

Contrasting Genetic Effects Within *STAT4* Define Susceptibility and Protection in *Leishmania guyanensis* Cutaneous Leishmaniasis

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Background. *Leishmania guyanensis*-associated cutaneous leishmaniasis (CL) is endemic in the Brazilian Amazonas. Despite similar exposure to the pathogen in endemic areas, only a subset of individuals develops disease, suggesting host-genetic control. *STAT4* triggers interleukin 12 (IL-12)-dependent Th1 differentiation and interferon- γ (IFN- γ) production, essential for macrophage-mediated parasite clearance.

Methods. We performed a case-control study of 802 patients with *L. guyanensis* CL and 842 endemic controls (ECs). Eleven *STAT4* variants (10 SNVs and 1 insertion/deletion) were genotyped. Associations were assessed using logistic regression adjusted for age and sex across multiple genetic models, with haplotype analysis adjusted for age and sex via omnibus and likelihood ratio tests.

Results. Two SNVs exerted contrasting associations: the rs1031509 T allele increased susceptibility (adjusted odds ratio [OR_{adj}] = 1.5; 95% CI, 1.3–1.7; adjusted *P* value, Bonferroni correction [Padj.B] = 1.3×10^{-5}), while the rs6434435 A allele conferred protection (OR_{adj} = 0.56; 95% CI, .43–.68; Padj.B = 2.6×10^{-8}). Omnibus haplotype analysis revealed significant global differences in haplotype distribution between *L. guyanensis* CL and ECs (omnibus unadjusted $\chi^2 = 55.65$, df = 13, *P* = 3.11×10^{-7} ; likelihood ratio test $\chi^2 = 67.81$, df = 13, *P* = 2.02×10^{-9}), with a specific high-risk combination (AIGAAAGCTGC) associated with a 9-fold increase in susceptibility (adjusted OR = 9.12; 95% CI, 4.22–19.70; *P* = 1.9×10^{-8} ; FDR *q* = 2.4×10^{-7}). Genotype-cytokines analyses revealed that carriers of the protected rs6434435 A allele had significantly higher TNF- α plasma levels under dominant model (+40.2%, FDR adjusted *P* = .026) and additive model, with a nominal trend toward elevated IFN- γ .

Conclusions. Haplotypic variation at this locus significantly shapes host inflammatory responses, highlighting *STAT4* as a key immunogenetic determinant of resistance to *Leishmania* infection.

Keywords. cutaneous leishmaniasis; *STAT4*; host genetic susceptibility; haplotype analysis; *Leishmania guyanensis*.

Leishmaniasis remains one of the world's most neglected tropical diseases, with 99 countries reporting endemic transmission in 2022. Globally, an estimated 205 986 new cases of cutaneous leishmaniasis (CL) and 12 842 cases of visceral leishmaniasis were reported that year [1]. In Brazil, *Leishmania guyanensis* is the predominant causative agent of CL in the state of Amazonas and is transmitted by the sand fly *Lutzomyia*

umbratilis. Despite similar socioeconomic and epidemiological exposures within endemic regions, only a subset of individuals develops clinical disease, underscoring the critical role of host genetic factors in disease susceptibility.

In regions endemic for *Leishmania braziliensis*, some individuals develop a positive delayed-type hypersensitivity response to *Leishmania* antigen without prior clinical disease, indicating subclinical or asymptomatic infection [2]. Family-based and population studies demonstrate a heritable susceptibility to leishmaniasis, with familial clustering of *L. braziliensis* CL and *L. braziliensis* mucosal leishmaniasis [3]. A major recessive gene has been proposed to influence susceptibility to *L. braziliensis* CL among migrants to endemic regions in Bolivia [4], and studies in Peru indicate that host genetics influences susceptibility to *Leishmania peruviana* CL, particularly early-onset cases [5].

Experimental infections in mice with *Leishmania major* demonstrate that resistant C57BL/6 strains elicit a Th-1 biased

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immune response involving interleukin 12 (IL-12), interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), whereas susceptible BALB/c mice develop a Th2-skewed response, resulting in uncontrolled parasite proliferation [6]. The balance between Th1 and Th2 immune responses critically influences disease outcomes. Th1 cytokines, such as IFN- γ and TNF- α , promote parasite clearance by activating macrophages and inducing nitric oxide production. Conversely, Th2 cytokines, including IL-4 and IL-10, facilitate parasite persistence.

STAT4, a member of the STAT transcription factor family, is activated by IL-12 and type 1 IFN to induce IFN- γ production, thereby promoting Th1 differentiation. Additionally, STAT4 activation by IL-23 drives Th17 differentiation [7–10]. STAT4 activation enhances expression of IFN- γ (encoded by *IFNG* gene) and T-bet (encoded by *TBX21* gene), which are key regulators of Th1 cell commitment. IL-12 signaling through STAT4 protein stabilizes T-bet, further promoting Th1 differentiation [11, 12]. T-bet initiates transcription of *IFNG*, the canonical Th1 cell cytokine, while repressing Th2 cytokines expression [13]. Furthermore, IFN- γ signaling enhances Th1 differentiation via STAT1-mediated gene transcription, which together with IL-12–driven STAT4 signaling sustains T-bet and IFN- γ expression [14, 15].

HiChIP analyses have demonstrated chromatin interactions within the *STAT4* locus, including intronic regulatory regions of *STAT4* that overlap genetic variants such as rs3821236, linking distal enhancers to the *STAT4* and *STAT1* promoters, implying potential transcriptional regulation [16]. Several *STAT4* variants have been associated with various human diseases [17].

Given the central role of the STAT4 protein in IL-12/IFN- γ signaling and Th1 effector differentiation, we hypothesized that *STAT4* variants might modulate susceptibility to *L. guyanensis* CL by altering cytokine responses. To test this hypothesis, we analyzed 11 *STAT4* variants spanning the gene in a large cohort from Amazonas, combining genetic association studies with cytokine profile.

METHODS

Study Population

The study included 802 primary treatment-naïve patients with *L. guyanensis* CL with recent active lesions (≤ 6 lesions, mostly single, with 3–5 weeks of evolution) and 842 endemic controls (ECs) without any history or clinical signs of *Leishmania* infection. All participants were recruited as unrelated individuals based on structured interviews from the same periurban endemic region of Manaus, Amazonas, and matched for socioeconomic and environmental exposure. All ECs underwent clinical evaluation to exclude prior or active leishmaniasis [18–23]. Participants were human immunodeficiency virus (HIV) negative with no chronic or immunosuppressive conditions and had

no history of cardiovascular, renal, or diabetic disease. Pregnant women were excluded. The research was conducted at the Fundação de Medicina Tropical Dr Heitor Vieira Dourado, Manaus, a regional reference center for tropical diseases. Ethical approval was under Certificado de Apresentação para Apreciação Ética 09995212.0.0000.0005. The study adhered to the STREGA guidelines for genetic association studies.

Sample Size Estimation

Sample size was estimated using the Harvard Genetic Power calculator. Assuming a 5% minor allele frequency, 5% disease prevalence, complete linkage disequilibrium (LD) between the marker and the trait, and 80% statistical power (type 1 error of 5%), 789 cases and 789 controls were required to detect an odds ratio of 1.5 (heterozygotes) and 2.0 (homozygotes).

Leishmania Species Identification

CL was confirmed by microscopic detection of parasites in Giemsa-stained lesion scrapings. *Leishmania* (*Viannia*)-infection and species identification were further confirmed by polymerase chain reaction (PCR) using DNA extracted from lesion biopsies, following established protocols [24, 25]. Species identification was performed by amplification and sequencing of heat shock protein 70 gene (233 bp) and mini exon genes (227 bp) on an ABI 3130XL Genetic Analyzer [22].

DNA Purification and Genotyping of *STAT4* Variants by PCR-RFLP

Peripheral blood (5 mL) was collected in Ethylenediaminetetraacetic acid-coated Vacutainer tubes (Becton Dickinson). Genomic DNA was extracted using proteinase K digestion followed by salting-out [26]. Genotyping was performed by PCR restriction fragment length polymorphism (PCR-RFLP) analysis. Primer sequences, PCR conditions, restriction enzymes, and expected fragment sizes are detailed in [Supplementary Table 1](#). Genotyping accuracy was validated by duplicate blind assays and direct sequencing of 5% of samples.

Plasma Cytokines Measurement

Plasma cytokines concentrations in 5 μ L were quantified in 400 patients with *L. guyanensis* CL and 400 ECs using the Human Cytokine Grp I Panel 27-Plex kit (Bio-Rad) on a Bio-plex 200 Protein Array System (Luminex Corporation).

Statistical Analysis

Hardy-Weinberg equilibrium (HWE) was assessed separately in patients with *L. guyanensis* CL and ECs using the χ^2 test. Variants with HWE $P < .05$ in ECs were prespecified for exclusion; none met this criterion. Single-variant associations were tested using logistic regression adjusted for age and sex under codominant, dominant, recessive, overdominant, and additive models (R version 4.3.1; SNPassoc), applying Bonferroni correction for 55 comparisons (significance threshold $P < 9.1 \times 10^{-4}$).

LD and haplotypes were inferred using Haploview version 4.2. Haplotypes were initially defined without excluding low-frequency combinations. For regression analyses, haplotypes with overall frequency < 1% were pooled into a single “other” category to preserve structure while avoiding unstable estimates. Global haplotype association was assessed using an unadjusted χ^2 omnibus test and age- and sex-adjusted haplotype logistic regression with likelihood ratio testing. Cytokine concentrations were natural-log transformed and analyzed using generalized linear models to estimate allele dosage effects, with false discovery rate (FDR) correction for pairwise comparisons.

Table 1 . Baseline Characteristics of the Study Population

Characteristic	<i>Leishmania guyanensis</i> CL (n = 802)	ECs (n = 842)	PValue
Age, y, mean $\pm$ SD	35.0 $\pm$ 13.8	41.1 $\pm$ 17.4	< .001
Age, y, median (IQR)	33 (24–45)	40 (27–55)	
Men, n (%)	605 (75.4)	578 (68.6)	.002
Women, n (%)	197 (24.6)	264 (31.4)	
Age in men, y, mean $\pm$ SD	34.4 $\pm$ 13.3	41.6 $\pm$ 17.7	< .001
Age in women, y, mean $\pm$ SD	37.1 $\pm$ 15.1	40.3 $\pm$ 16.9	.036

P values were calculated using Student *t* test for continuous variables and χ^2 test for categorical variables. Sex-stratified age comparisons were performed using Student *t* test. All tests were 2-sided.

Abbreviations: CL, cutaneous leishmaniasis; EC, endemic control.

RESULTS

Basic Characteristics of the Study Population

A total of 802 patients with *L. guyanensis* CL and 842 ECs were included (Table 1). Patients with *L. guyanensis* CL were significantly younger than ECs (mean, 35.0 [SD 13.8] vs 41.1 [SD 17.4] years; $P < .001$). Median age was also lower among patients with *L. guyanensis* CL (33; interquartile range [IQR], 24–45 years) than ECs (40; IQR, 27–55 years). When stratified by sex, male patients with *L. guyanensis* CL were younger than male controls (mean, 34.4 [SD 13.3] vs 41.6 [SD 17.7] years; $P = < .001$), and female cases were likewise younger than female ECs (mean, 37.1 [SD 15.1] vs 40.3 [SD 16.9] years; $P = .036$). Approximately 80% of both patients with *L. guyanensis* CL and ECs were agricultural or rural workers, reflecting similar occupational exposure.

Minor Allele Frequencies and Hardy-Weinberg Equilibrium

The genomic positions of the 11 *STAT4* variants are shown Figure 1A. *STAT4* is located on the minus strand of chromosome 2, spanning approximately 220 kilobases. All variants conformed to HWE in ECs and patients with *L. guyanensis* CL ($P > .05$). Minor allele frequencies ranged from 0.14 to 0.48 (Supplementary Table 2). Given the admixture structure of the study population (50%–60% Native American, 40%–50% European, and approximately 10% African ancestry

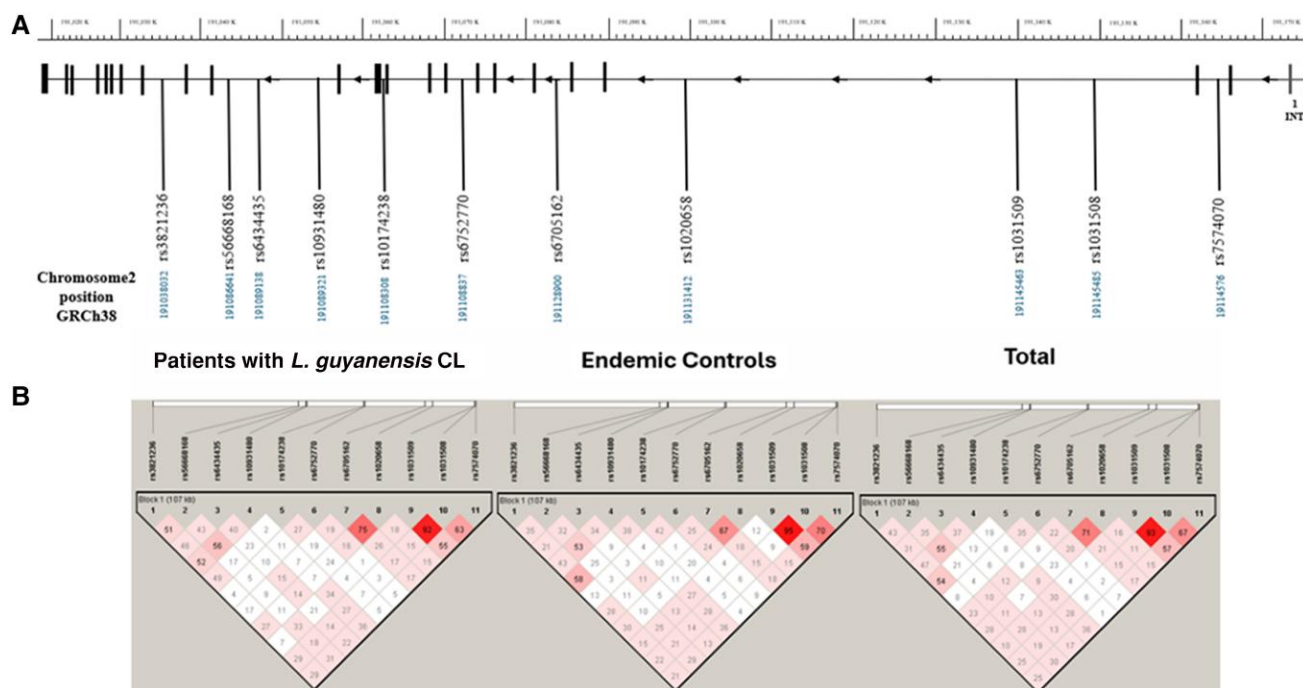


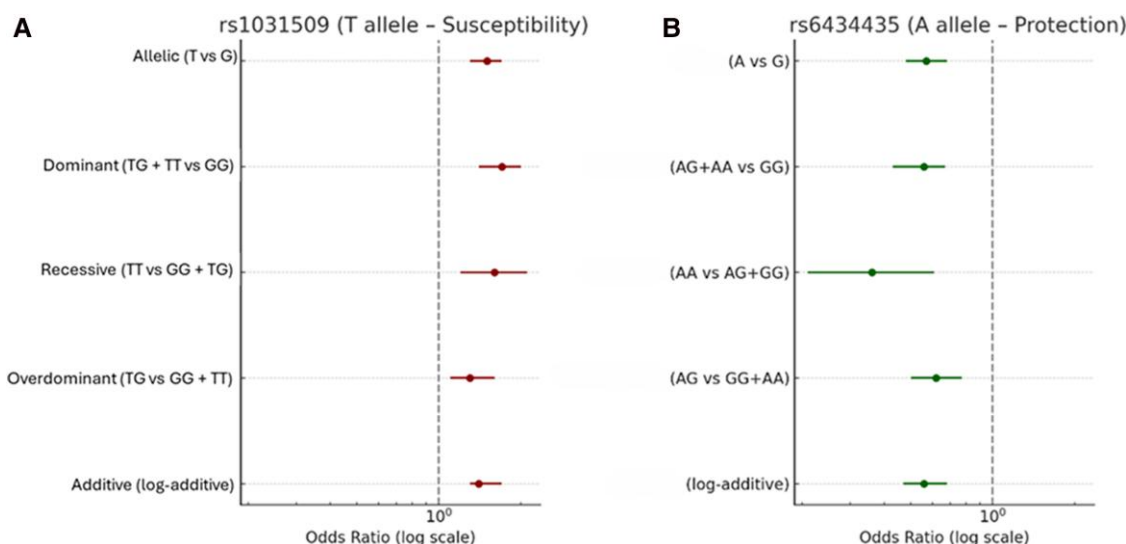
Figure 1. Structure of the *STAT4* gene and linkage disequilibrium patterns of the eleven *STAT4* variants analyzed. (A) Schematic representation of the *STAT4* gene modified from the National library of medicine (<https://ncbi.nlm.nih.gov>), showing the position of the eleven variants according to GRCh38 assembly. (B) Pairwise linkage disequilibrium among *STAT4* variants in Lg-CL patients, endemic controls and the combined dataset (Total). LD is expressed in *D'* values. Abbreviations: CL, cutaneous leishmaniasis; LD, linkage disequilibrium.

Table 2. Genotype Frequencies and Genetic Model Analyses for rs1031509 and rs6434435 in Patients With *Leishmania guyanensis* CL and Endemic Controls

	<i>Leishmania guyanensis</i> CL, No. (%)	ECs, No. (%)	Comparisons	ORadj (95% CI)	Padj	Padj-B	AIC
rs1031509	n = 802	n = 842					
G/G	274 (34)	389 (46)		1.00	1.6×10^{-6}	8.8×10^{-5}	2192
T/G	382 (48)	351 (42)	GG vs TG	1.5 (1.3–1.9)	6.5×10^{-4}	.036	...
T/T	146 (18)	102 (12)	GG vs TT	2.0 (1.5–2.7)	3.5×10^{-6}	2.0×10^{-4}	...
log-Additive							
0,1,2	802 (49)	842 (51)		1.4 (1.3–1.7)	2.4×10^{-7}	1.3×10^{-5}	2190
rs6434435	n = 802	n = 842					
G/G	587 (73)	506 (60)		1.00	2.7×10^{-9}	1.5×10^{-7}	...
A/G	195 (24)	282 (34)	GG vs AG	0.58 (.45–.72)	8.5×10^{-9}	4.7×10^{-7}	2179
A/A	20 (3)	54 (6)	GG vs AA	0.30 (.18–.52)	9.5×10^{-6}	5.2×10^{-4}	...
log-Additive							
0,1,2	802 (49)	842 (51)		0.56 (.47–.68)	4.7×10^{-10}	2.6×10^{-8}	2177

To account for the multiple testing (11 SNVs $\times$ 5 models), we applied for a Bonferroni correction for 55 comparisons.

Abbreviations: %, genotype frequency; AIC, Akaike Information Criterion; CL, cutaneous leishmaniasis; EC, endemic control; ORadj, adjusted odds ratio; Padj, *P* value adjusted for age and sex; Padj-B, age/sex-adjusted *P* value after Bonferroni correction.

**Figure 2.** Forest plot of odds ratios for (A) rs1031509 (susceptibility) and (B) rs6434435 (protection) across different genetic models. Forest plots show adjusted odds ratios and 95% confidence intervals (CI) under dominant, recessive, overdominant, and log-additive models.

[27]), observed frequencies were compared with 1000 Genomes reference populations. Overall frequencies were closest to the Latin American 2 (AMR) panel (Supplementary Table 2). For some variants, however, frequencies were closer to East Asian populations, consistent with documented shared ancestry between Native American and East Asian populations during the initial peopling of the Americas [28].

Single-Variant Association

Among the variants studied, rs1031509 and rs6434435 showed significant association with susceptibility and protection, respectively (Table 2). Detailed statistical comparisons are shown

in Supplementary Table 3. Genotypes distributions of all variants studied are shown in Supplementary Table 4.

The rs1031509 T allele was significantly overrepresented among patients with *L. guyanensis* CL (adjusted odds ratio [ORadj] = 1.5; 95% CI, 1.3–1.7; adjusted *P* value, Bonferroni correction [Padj-B] = 1.3×10^{-5}). In the codominant model, TT homozygotes had ORadj = 2.0; 95% CI, 1.5–2.7; Padj-B = 2.0×10^{-4} , while TG heterozygote had ORadj = 1.5; 95% CI, 1.3–1.9; Padj-B = 0.036. The log-additive models reinforced this association (ORadj = 1.4; 95% CI, 1.3–1.7, Padj-B = 1.3×10^{-5}). The increasing ORs (1.5 to 2.0) demonstrates an additive genetic model; strengthening each copy of the T allele increases the risk. Figure 2A shows a forest plot of the ORs of the

Table 3. Age- and Sex-Adjusted Haplotype Associations of *STAT4* in *Leishmania guyanensis* Cutaneous Leishmaniasis

	Haplotype, 11 Loci	ECs, No. (%)	CL, No. (%)	ORadj (95% CI)	P (Wald)	FDR q
1	AIGAAAGCGGC (Reference)	208.0 (12.35)	205.9 (12.83)	1.00	...	...
2	GIGAAAGCGGC	188.3 (11.18)	161.0 (10.03)	0.93 (.70–1.24)	.637	0.69
3	AIGAGGGCGGC	92.5 (5.49)	71.5 (4.46)	0.77 (.51–1.17)	.223	0.444
4	AIGAGAGCGGC	57.8 (3.43)	60.0 (3.74)	1.38 (.86–2.21)	.181	0.444
5	AIGAAAGCTGC	21.3 (1.27)	65.8 (4.10)	9.12 (4.22–19.70)	1.9×10^{-8}	2.4×10^{-7}
6	GIGAAGGCGGC	38.9 (2.31)	40.4 (2.52)	1.38 (.74–2.55)	.307	0.444
7	AIGAAGGCGGC	32.8 (1.95)	42.2 (2.63)	2.12 (1.08–4.16)	.0293	0.134
8	GIAAAAGCGGC	36.0 (2.14)	17.5 (1.09)	0.42 (.19–0.92)	.0309	0.134
9	GIGAAAGCTGC	21.0 (1.25)	29.6 (1.85)	1.50 (.61–3.71)	.38	0.45
10	GIGAAGAGGGC	24.2 (1.44)	21.5 (1.34)	0.94 (.41–2.16)	.883	0.883
11	GIGAAAGCTAA	24.4 (1.45)	16.2 (1.01)	0.48 (.19–1.23)	.127	0.412
12	GIGAAAGCGGA	19.9 (1.18)	18.9 (1.18)	1.59 (.66–3.83)	.306	0.444
13	AIGAGGGCTGC	14.9 (0.88)	20.5 (1.28)	1.59 (.61–4.17)	.341	0.444
14	AIGAAAGCTAA	17.1 (1.01)	17.1 (1.06)	1.64 (.61–4.43)	.33	0.444
15	Other, <1% pooled	887.0 (52.67)	815.9 (50.87)	...	...	...

SNVs are ordered as: SNV1 rs3821236 A > G, SNV2 rs56668168 I > D, SNV3 rs6434435 A > G, SNV4 rs10931480 A > G, SNV5 rs10174238 A > G, SNV6 rs6752770 A > G, SNV7 rs6705162 A > G, SNV8 rs1020658 C > G, SNV9 rs1031509 T > G, SNV10 rs1031508 A > G, and SNV11 rs7574070 A > C. Haplotype associations were evaluated using haplotype-based logistic regression models adjusting for age and sex, using the most frequent haplotype as the reference category. Rare haplotypes (<1% overall frequency) were pooled into a single "Other" category for regression analyses and inclusion in the global model. ORadj adjusted for age and sex are presented relative to the reference haplotype. Padj correspond to age- and sex-adjusted models, and FDR q values applied to Padj were calculated using the Benjamini-Hochberg procedure across haplotype-specific tests.

Abbreviations: CL, cutaneous leishmaniasis; EC, endemic control; FDR, false discovery rate; ORadj, adjusted odds ratio; SNV, single-nucleotide variant.

different genetic models of association with susceptibility of rs1031509.

Genotypes distribution of rs6434435 Genotype distribution of rs6434435 also differed significantly between the patients with *L. guyanensis* CL and ECs (Padj-B = 1.5×10^{-7} ; [Supplementary Table 3](#)). Individuals with the AA genotype showed 70% lower risk compared with GG (ORadj = 0.30; 95% CI, .18–.52; Padj-B = 5.2×10^{-4}). Heterozygous individuals (AG) also showed protection (ORadj = 0.58; 95% CI, .45–.72; Padj-B = 4.7×10^{-7}). The log-additive model also confirmed a strong allele-dose protective effect (ORadj = 0.56; 95% CI, .47–.68; Padj-B = 2.6×10^{-8}). [Figure 2B](#) shows a forest plot of the odds ratios of the different genetic models of association with protection of rs6434435.

Linkage Disequilibrium and Haplotype Analysis

[Figure 1B](#) shows the LD analysis among the 11 variants based on D' values, while corresponding r^2 estimates are presented in [Supplementary Figure 1](#). LD was generally low, with only rs1031509 showing high LD with rs1031508. Given the limited LD structure, haplotype inference was restricted to those with an estimated frequency $\geq 1\%$ to avoid the inclusion of rare, noninformative combinations, yielding 10 common haplotypes ([Supplementary Table 5](#)). In the unadjusted Haploview comparison, haplotype AIGAAAGCTGC was overrepresented in patients with *L. guyanensis* CL relative to ECs (61:1353 vs 20:1449), consistent with a susceptibility effect (crude OR = 3.3; 95% CI, 2.0–5.4; $P = 1.6 \times 10^{-6}$), whereas haplotype GIAAAAGCGGC was more frequent in controls than in patients with *L. guyanensis* CL (34:1435 vs 14:1400), consistent

with protection (crude OR = 0.4; 95% CI, .23–.8; $P = .005$). The remaining haplotypes showed no evidence of association in crude analyses, although haplotype AIGAAGGCGGC suggested a borderline risk trend (crude OR = 1.6; 95% CI, 1.0–2.6; $P = .06$).

Global differences in haplotype distribution were highly significant between the patients with *L. guyanensis* CL and ECs. Unadjusted haplotype distributions differed markedly between *L. guyanensis* CL cases and endemic controls (Wald omnibus test, $\chi^2 = 55.65$, df = 13, $P = 3.11 \times 10^{-7}$). In haplotype-based logistic regression adjusting for age and sex, haplotypes remained globally associated with disease (likelihood ratio test, $\chi^2 = 67.81$, d = 13, $P = 2.02 \times 10^{-9}$; [Table 3](#)). Subsequent haplotype-specific logistic regression (adjusted for age and sex) was analyzed by pooling rare haplotypes (<1% overall frequency) into a single category rather than excluded, and retaining complete haplotypic structure in the global comparison. Haplotype-specific regression identified AIGAAAGCTGC as strongly associated with susceptibility (ORadj = 9.12; 95% CI, 4.22–19.70; $P = 1.9 \times 10^{-8}$; FDR q = 2.4×10^{-7}), whereas GIAAAAGCGGC was associated with reduced odds of disease (ORadj = 0.42, 95% CI, .19–.92; $P = .0309$; FDR q = 0.134). In this framework ([Table 3](#)), haplotype-specific estimates remained directionally consistent with the crude Haploview results ([Supplementary Table 5](#)).

Conditional modeling, including both the top-risk haplotype and rs6434435 in the same multivariable model, showed that AIGAAAGCTGC remained strongly associated with disease (OR = 7.9; 95% CI, 3.6–17.0; $P = 1.4 \times 10^{-7}$). rs6434435 also retained an independent allele-dose effect, with the A allele

conferring protection (OR per A copy = 0.60; 95% CI, .50–.70; $P = 2.7 \times 10^{-7}$). These findings indicate that the haplotypic signal is not fully explained by rs6434435 and support a multivariate contribution within the *STAT4* locus.

Plasma Cytokine Correlation With *STAT4* Genotypes

Previously, we showed that plasma IFN- γ , TNF- α , IL-12, and IL-17 concentrations were higher in patients with *L. guyanensis* CL than in ECs [29]. Comparison of circulating cytokines between patients and ECs is shown in Figure 3A. Patients exhibited significantly higher circulating concentrations of all evaluated cytokines (IL-12, IL-17, IFN- γ , and TNF- α) compared with controls after adjustment for age and sex, and FDR correction (all FDR-adjusted $P < .001$) (Supplementary Table 6). The largest relative differences were observed for IL-12, IL-17, and TNF- α , for which controls showed approximately 30%–40% lower levels compared with patients on the log-transformed scale. These findings indicate a broad activation of inflammatory and Th1/Th17-related immune pathways in affected individuals, consistent with heightened immune activation during disease. No significant variation in plasma IFN- γ , TNF- α , IL-12, and IL-17 concentrations was observed across rs1031509 genotypes ($P > .37$) as shown in Supplementary Figure 2.

The *STAT4* rs6434435 genotype correlation under the co-dominant model showed nominal associations on IFN- γ and TNF- α production that did not remain significant after FDR correction (Figure 3B). The concentrations of plasma IFN- γ and TNF- α under different genetic models for rs6434435 are shown in Supplementary Table 7. Associations between *STAT4* rs6434435 and circulating IFN- γ and TNF- α levels were evaluated using natural log-transformed cytokine concentrations under pairwise, dominant, and additive genetic models (Supplementary Table 7). In pairwise analyses using GG homozygotes as the reference group, IFN- γ levels were modestly higher in AG heterozygotes compared with GG homozygotes (+13.7%, P value adjusted for age and sex (P_{adj}) = .036), whereas no difference was observed between AA and GG genotypes. However, after correction for multiple testing across all pairwise genotype-cytokine comparisons, none of the IFN- γ pairwise associations remained statistically significant when FDR correction was applied to P_{adj} ($FDR\ P_{adj} \geq .144$). Similar pairwise analyses for TNF- α showed higher levels in AG heterozygotes compared with GG homozygotes (+40.2%, P_{adj} = .0139), while the AA versus GG comparison was not significant; again, these pairwise associations did not survive FDR correction.

Given the low frequency of AA homozygotes and to increase statistical power, dominant and additive genetic models were subsequently evaluated. Under a dominant model combining AA and AG genotypes, carriers of the rs6434435 A allele exhibited higher TNF- α levels compared with GG homozygotes

(+40.2%, P_{adj} = .0065), and this association remained significant after FDR correction across the 4 cytokines analyzed ($FDR\ P_{adj}$ = .026). In contrast, the corresponding dominant association with IFN- γ was weaker (+13.8%, P_{adj} = .0277) and did not remain significant after correction for multiple testing ($FDR\ P_{adj}$ = .055). Consistent with these findings, additive models demonstrated a significant per-allele increase in TNF- α levels (+33.2% per A allele, P_{adj} = .00674; $FDR\ P_{adj}$ = .027), whereas the per-allele association with IFN- γ was nominal ($FDR\ P_{adj}$ = .071). Collectively, these results indicate a selective and significant association between rs6434435 and TNF- α , with weaker and nonrobust effects on IFN- γ .

DISCUSSION

L. guyanensis CL continues to pose a significant public health challenge where exposure to infected phlebotomine vectors is common. However, only a subset of exposed individuals develops clinical disease, reflecting complex interactions among host genetic factors, parasite virulence, and environment.

Here, we demonstrate that *STAT4* variation is associated with both susceptibility and protection against *L. guyanensis* CL. The rs1031509 T allele was associated with increased risk, whereas rs6434435 A allele conferred protection, with effects remaining robust after age and sex adjustment across genetic models. These contrasting effects highlight the immunogenetic complexity within the IL-12/IFN- γ axis and underscore its critical role in modulating individual susceptibility to disease progression.

A key finding is the substantial cumulative risk captured by haplotype analysis, which exceeded the effects of individual single-nucleotide variants (SNVs). The haplotype AIGAAAGCTGC revealed a 9-fold increase in susceptibility, exceeding the 2-fold risk associated with rs1031509 T/T homozygosity alone. The highly significant global omnibus haplotype test further supports that disease risk is driven by variants combination, rather than by isolated SNVs. Conversely, alternative haplotypes exhibited susceptibility or protection, supporting a polygenic and haplotype-driven model, consistent with *STAT4* functioning as a multisignal regulatory hub during intracellular infection.

STAT4 is a pivotal transcription factor directing Th1 differentiation through IL-12 engagement with IL-12R β , promoting IFN- γ production and macrophage activation. Activation of *STAT4* enhances T-bet expression and sustains *IFNG* transcription, thereby reinforcing Th1 polarization, promoting macrophage activation and nitric oxide-mediated parasite clearance. The contrasting effects of rs1031509T > G and rs6434435A > G suggest that distinct intronic regions within *STAT4* may exert opposing regulatory influence. The autoimmune-associated intronic variant rs7574865 tags a large LD block extending toward the 3' end of *STAT4* [30], and has been linked to context-dependent modulation of IL-12-induced

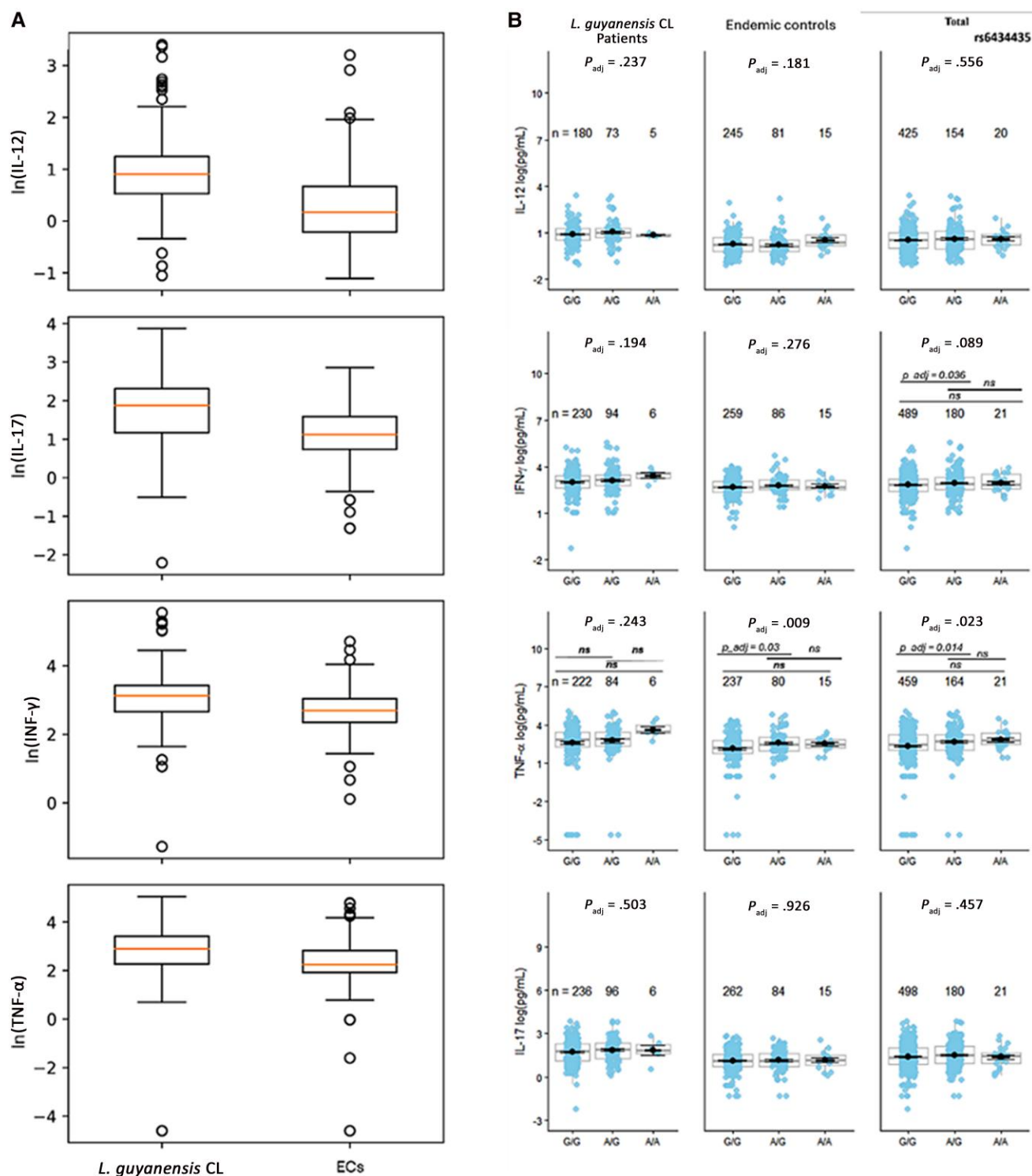


Figure 3. Cytokine profiles in patients with *Leishmania guyanensis*-CL and ECs, and genotype–cytokine associations. *A*, Comparison of circulating cytokine concentrations between patients with *L. guyanensis* CL and ECs without clinical disease. Cytokine levels were naturally transformed to improve normality and are presented as box-and-whisker plots, where boxes indicate the IQR, horizontal lines denote the median, and whiskers represent 1.5 $\times$ the IQR; individual data points are shown. *B*, Associations between host genotypes and cytokine levels, stratified by clinical group (*L. guyanensis* CL, ECs, and total population). Genotype groups are displayed as box-and-whisker plots with individual observations overlaid. Sample sizes for each genotype are indicated above the corresponding plots. Across panels, statistical comparisons were performed using linear regression models adjusted for age and sex, with cytokine concentrations analyzed on the log scale. P_{adj} are shown for each comparison, and FDR correction was applied where indicated. Abbreviations: CL, cutaneous leishmaniasis; EC, endemic control; FDR, false discovery rate; IFN- γ , interferon- γ ; IL, interleukin; IQR, interquartile range; ns, not significant; P_{adj} , adjusted P value; TNF- α , tumor necrosis factor- α .

STAT4 phosphorylation and IFN- γ [31], supporting functional heterogeneity. Accordingly, rs6434435 lies within the same broader STAT4 regulatory landscape and may tag a potentially distinct intronic configuration within a locus known to harbor multiple independent regulatory effects.

The genomic location of the associated variants investigated here further supports functional divergence. rs1031509 (GRCh38 chr2: 191,145,463) lies more proximal to the 5' end of *STAT4* than rs7574865 (GRCh38 chr2: 191,099,907), whereas rs6434435 (GRCh38 chr2: 191,089,138) is positioned more distally within the gene structure. Fine-mapping studies have identified at least 3 independent *STAT4* association signals, supporting a model in which distinct intronic variants, or variants in high LD with them, differentially regulate *STAT4* [16]. IFN- γ expression is controlled by an early T-bet-dependent program responsible for Th1 lineage commitment and a later IL12/STAT4-dependent phase that sustains transcriptional activity [11].

Integrating our findings with the 2-step model proposed by Mullen et al [11], the distal rs6434435 may influence regulatory elements involved in early T-bet-mediated epigenetic priming of the *STAT4* locus, while the more proximal rs1031509 modulates the magnitude cytokine production in activated cells. In our cohort, patients exhibited a robust inflammatory profile with markedly higher circulating levels of IL-12, IL-17, IFN- γ , and TNF- α compared to ECs. Notably, rs6434435 demonstrated an association with TNF- α levels under dominant and additive models. Given the central role of TNF- α in macrophage activation and parasite control, these findings suggest that *STAT4* variation contributes to interindividual heterogeneity in inflammatory effector pathways, while the overall cytokine milieu is primarily driven by disease status. HiChIP analyses have identified intronic regulatory chromatin interactions within *STAT4* linking distal enhancers to the *STAT4* and *STAT1* promoters, including regions overlapping rs3821236 [16]. Although rs6434435 is not in LD with rs3821236 in our cohort, it lies approximately 51 kb away within the same neighborhood (Figure 1).

The lack of LD between these SNVs suggests that rs6434435 may represent an independent functional signal or tag a distinct regulatory element. This supports a multisignal regulatory model in which nonlinked variants within the *STAT4* locus interact through chromatin interactions to modulate gene expression. Accordingly, the protective rs6434435 A allele may increase transcriptional accessibility, enhancing STAT4 responsiveness to IL-12 signaling and promoting Th1-mediated clearance of *Leishmania*.

In macrophages, STAT4-driven IFN- γ signaling upregulates inducible nitric oxide synthase (iNOS) facilitating NO-dependent parasite killing. The directionally consistent increases in TNF- α , and to a lesser extent IFN- γ , among carriers of the rs6434435 A allele suggest a potential Th1-skewed tendency that may contribute to parasite control, concordant with prior findings

involving *IFNG-AS1* and *IFNG* [23, 32]. The biological relevance of these findings is supported by murine studies, where *STAT4*-deficient mice have impaired Th1 and heightened susceptibility to *Trypanosoma cruzi*, *L. major*, and *Leishmania mexicana* [32–34].

Conversely, rs1031509T allele may impair STAT4-mediated immunity. Its association with increased susceptibility, demonstrated by dose-dependent odds ratios, combined with a lack of significant effect on plasma cytokine levels, suggests a functional effect distinct from that of rs6434435. Interestingly, the rs1031509G allele was previously associated with tuberculosis susceptibility in a Moroccan population [35]. In that study, the rs1031509G allele frequency was 59% in tuberculosis cases, which is nearly identical to the frequency observed in our patients with *L. guyanensis* CL (58%).

These results are consistent with our previous findings, indicating that IFN- γ plays a key role in the initiation of infection [23, 36, 37]. Collectively, these results reinforce the central role of the IL-12/IFN- γ axis in mediating resistance to *L. guyanensis* infection. Protective rs6434435 A allele mirrors findings for *IFNG* haplotypes linked to higher IFN- γ plasma levels and resistance to disease [24], supporting a model where *STAT4* genetic variation modulates the efficacy of the IL-12/IFN- γ signaling pathway. However, while our analysis identified nominally significant correlations between the rs6434435 genotypes and plasma IFN- γ and TNF- α , these associations did not maintain significance after FDR correction. Nevertheless, the consistent directionality of the effect suggests a potential dosage effect on the Th1 axis that warrants further validation.

The genetic admixture of the Amazonas population [27] may have preserved functionally diverse *STAT4* haplotypes under long-standing selective pressures from endemic pathogens. The opposing associations of rs1031509 and rs6434435 likely reflect distinct evolutionary adaptations to pathogen exposure. Our findings suggest that these variants tag regulatory haplotypes influencing the efficacy of the IL-12/IFN- γ axis.

Despite its large sample size and pathogen-specific focus, the study has limitations. Although the similar LD structure between patients with *L. guyanensis* CL and ECs, together with adjustment for age and sex, reduces the likelihood of confounding, the absence of genome-wide ancestry markers means that population stratification or cryptic relatedness cannot be fully excluded. Participants were recruited from the same endemic area with comparable occupational exposure. However, detailed peridomestic environmental variables (eg, domestic animals and standing water near the household) were not collected and could not be included as covariates. The associated *STAT4* variants likely tag regulatory haplotypes rather than causal variants and warrant external validation. Plasma cytokines provide a surrogate of immune status but may not fully reflect the local cellular dynamics at the cutaneous lesion sites, and the absence of longitudinal data precludes genotypes-healing correlations.

Altogether, our data reveal *STAT4* as a key determinant of protection or susceptibility to *L. guyanensis* CL, underscoring the pivotal role of *STAT4*-mediated signaling to intracellular pathogens and reinforce evidence that the IL-12/IFN- γ axis is critical for resistance. Defining the underlying molecular mechanisms may facilitate biomarker development, improve risk stratification in endemic areas, and identify novel immunomodulatory targets for therapeutic intervention.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Data availability. The data underlying this article are available in the article and its online [Supplementary Material](#).

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