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# Polymorphism rs8057341 in the *NOD2* gene and its clinical-laboratory association with visceral leishmaniasis

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**ABSTRACT.** Visceral leishmaniasis (VL) is a chronic and debilitating disease with high lethality, characterized by diverse clinical and epidemiological features. Among these, the parasite-host relationship is known to influence disease progression, which can be affected by single nucleotide polymorphisms (SNPs) in genes associated with the immune response. The objective of this study was to evaluate the SNP rs8057341 in the *NOD2* gene and its clinical-laboratory association with VL. For this purpose, patients diagnosed with VL (n = 28) were studied. The SNP rs8057341 in the *NOD2* gene was genotyped using real-time PCR, and patient data were collected from medical records. Our results showed no association between genotypes and the main symptoms of the disease. However, regarding disease severity, most patients had a severe condition (n = 21), a factor influenced by the AG (p = 0.0455) and GG (p = 0.0110) genotypes, compared to the AA genotype. Based on these findings, we hypothesize that the AA genotype of rs8057341 in the *NOD2* gene is associated with susceptibility to *L. infantum*, possibly by impairing the antiparasitic immune response involved in disease control, with probable modulation of IL-17. However, because it may promote a less intense inflammatory response, it could be associated with milder disease manifestations.

**Keywords**: NOD2 Signaling Adaptor Protein; Polymorphism; Single Nucleotide; Leishmania; Disease Severity.

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## Introduction

Leishmaniasis is a neglected tropical disease that affects vulnerable populations in over 90 countries, with a risk association linked to poverty, population migration, malnutrition, lack of hygiene, and the immunosuppressed state of its host (World Health Organization [WHO], 2023).

The immune response to the protozoan is complex, and different profiles of helper T cells (Th) take part in the process. The Th1 profile, characterized by the production of IFN-γ, is associated with stimulating the microbicidal action of macrophages, granuloma formation, and parasite destruction (Dayakar et al., 2019). Another profile, Th17, characterized by the production of IL-17, assists in stimulating anti-leishmania molecules in infected macrophages (Nascimento et al., 2015). Some authors have demonstrated that the Th17 and Th1 profiles perform complementary functions in protection against *L. donovani* infection (Sacks & Anderson, 2004).

The innate immune system can modulate adaptive responses through the induction of signaling cascades via pattern recognition receptors, such as NOD2 (nucleotide-binding oligomerization domain-containing 2) (Takeuchi & Akira, 2010; Liu et al., 2013). A study demonstrated that NOD2 can influence both Th1 and Th17 responses, after evaluating *L. infantum* infection in gene-knockout mice (Nascimento et al., 2016).

The assessment of single nucleotide polymorphisms (SNPs) in the *NOD2* gene in patients with ocular toxoplasmosis showed an impact on IL-17A production (Dutra et al., 2013). Other authors found associations between SNPs in *NOD2* and visceral leishmaniasis, suggesting their potential as risk markers for infection in the Brazilian population (Mesquita et al., 2023). Thus, genetic variability may play an important role in susceptibility or resistance to *L. infantum*, as most residents of endemic regions for the disease do not exhibit active infection (Duthie et al., 2014; Gatto et al., 2015).

The immune response to leishmaniasis is complex, and *NOD2* is considered a key component in this process. However, few studies have assessed SNPs in this gene in the Brazilian population and their relationship with visceral leishmaniasis.Therefore, investigating the potential relationship between SNPs in the *NOD2* gene and clinical-laboratory manifestations of the disease may contribute to a better understanding of VL susceptibility and progression.In this context, the present study aims to assess the relationship between the SNP rs8057341 in the *NOD2* gene and visceral leishmaniasis, analyzing the allelic and genotypic frequency in patients, and its association with clinical and laboratory aspects of the disease.

## Material and methods

### Study population

This was a prospective, cross-sectional study involving 28 patients with VL, of whom 19 were men (mean age: 50.58 years; range: 20–82 years), and 9 were women (mean age: 46.22 years; range: 19–82 years. All patients were treated at the Infectious Diseases Outpatient Clinic of the Regional Hospital of Presidente Prudente and had a confirmed diagnosis of VL based on clinical-epidemiological presentation and/or immunological laboratory testing, including enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence (IFI).

At the time of blood collection for SNP analysis, all patients were considered cured of the parasitic infection and had completed treatment, with an average post-treatment period of 59.48 months (range: 12–108 months). Clinical and laboratory data used for the analyses were collected through medical record review during each patient's diagnostic period. Three criteria were employed to assess disease severity: hepatomegaly, splenomegaly, and liver dysfunction (defined as Aspartate Transaminase [AST] > 100 $UL^{-1}$ and/or Alanine Transaminase [ALT] > 100 $UL^{-1}$). Patients were classified as having severe VL if they met at least two out of these three criteria (Pelloso et al., 2020).

Only patients who agreed to participate after receiving appropriate information and signing the informed consent form were included in the study. The research was approved by a Research Ethics Committee (67043423.1.0000.5515) and conducted in accordance with the Declaration of Helsinki of 1964.

### Genotyping of rs8057341 in the *NOD2* gene

For genotyping, 5 mL samples of peripheral blood were collected in EDTA tubes from patients with VL at a single time point. The blood was centrifuged for 20 minutes at 1500 rpm, and the leukocyte-rich buffy coat was transferred into a new 1.5 mL tube and storedat -80 °C until the time of extraction. Genomic DNA was extracted from leukocytes using a commercial DNAzol reagent (Invitrogen, Carlsbad, CA, USA) and storedat -80 °C until genotyping, following the manufacturer's instructions.

The concentration and purity of the extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Genotyping was performed using the allelic discrimination technique with the TaqMan system (Applied Biosystems) on a StepOnePlus real-time PCR instrument (Applied Biosystems). A DNA concentration of 20 $\mu g \mu L^{-1}$ per sample was used, following the manufacturer's instructions. After genotyping the SNP rs8057341 in the *NOD2* gene, results were analyzed based on allele distribution (A/G) or genotypes (AA/AG/GG).

### Data analysis

The association between different alleles and genotypes and the clinical manifestations and severity of VL was analyzed using the $\chi 2$ test or Fisher's exact test. Significance was considered for $p< 0.05$.

## Results

Patients with VL showed a higher frequency of the G allele (n = 32; 69.57%), with more than half presenting the GG genotype (n = 16; 57.14%), followed by the AG genotype (n = 10; 35.71%) and the AA genotype (n = 2; 7.14%).

The presence or absence of the main clinical manifestations at the time of diagnosis, in association with the corresponding alleles and genotypes of each patient, is presented in Figure 1. When assessing signs and symptoms by genotype distribution, a non-significant trend was observed toward a higher frequency of hepatomegaly among carriers of the G allele. No association was found between genotypes and splenomegaly, weight loss, or fever. This assessment was conducted in only 27 patients due to missing information in one medical record.

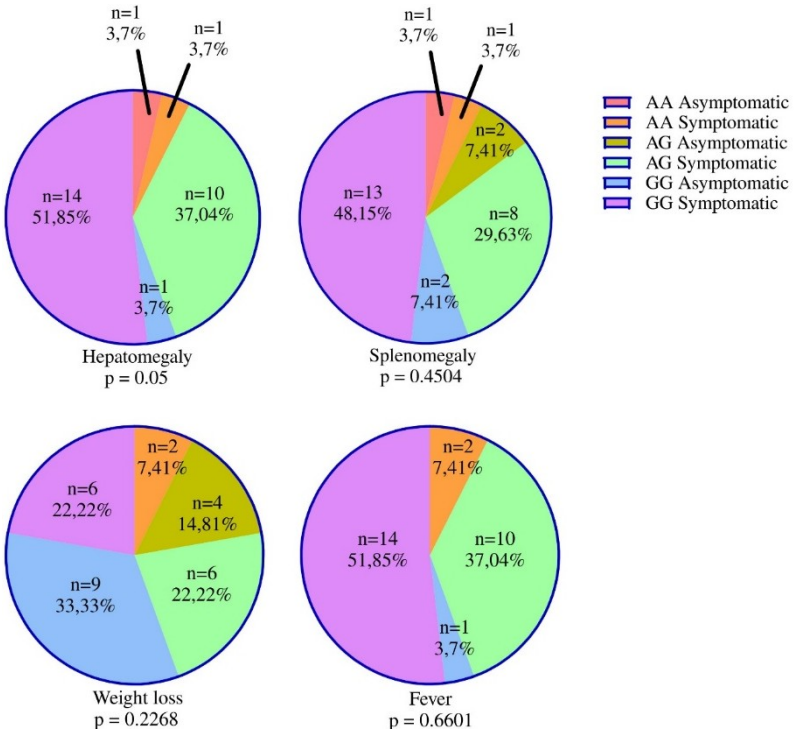


**Figure 1.** Profile of the main clinical manifestations of patients with visceral leishmaniasis (n = 27) according to the distribution of rs8057341 genotypes in the *NOD2* gene.

When evaluating the severity of VL, most patients had a severe condition (n = 21), which was influenced by the AG and GG genotypes compared to the AA genotype (Table 1). This assessment was conducted in only 24 patients due to incomplete medical records.

**Table 1.** Association between visceral leishmaniasis severity and rs8057341 genotypes in the *NOD2* gene (n = 24).

| *NOD2* (rs8057341) | Severity of visceral leishmaniasis | | Fisher | p |
|---|---|---|---|---|
| | Severe | Not severe | | |
| AA | 0 | 2 | AA x AG | 0.0455 |
| AG | 9 | 1 | AA x GG | 0.0110 |
| GG | 12 | 0 | AG x GG | 0.4545 |

## Discussion

Visceral leishmaniasis is a chronic and debilitating disease with high lethality, characterized by distinct clinical and epidemiological aspects specific to each region, including Brazil (Ministério da Saúde [MS], 2022). The progression of VL is influenced by the host-parasite relationship, which can be modulated by SNPs in immune-related genes (Nascimento et al., 2016; Vieira et al., 2024). The present study demonstrated an association between severe VL and the AG and GG genotypes, suggesting that the AA genotype of rs8057341 in the *NOD2* gene might be related to a better prognosis of disease progression.

Signaling through pattern recognition receptors contributes to the stimulation of adaptive immunity. Nascimento et al. (2016) demonstrated that *NOD2* is involved in the intracellular recognition of *L. infantum* by dendritic cells, promoting a Th1 immune response and suppressingthe Th17 profile.Furthermore, the authors observed a negative modulation of genes associated with the Th17 profile, indicating that post-infection IL-17A production may occur through an RORγt-independent pathway involving an unidentified cell type.

Recent studies conducted in Brazil have identified variants of the *NOD2* gene associated with susceptibility to different forms of leishmaniasis. Mesquita et al. (2023) observed that polymorphisms in *NOD2* correlated with altered levels of pro-inflammatory cytokines, suggesting that genetic variability in this receptor influences immune responses to *Leishmania* infection. Similarly, Nascimento et al. (2016) demonstrated that the activation of *NOD2* modulates the Th1/Th17 balance, contributing to the control of parasite replication. These findings support the hypothesis that variations in *NOD2* signaling may regulate IL-23 and IL-17 pathways, affecting nitric oxide production by macrophages and the efficiency of the Th1 response in eliminating *Leishmania* (Afonso et al., 2013; Nascimento et al., 2015; Nascimento et al., 2016).

Santos et al. (2017) reported that individuals heterozygous for rs2066847 in *NOD2* produced lower levels of inflammatory cytokines (e.g., IFN-γ, TNF-α, IL-6, IL-1β, and IL-8) after peripheral blood mononuclear cell (PBMC) stimulation with different species associated with cutaneous leishmaniasis, *L. amazonensis* and *L. braziliensis*. Interestingly, IL-17 production was species-dependent, being induced only in response to *L. amazonensis*, demonstrating that *NOD2* may shape the immune response against *Leishmania* spp. Thus, the authors suggest that genetic variations in *NOD2* may lead to dysfunctional receptor activity, affecting the recognition and control of *Leishmania*.

*Leishmania* parasites replicate within the phagolysosomes of macrophages mainly found in the liver, spleen, bone marrow, and lymph nodes. This can lead to organ enlargement due to hyperplasia of the mononuclear phagocytic system, which in turn can contribute to the presence of other symptoms, such as hematological changes resulting from splenomegaly (Varma & Naseem, 2010; Middib & Al-Mouktar, 2014).

A study using mice infected with *L. infantum* demonstrated that IL-17 is involved in controlling parasite growth, however, it may also contribute to hepatosplenomegaly. The authors observed lower levels of ALT and AST in IL-17A receptor knockout mice, indicating less liver damage in these animals. This suggests that IL-17 contributes to parasite control while also exacerbating the inflammatory response, contributing to tissue damage (Nascimento et al., 2015).

Another experimental study with *L. donovani* demonstrated a contradictory role for IL-17, as animals treated with IL-17 neutralizing antibodies showed a decrease in parasite loads, indicating a detrimental role of this cytokine in the initial control of infection in the animals' liver. The authors attributed this phenomenon to two possible explanations. The first is that IL-17 inhibits the initial activation of macrophages and parasite death. The second is that there is insignificant parasite death in the early stages of infection, with IL-17 contributing to increased parasite growth within hepatic macrophages, resulting in higher parasite loads (Sheel et al., 2015).

The present study presents limitations in terms of the number of patients studied and the inability to assess the immune response during infection. Thus, future studies are necessary to better clarify the relationship between *NOD2* and VL.

## Conclusion

Given the presented context, we hypothesize that the AA genotype of rs8057341 in the *NOD2* gene may be associated with susceptibility to *L. infantum*, possibly by impairing antiparasitic immune responses associated with disease control and modulating IL-17 production. However, by promoting a less intense inflammatory response, it might be related to milder disease manifestations.

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