. https://doi.org/10.1016/S0140-6736(18)31204-2 PMID: 30126638

3. Kevric I, Cappel MA, Keeling JH. New World and Old World Leishmaniasis Infections: A Practical Review. Dermatol Clin. 2015; 33(3): 579–93. https://doi.org/10.1016/j.det.2015.03.018 PMID: 26143433

4. Anversa L, Tiburcio MGS, Richini-Pereira VB, Ramirez LE. Human leishmaniasis in Brazil: A general review. Rev Assoc Med Bras (1992). 2018; 64(3): 281–289. https://doi.org/10.1590/1806-9282.64.03.281 PMID: 29641786

5. Benício E, Cordeiro M, Monteiro H, Moura MA, Oliveira C, Gadelha EP, et al. Sustained Presence of Cutaneous Leishmaniasis in Urban Manaus, the Largest Human Settlement in the Amazon. Am J Trop Med Hyg. 2015; 93(6):1208 –13. https://doi.org/10.4269/ajtmh.14-0164 PMID: 26483119

6. Gabriel A, Valério-Bolas A, Palma-Marques J, Mourata-Gonçalves P, Ruas P, Dias-Guerreiro T, et al. Cutaneous Leishmaniasis: The Complexity of Host’s Effective Immune Response against a Polymorphic Parasitic Disease. J Immunol Res. 2019; 2019: 2603730. https://doi.org/10.1155/2019/2603730 PMID: 31871953

7. Hurdayal R, Brombacher F. The role of IL-4 and IL-13 in cutaneous Leishmaniasis. Immunol Lett. 2014; 161(2): 179–83. https://doi.org/10.1016/j.imlet.2013.12.022 PMID: 24412597

8. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136(2): 215–33. https://doi.org/10.1016/j.cell.2009.04.002 PMID: 19167326

9. Gerald NS, Tan JC, McDowell MA. Characterization of microRNA expression profiles in Leishmania infected human phagocytes. Parasite Immunol. 2015; 37(1): 43–51. https://doi.org/10.1111/1877-0161.PBI.2015.00736 PMID: 25376316

10. Krol J, Łedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010; 11(9): 597–610. https://doi.org/10.1038/nrg2643 PMID: 20661255

11. Kabekkodu SP, Shukla V, Varghese VK, D’ Souza J, Chakrabarty S, Satyamoorthy K. Clustered miRNAs and their role in biology and diseases. Biol Rev Camb Philos Soc. 2018; 93(4): 1955–1986. https://doi.org/10.1111/brv.12426 PMID: 29797774

12. Tahamtan A, Teymoori-Rad M, Nakstad B, Salimi V. Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. Front Immunol. 2018; 9: 1377. https://doi.org/10.3389/fimmu.2018.01377 PMID: 29986529

13. Lima-Junior DS, Costa DL, Carregaro V, Cunha LD, Silva AL, Mineo TW, et al. Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to leishmania. Nat. Med. 2013; 19: 909–915. https://doi.org/10.1038/nm.3221 PMID: 23749230
14. Aoki JI, Muxel SM, Zampieri RA, Müller KE, Nerland AH, Floeter-Winter LM. Differential immune response modulation in early leishmaniasis amazonensis infection of balb/c and c57bl/6 macrophages based on transcriptome profiles. Sci. Rep. 2019; 9: 19841. https://doi.org/10.1038/s41598-019-56305-1 PMID: 31882833

15. Ghosh J, Bose M, Roy S, Bhattacharyya SN. Leishmania donovani targets dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. Cell Host Microbe 2013; 13: 277–288. https://doi.org/10.1016/j.chom.2013.02.005 PMID: 23498953

16. Muxel SM, Laranjeira-Silva MF, Zampieri RA, Floeter-Winter LM. Leishmania (leishmania) amazonensis induces macrophage miR-294 and mir-721 expression and modulates infection by targeting nos2 and l-arginine metabolism. Sci. Rep. 2017; 7: 44141. https://doi.org/10.1038/srep44141 PMID: 28276497

17. Guerfali FZ, Laouini D, Guizani-Tabbane L, Ottones F, Ben-Aissa K, Benchallal A, et al. Simultaneous gene expression profiling in human macrophages infected with leishmania major parasites using sace. BMC Genom. 2008; 9: 238. https://doi.org/10.1186/s12867-008-0048-0 PMID: 18495030

18. Das S, Mukherjee S, Ali N. Super enhancer-mediated transcription of mir146a-5p drives M2 polarization during Leishmania donovani infection. PLoS Pathog. 2021; 17(2): e1009343 https://doi.org/10.1371/journal.ppat.1009343 PMID: 33630975

19. Saba R, Sorensen DL, Booth SA. MicroRNA-146a: A Dominant, Negative Regulator of the Innate Immune Response. Front Immunol. 2014; 5: 578. https://doi.org/10.3389/fimmu.2014.00578 PMID: 25484682

20. Toraih EA, Hussein MH, Al Ageel E, Riad E, AbdAllah NB, Helal GM, Fawzy MS. Structure and functional impact of seed region variant in MIR-499 gene family in bronchial asthma. Respir Res. 2017; 18 (1): 169. https://doi.org/10.1186/s12931-017-0648-0 PMID: 28886711

21. Hashemi M, Eskandari-Nasab E, Zakeri Z, et al. Association of pre-miRNA-146a rs2910164 and pre-miRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis. Mol Med Rep. 2013; 7: 287–91. https://doi.org/10.3892/mmr.2012.1776 PMID: 23138379

22. Kroliczewski J, Sobolewska A, Lejnowski D, Collawn JF, Bartoszewski R. microRNA single nucleotide polymorphism influences on microRNA biogenesis and mRNA target specificity. Gene. 2018; 640: 66–72. https://doi.org/10.1016/j.gene.2017.10.021 PMID: 29302146

23. Nicoloso MS, Sun H, Spizzo R, et al. Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. Cancer Res. 2010; 70(7): 2789–2798. https://doi.org/10.1158/0008-5472.CAN-09-3541 PMID: 20332227

24. Moszyńska A, Gebert M, Collawn JF, Bartoszewski R. SNPs in microRNA target sites and their potential role in human disease. Open Biol. 2017; 7(4): 170019. https://doi.org/10.1098/rsob.170019 PMID: 28381629

25. Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. Proc Natl Acad Sci U S A. 2008; 105(20): 7269–7274. https://doi.org/10.1073/pnas.0802682105 PMID: 18473871

26. Zhang H, Zhang Y, Yan W, Wang W, Zhao X, Ma X, et al. Association between three functional microRNA polymorphisms (miR-499 rs3746444, miR-196a rs11614913 and miR-146a rs2910164) and breast cancer risk: a meta-analysis. Oncotarget. 2017; 8: 393–407. https://doi.org/10.18632/oncotarget.13426 PMID: 27880723

27. Mi Y, Ren K, Zou J, Bai Y, Zhang L, Zuo L, et al. The Association Between Three Genetic Variants in MicroRNAs (Rs11614913, Rs2910164, Rs3746444) and Prostate Cancer Risk. Cell Physiol Biochem. 2018; 48: 149–157. https://doi.org/10.1007/s00975-018-1225-1 PMID: 30001553

28. Gong HB, Zhang SL, Wu XJ, Pu XM, Kang XJ. Association of rs2910164 polymorphism in MiR-146a gene with psoriasis susceptibility: A meta-analysis. Medicine (Baltimore). 2019; 98(6): e14401. https://doi.org/10.1097/MD.0000000000014401 PMID: 30732186

29. Fattah SA, Ghattas MH, Saleh SM, Abo-Elfatty DM. Pre-micro RNA-499 Gene Polymorphism rs3746444 T>C is Associated with Susceptibility to Rheumatoid Arthritis in Egyptian Population. Indian J Clin Biochem. 2018; 33(1): 96–101. https://doi.org/10.1007/s12291-017-0652-7 PMID: 29371777

30. de Araújo Santos FJ, da Silva LS, Júnior JDES, Ramos de Mesquita TG, de Souza MLG, de Andrade Júnior MC, et al. Single nucleotide polymorphisms of the genes IL-2, IL-2RB, and JAK3 in patients with cutaneous leishmaniasis caused by Leishmania (V.) guyanensis in Manaus, Amazonas, Brazil. PLoS One. 2019; 14(8): e0220572. https://doi.org/10.1371/journal.pone.0220572 PMID: 31393896

31. da Silva LS, Santo JDE, de Mesquita TGR, Santos VAM, de Souza JL, de Araújo FJ, et al. IL-23R variant rs11805303 is associated with susceptibility to the development of cutaneous leishmaniasis in Leishmania guyanensis-infected individuals. J Infect Dis. 2021;jiab320. https://doi.org/10.1093/infdis/jiab320 PMID: 34139757
32. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the minixeon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003; 41(7): 3147–53. https://doi.org/10.1128/JCM.41.7.3147-3153.2003 PMID: 12843055

33. Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, De Doncker S, Arevalo J, et al. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J Clin Microbiol. 2004; 42(5): 2284–7. https://doi.org/10.1128/JCM.42.5.2294-2297.2004 PMID: 15131217

34. da Silva GAV, de Mesquita TGR, de Souza Encarnação HV, do Espirito Santo Junior J, da Costa Sabino K, de Aguilar Neres I, et al. A polymorphism in the *IL1B* gene (rs16944 T/C) is associated with cutaneous leishmaniasis caused by *Leishmania guyanensis* and plasma cytokine interleukin receptor antagonist. Cytokine. 2019; 123: 154788. https://doi.org/10.1016/j.cyto.2019.154788 PMID: 31357078

35. da Silva GAV, Mesquita TG, Souza VC, Junior JDES, Gomes de Souza ML, Talhari AC, et al. A Single Haplotype of *IFNG* Correlating with Low Circulating Levels of Interferon-γ Is Associated with Susceptibility to Cutaneous Leishmaniasis Caused by Leishmania guyanensis. Clin Infect Dis. 2020; 71(2): 274–281. https://doi.org/10.1093/cid/ciaz110 PMID: 31722386

36. Sambrook J., Fritsch E.F., Maniati T., Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, 1989.

37. Bhaumik D, Scott GK, Schokrpu r S, Patil CK, Campisi J, Benz CC. Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. Oncogene. 2008; 27: 5643–5647. https://doi.org/10.1038/onc.2008.171 PMID: 18504431

38. Zhang QB, Qing YF, Yin CC, Zhou L, Liu XS, Mi QS, et al. Mice with miR-146a deficiency develop severe gouty arthritis via dysregulation of TRAF 6, IRAK 1 and NALP3 inflammasome. Arthritis Res Ther. 2018; 20(1): 45. https://doi.org/10.1186/s13075-018-1546-7 PMID: 29544526

39. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. J Immunol. 2008; 180: 5689–5698. https://doi.org/10.4049/jimmunol.180.8.5689 PMID: 18390754

40. Lu LF, Gasteiger G, Yu IS, Chaudhry A, Hsin JP, Lu Y, et al. A Single miRNA-mRNA Interaction Affects the Immune Response in a Context- and Cell-Type-Specific Manner. Immunity. 2015; 43(1): 52–64. https://doi.org/10.1016/j.immuni.2015.04.022 PMID: 26163372

41. Forster SC, Tate MD, Hertzog PJ. MicroRNA as Type I Interferon-Regulated Transcripts and Modulators of the Innate Immune Response. Front Immunol. 2015; 6: 334. https://doi.org/10.3389/fimmu.2015.00334 PMID: 26217335

42. Cezar-de-Melo PF, Toledo-Pinto TG, Marques CS, Arnez LE, Cardoso CC, Guerreiro LT, et al. Pre-miR-146a (rs2910164 G>C) single nucleotide polymorphism is genetically and functionally associated with leprosy. PLoS Negl Trop Dis. 2014; 8(9): e3099. https://doi.org/10.1371/journal.pntd.0003099 PMID: 25187983

43. Maharaj AB, Naidoo P, Ghazi T, Abdulk NS, Dhani S, Docrat TF, et al. MiR-146a G/C rs2910164 variation in South African Indian and Caucasian patients with psoriatic arthritis. BMC Med Genet. 2011; 20(1): 48. https://doi.org/10.1186/1471-2350-12-48 PMID: 22407689

44. Forstner T, Hauck M, Hertzog PJ. MicroRNA Polymorphisms and Susceptibility to Tuberculosis. Front Immunol. 2015; 6: 334. https://doi.org/10.3389/fimmu.2015.00334 PMID: 26217335

45. Li C, Fu W, Zhang Y, Zhou L, Mao Z, Lv W, et al. Meta-analysis of microRNA-146a rs2910164 G>C polymorphism association with autoimmune diseases susceptibility, an update based on 24 studies. PLoS One. 2015; 10(4): e0121918. https://doi.org/10.1371/journal.pone.0121918 PMID: 25830862

46. Li CX, Weng H, Zheng J, Feng ZH, Ou J., Liao WJ. Association Between MicroRNAs Polymorphisms and Risk of Ischemic Stroke: A Meta-Analysis in Chinese Individuals. Front Aging Neurosci. 2018; 10: 82. https://doi.org/10.3389/fnagi.2018.00082 PMID: 29643803

47. Yang B, Zhang JL, Shi YY, Li DD, Chen J, Huang ZC, et al. Association study of single nucleotide polymorphisms in pre-miRNA and rheumatoid arthritis in a Han Chinese population. Mol Biol Rep. 2011; 38 (8): 4913–9. https://doi.org/10.1007/s11033-010-0633-x PMID: 21181275

48. Naderi M, Hashemi M, Khorgami P, Koshki M, Ebrahimi M, Amininia S, et al. Lack of Association between miRNA-146a rs2910164 and miRNA-499 rs3746444 Gene Polymorphisms and Susceptibility to Pulmonary Tuberculosis. Int J Mol Cell Med. Winter 2015; 4(1): 40–5. PMID: 25815281

49. Li D, Wang T, Song X, Qucou M, Yang B, Zhang J, et al. Genetic study of two single nucleotide polymorphisms within corresponding miRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population. Hum Immunol. 2011; 72(7): 598–602. https://doi.org/10.1016/j.humimm.2011.03.004 PMID: 21524676
50. Xu HY, Wang ZY, Chen JF, Wang TY, Wang LL, Tang LL, et al. Association between ankylosing spondylitis and the miR-146a and miR-499 polymorphisms. PLoS One. 2015; 10: e0122055. https://doi.org/10.1371/journal.pone.0122055 PMID: 25836258
51. Wang J, Bi J, Liu X, Li K, Di J, Wang B. Has-miR-146a polymorphism (rs2910164) and cancer risk: a meta-analysis of 19 case-control studies. Mol Biol Rep. 2012; 39(4): 4571–9. https://doi.org/10.1007/s11033-011-1247-7 PMID: 21947843
52. Tan SC, Lim PY, Fang J, Mokhtar FFM, Jamal R. Association between MIR499 A rs3746444 polymorphism and breast cancer susceptibility: a meta-analysis. Sci Rep. 2020; 10(1): 3508. https://doi.org/10.1038/s41598-020-60442-3 PMID: 32103099
53. Chu YH, Hsieh MJ, Chiou HL, Liou YS, Yang CC, Yang SF, et al. MicroRNA gene polymorphisms and environmental factors increase patient susceptibility to hepatocellular carcinoma. PLoS ONE. 2014; 9(2): e89930. https://doi.org/10.1371/journal.pone.0089930 PMID: 24587132
54. Tang W, Wang Y, Pan H, Qiu H, Chen S. Association of miRNA-499 rs3746444 A>G variants with adenocarcinoma of esophago gastric junction (AEG) risk and lymph node status. Onco Targets Ther. 2019; 12: 6245–6252. https://doi.org/10.2147/OTT.S209013 PMID: 31496728
55. Toraih EA, Ismail NM, Toraih AA, Hussein MH, Fawzy MS. Precursor miR-499a variant but not miR-196a2 is associated with rheumatoid arthritis susceptibility in an Egyptian population. Mol Diagn Ther. 2016; 20: 279–95. https://doi.org/10.1007/s40291-016-0194-3 PMID: 27002721
56. Oner T, Yenmis G, Tomburturk K, Cam C, Kucuk OS, Yakicier MC, et al. Association of pre-miRNA-499 rs3746444 and pre-miRNA-146ª rs2910164 polymorphisms and susceptibility to Behcet's disease. Genet Test Mol Biomarkers. 2015; 19: 424–30. https://doi.org/10.1089/gtmb.2015.0016 PMID: 26053525
57. Chen C, Hong H, Chen L, Shi X, Chen Y, Weng Q. Association of microRNA polymorphisms with the risk of myocardial infarction in a Chinese population. Tohoku J Exp Med. 2014; 233(2): 89–94. https://doi.org/10.1620/tjem.233.89 PMID: 24850191
58. Zhi H, Wang L, Ma G, Ye X, Yu X, Zhu Y, et al. Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease. Clin Res Cardiol. 2012; 101: 289–96. https://doi.org/10.1007/s00392-011-0391-3 PMID: 22159951
59. Fawzy MS, Toraih EA, Hamed EO, Hussein MH, Ismail HM. Association of MIR-499 expression and seed region variant (rs3746444) with cardiovascular disease in Egyptian patients. Acta Cardiol. 2018; 73(2): 131–140. https://doi.org/10.1080/00015385.2017.1351243 PMID: 28786773
60. Reithinger R, Mohsen M, Aadil K, Sidiqi M, Erasmus P, Coleman PG. Anthropogenic cutaneous leishmaniasis, Kabul, Afghanistan. Emerg Infect Dis. 2003; 9(6): 727–9. https://doi.org/10.3201/eid9006.030026 PMID: 12781016
61. Abidoli A, Maspi N, Ghaffarifar F, Nasiri V. Viscerotrophic leishmaniasis: a systematic review of the case reports to highlight spectrum of the infection in endemic countries. Parasitology Open. 2018; 4: 1–14.
62. Cañeda-Guzmán IC, Salaza-Suazo N, Fernández-Figueroa EA, Carrada-Figueroa G, Aguirre-García M, Becker I. NK cell activity differs between patients with localized and diffuse cutaneous leishmaniasis infected with *Leishmania mexicana*: a comparative study of TLRs and cytokines. PLoS One. 2014; 9(11): e112410. https://doi.org/10.1371/journal.pone.0112410 PMID: 25397678
63. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol. 2016; 16(10): 626–38. https://doi.org/10.1038/nri.2016.90 PMID: 27546235
64. Sánchez-García L, Wilkins-Rodriguez A, Salaza-Suazo N, Morales-Montor J, Becker I. Dihydrotestosterone enhances growth and infectivity of *Leishmania mexicana*. Parasite Immunol. 2018; 40(3). https://doi.org/10.1111/pim.12512 PMID: 28272044
65. Stickel N, Hanke K, Marschner D, Prinz G, Köhler M, Melchinger W, et al. MicroRNA-146a reduces MHC-II expression via targeting JAK/STAT signaling in dendritic cells after stem cell transplantation. Leukemia. 2017; 31(12): 2732–2741. https://doi.org/10.1038/leu.2017.137 PMID: 28484267
66. Xu T, Zhu Y, Wei QK, Yuan Y, Zhou F, Ge YY, et al. A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma. Carcinogenesis. 2008; 29(11): 2126–31. https://doi.org/10.1093/carcin/bgn195 PMID: 18711148
67. Kogo R, Mimori K, Tanaka F, Komune S, Mori M. Clinical significance of miR-146a in gastric cancer cases. Clin Cancer Res. 2011; 17(13): 4277–84. https://doi.org/10.1158/1078-0432.CCR-10-2866 PMID: 21632853
69. Lofgren SE, Frostell J, Truedsson L, Pons-Estel BA, D’Alfonso S, Witte T, et al. Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene. Genes Immun. 2012; 13(3): 268–74. https://doi.org/10.1038/genes.2011.84 PMID: 22218224

70. Shao Y, Li J, Cai Y, Xie Y, Ma G, Li Y, et al. The functional polymorphisms of miR-146a are associated with susceptibility to severe sepsis in the Chinese population. Mediators Inflamm. 2014; 2014: 916202. https://doi.org/10.1155/2014/916202 PMID: 24701036

71. Srivastava A, Nikamo P, Lohcharoenkal W, Li D, Meisgen F, Xu Landén N, et al. MicroRNA-146a suppresses IL-17-mediated skin inflammation and is genetically associated with psoriasis. J Allergy Clin Immunol. 2017; 139(2): 550–561. https://doi.org/10.1016/j.jaci.2016.07.025 PMID: 27568078

72. Meisgen F, Xu Landén N, Wang A, Réthi B, Bouez C, Zuccolo M, et al. MiR-146A Negatively Regulates TLR2-Induced Inflammatory Responses in Keratinocytes. J. Invest. Dermatol. 2014; 134: 1931–1940. https://doi.org/10.1038/jid.2014.89 PMID: 24670381

73. Nimlsarkar P, Ingale P, Singh S. Systems Studies Uncover miR-146a as a Target in Leishmania major Infection Model. ACS Omega. 2020; 5(21): 12516–12526. https://doi.org/10.1021/acsomega.0c01502 PMID: 32548436

74. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. J Exp Med. 2011; 208(6): 1189–201. https://doi.org/10.1084/jem.20101823 PMID: 21555486

75. Zhao JL, Rao DS, Boldin MP, Taganov KD, O’Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A. 2011; 108(22): 9184–9. https://doi.org/10.1073/pnas.1105398108 PMID: 21576471

76. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006; 103: 12481–12486. https://doi.org/10.1073/pnas.0605298103 PMID: 16855212

77. Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH, Chan EK. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis Res Ther. 2008; 10(4): R101. https://doi.org/10.1186/ar2493 PMID: 18759964

78. Nahid MA, Pauley KM, Satoh M, Chan EK. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN AUTOIMMUNITY. J Biol Chem. 2009; 284(50): 34590–9. https://doi.org/10.1074/jbc.M109.056317 PMID: 19840932

79. Hou J, Wang P, Lin L, Liu X, Ma F, An H, et al. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol. 2009; 183(3): 2150–6. https://doi.org/10.4049/jimmunol.0900707 PMID: 19596990

80. Tang Y, Luo X, Cui H, Ni X, Yuan M, Guo Y, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis Rheum. 2009; 60(4): 1065–75. https://doi.org/10.1002/art.24436 PMID: 19333922

81. Pfalffer D, Roßmannith E, Lang I, Falkenhagen D. miR-146a, miR-146b, and miR-155 increase expression of IL-6 and IL-8 and support HSP10 in an In vitro sepsis model. PLoS One. 2017; 12(6): e0179850. https://doi.org/10.1371/journal.pone.0179850 PMID: 28662100

82. Liew FY, Patel M, Xu D. Toll-like receptor 2 signalling and inflammation. Ann Rheum Dis. 2005; 64 Suppl 4: iv104–105. https://doi.org/10.1136/ard.2005.042515 PMID: 16239376

83. Alishawi AA, Shafi G, Hasan TN, Syed NA, Al-Hazzani AA, Alsaif MA, et al. Differential expression profile and genetic variants of microRNAs sequences in breast cancer patients. PLoS One. 2012; 7(2): e30049. https://doi.org/10.1371/journal.pone.0030049 PMID: 22363415

84. Bacellar O, Faria D, Nascimento M, Cardoso TM, Gollob KJ, Dutra WO, et al. Interleukin 17 production among patients with American cutaneous leishmaniasis. J Infect Dis. 2009; 200(1): 75–78. https://doi.org/10.1086/599380 PMID: 19476435

85. Gonzalez-Lombana G, Gimblet B, Bacellar O, Oliveira WW, Passos S, Carvalho LP, et al. IL-17 mediates immunopathology in the absence of IL-10 following Leishmania major infection. PLoS Pathog. 2013; 9(3): e1003243. https://doi.org/10.1371/journal.ppat.1003243 PMID: 23552526

86. Lopez Kostka S, Dinges S, Griewank K, Iwakura Y, Udey MC, von Stebut E. IL-17 promotes progression of cutaneous leishmaniasis in susceptible mice. J Immunol. 2009; 182(5): 3039–46. https://doi.org/10.4049/jimmunol.0713598 PMID: 19234200

87. Hatzigeorgiou DE, He S, Sobel J, Grabstein KH, Hafner A, Ho JL. IL-6 down-modulates the cytokine-enhanced antileishmanial activity in human macrophages. J Immunol. 1993; 151(7): 3682–92. PMID: 8397259

88. Santiago HC, Oliveira CF, Santiago L, Ferraz FO, de Souza D G, de-Freitas LA, et al. Involvement of the chemokine RANTES (CCL5) in resistance to experimental infection with Leishmania major. Infect Immun. 2004; 72(8): 4918–23. https://doi.org/10.1128/IAI.72.8.4918-4923.2004 PMID: 15271961