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2 **Analysis of the frequency of the Toll-like 2 gene**
3 **polymorphism in leprosy**

4 **Marcos Jessé Abrahão Silva¹, Everalina Cordeiro dos Santos¹, Yan**
5 **Corrêa Rodrigues¹, Karla Valéria Batista Lima¹, Luana Nepomuceno**
6 **Gondim Costa Lima^{1*}**

7 ¹*Evandro Chagas Institute (IEC) / Ministry of Health, Rodovia BR-316 km 7 s/n - Levilândia –*
8 *Zip code: 67030-000 - Ananindeua / Pará / Brazil.*
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13 **ABSTRACT**
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Polymorphisms in genes that are responsible for encoding cytokines and receptors involved in the immune response, such as Toll-like Receptor (TLR) 2 in leprosy, are of great interest for immunogenetic studies. This work aimed to analyze the possible association of single nucleotide polymorphism (SNP), synonymous, rs3804100 of the TLR2 gene with leprosy. The study was conducted in Bacteriology and Mycology section of Evandro Chagas Institute, Brazil between August 2020 and July 2021. The scope of the study consisted of 122 subjects from cities of Goianésia, Rondon, Curionópolis, Altamira, Parauapebas and Redenção of the State of Pará, Brazil. Genotyping was performed by conventional PCR and sequencing in the ABI 3130 Genetic Analyzer (Applied Biosystems®) using primer nucleotides designed by the Primer3Plus program from the genomic region “Homo sapiens toll like receptor 2 (TLR2) transcript variant X6, mRNA”, deposited in GenBank with reference XM_011532216.2. The analyzes were performed based on Fisher's exact test. It was managed in accordance with Helsinki Declaration and the Brazilian National Health Council and with approval of the ethics committee at Evandro Chagas Institute, under opinion number: 3.950.570. No associations between gender and leprosy were possible ($P > 0.05$). However, associations were observed between age groups, which were significant between those over 46 years old ($P=0.004$) and the 2nd dose of BCG as a more protective agent between the groups analyzed ($P=0.004$). For the subjects with the typed genotypes, 68 contacts had T/T genotype and only 4 T/C genotypes, while in multibacillary (MB) group only 1 T/C genotype was found and none in paucibacillary (PB) ($P > 0.05$). We conclude that there is no association between the TLR2 SNP rs3804100 and leprosy in the Pará population, which still indicates the need for new immunogenetic studies with other genes involved in the immune response and a greater number of polymorphisms.

15
16 *Keywords: Epidemiology; Leprosy; Genetic polymorphism; Immunogenetics.*
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18 **1. INTRODUCTION**
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20 Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, a bacillus-shaped mycobacterium that acts as
21 an obligate intracellular parasite, mainly affecting the skin and nervous tissues [1]. Moreover, *M. leprae* infects
22 predominantly macrophages, dendritic cells, and Schwann cells, which may lead to nerve damage and disability [2].

23 Epidemiological data position Brazil as the second in prevalence of cases globally, surpassed only by India [3]. According
24 to data from the Brazilian Ministry of Health, high incidence of leprosy cases occurs in the Midwest, Northeast and North
25 regions, and with the state of Pará as the 3rd in number of detected cases in 2020 within the Brazilian territory [4].

26 Leprosy progression is linked to immunogenetic factors that orientate the host's immune response against the pathogen.
27 The tuberculoid form (TT) (paucibacillary) is understood by the high host resistance to infection, which is characterized by
28 a T helper 1 (Th1) lymphocyte response. Oppositely, the lepromatous form (LL) (multibacillary) a reduced Th1 response is

29 observed, causing infection spread, bacillary multiplication, and a Th2-based immune response. Finally, transitions clinical
30 manifestations between the polar clinical (TT and LL) forms, correspond to the borderline group [5]

31 The innate immune response is the first line of defense against *M. leprae*, being a crucial step for the development of the
32 response against the bacillus, as it has essential effector components in combating the pathogen, and is capable of
33 directing adaptive immunity. Several cells of the immune system are activated through their receptors to generate an
34 antigenic response, such as Natural Killer cells, lymphocytes, among others. Among the receptors expressed by Natural
35 Killer cells, the Toll-Like receptors (TLR) on the cell surface and cytoplasm stand out [6].

36 The TLRs are a group of transmembrane receptors present from *Drosophila* to mammals. In humans, the group of these
37 receptors consists of 10 protein subtypes. These proteins are specific cells receptors for virus, bacterial and fungal
38 components, and to initiate the activation of the cytokine production and adaptive immune response [7].

39 Each member of the TLR family is activated by specific antigens that lead to different transcriptional activation profiles,
40 initiating an adequate immune response to the pathogen. These polymorphic receptors recognize lipoproteins present in
41 the bacillus and promote the differentiation of monocytes into macrophages and CD1b+ dendritic cells, which in turn
42 activate lymphocytes that release cytokines such as TNF- α and IL-12, promoting an antimicrobial activity. Of all
43 mammalian TLRs, TLR2 detects the broadest repertoire of molecular patterns from a wide variety of pathogens, including
44 mycobacteria, making it of particular clinical importance [8]. Just because *M. leprae*, in the host, initiates cell signaling
45 through activation of the TLR1/TLR2 heterodimer, single nucleotide polymorphisms (SNP) in these genes may be able to
46 confer susceptibility or protection to leprosy [9].

47 Kang and Chae, 2001 studied the SNP rs121917864 (C to T substitution) at nucleotide 2029 from the start codon of the
48 TLR2. The mutation would substitute Arg to Trp at amino acid residue 677, one of the conserved regions of TLR2. They
49 only found the mutated base in lepromatous leprosy individuals [10].

50 Another SNP in the TLR2 gene, the rs3804100 is a synonymous mutation that does not lead to a substitution of amino
51 acid serine (Ser) at residue 450 and was predicted to have a functional effect in decreasing the number of exonic splicing
52 enhancing motifs [11]. This SNP has been extremely associated with several infectious diseases [12–17], including
53 tuberculosis, in which the infectious agent is of the same genus as leprosy and has a host cellular immune response
54 similar to leprosy [18]. However, the only work in the literature that studied the relationship of the SNP rs3804100 with
55 leprosy was by Bochud P-Y et al., 2008, with samples from a population from Africa. However, SNPs change from one
56 population to another (genetic makeup) and so do results on disease associations [19].

57 Then, the present study aimed to verify the relationship between the SNP rs3804100 of the TLR2 gene and leprosy by
58 comparing the genotypic and allelic frequencies among leprosy cases and their healthy contacts from an endemic area of
59 Brazil.

60 2. MATERIAL AND METHODS

61 2.1 Sampling and Study Location

62 This study involved patients in health care centers in the cities of Altamira, Goianésia, Rondon, Parauapebas, Redenção
63 and Curionópolis of the State of Pará, Brazil. The sample consisted 50 patients, including 25 TT and 25 LL, which were
64 diagnosed according to the Brazilian Ministry of Health criteria [20]. Additionally, the patients' household contacts were
65 invited to participate in the study, forming a group of 72 subjects, which have no genetic link with patients and did not
66 present clinical symptoms of leprosy. This group was called control. Blood samples were collected from patients and
67 contacts by venipuncture in 5ml tubes and stored at -20°C for laboratory procedures at the Bacteriology and Mycology
68 section, Evandro Chagas Institute. Household contacts with a history or suspicion of leprosy were excluded.
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70 2.2 SNP rs3804100 typing of the TLR2 gene

71 The SNP under analysis is rs3804100 from Thymine to Cytosine (T>C) at position 450 of the extracellular domain of TLR2
72 at nucleotide 1350 (1350T/C). The typing was based on two PCR primer nucleotides: Primer 1 - F:
73 5'ATTCAGCCTGTGAGGATGCC 3' and R: 5' TTGCTGCTCCTGAGTGAAGG 3' generating a 512 bp fragment and
74 Primer 2 - F: 5' ACCGGAGAGACTTTGCTCAC 3' and R: 5' GCTTGCTGCTCCTGAGTGAA 3' generating fragment of
75 437 bp. Primers used in PCR essays were generated on the Primer3Plus program based on the genomic region "Homo
76 sapiens toll like receptor 2 (TLR2) transcript variant X6, mRNA", deposited in GenBank with the reference
77 XM_011532216.2.
78

DNA extractions were performed using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany), following the manufacturer's guidelines. Conventional PCR essays for TLR2 gene amplification were performed using PlatinumTaq (Invitrogen, Carlsbad, CA, U.S.A) on the Veriti Thermocycler (Applied Biosystems, Foster City, CA, U.S.A.), under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Amplified products were submitted to electrophoresis in 3,6% agarose gel stained with 3,0 µL of SybrSafe for visualization of the amplified DNA fragments. Reaction products were purified and bidirectionally sequenced using Big Dye Terminator v3.1 chemistry on ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were analyzed and compared to those available at BLAST on the National Center for Biotechnology Information (NCBI) website.

2.3 Data Analysis

The results were organized in a database of the Epi info™ 7 program. The observed proportions of polymorphism presence within each studied group were analyzed using Fisher's exact test. The analysis for SNPs prospection considering the dominant and variant alleles of the searched nucleotide base was performed by means of a bioinformatics program, Bioedit™ v7.2.5.

2.4 Ethical Considerations

The present study was conducted in accordance with Helsinki Declaration and the Brazilian National Health Council and with approval of the ethics committee at Evandro Chagas Institute, under opinion number: 3.950.570.

3. RESULTS

From a total of 122 individuals with the defined genotypes, 39 were from Altamira (31,97%), followed by Redenção (33 – 27,05%), Goianésia (19 - 15,57%), Parauapebas (12- 9,84%), Rondon (10 - 8,20%), and Curionópolis (9 - 7,38%). Regarding household contacts individuals, 32 were from Altamira (44,44%), followed by Redenção (21 - 29,17%) and Goianésia (8 - 11,11%). Most of the patients group came from Redenção with 12 (24,00%), then from Goianésia with 11 (22,00%) and Parauapebas with 10 (20,00%). Distribution of individuals according to location and clinical manifestation is presented in Table 1.

Table 1. Distribution of number and frequency of sampling of contacts, LL, TT, and patients according to municipalities.

MUNICIPALITIES	CONTACTS N (%)	LL N (%)	TT N (%)	PATIENTS N (%)	TOTAL N (%)
ALTAMIRA	32 (44,44%)	6 (24,00%)	1 (4,00%)	7 (14,00%)	39 (31,97%)
REDENÇÃO	21 (29,17%)	6 (24,00%)	6 (24,00%)	12 (24,00%)	33 (27,05%)
GOIANÉSIA	8 (11,11%)	1 (4,00%)	10 (40,00%)	11 (22,00%)	19 (15,57%)
PARAUPEBAS	2 (2,78%)	9 (36,00%)	1 (4,00%)	10 (20,00%)	12 (9,84%)
RONDON	2 (2,78%)	3 (12,00%)	5 (20,00%)	8 (16,00%)	10 (8,20%)
CURIONÓPOLIS	7 (9,72%)	0 (0,00%)	2 (8,00%)	2 (4,00%)	9 (7,38%)
TOTAL	72 (100,00%)	25 (100,00%)	25 (100,00%)	50 (100,00%)	122 (100,00%)

Socio-epidemiological data revealed that most study subjects were women (78 - 63,93%), over 46 years of age (41,80%), and having taken only 1 dose of BCG (bacillus *Calmettê-Guerin*) vaccine (63 - 51,64%). Among the group of household contacts, 49 were females (68,08%), over 46 years of age (27 - 37,50%), and having taken only 1 dose of BCG vaccine (40 - 55,56%). The group of patients was also mostly female, 29 (58%), with a predominance of 24 of them older than 46 years (48%), and 25 of them had not had a BCG vaccination (50%) (Table 2).

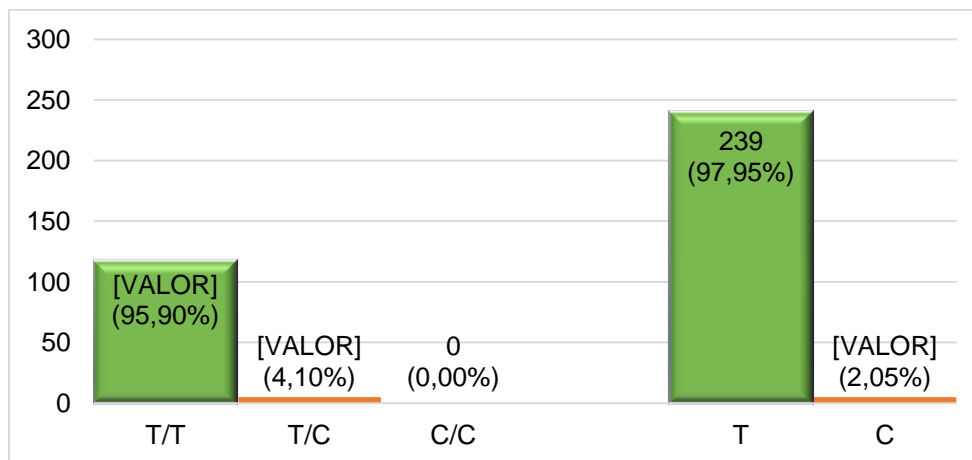
Table 2. Demographics of the subjects as to sex, age, and BCG.

	CONTACTS N (%)	LL N (%)	TT N (%)	PATIENTS N (%)	TOTAL N (%)
Sex					
Male	23 (31,94%)	12 (48,00%)	9 (36,00%)	21 (42,00%)	44 (36,07%)
Female	49 (68,06%)	13 (52,00%)	16 (64,00%)	29 (58,00%)	78 (63,93%)
Age					
0-15	15(20,83%) ^{a,b,c,d}	1 (4,00%) ^{a,c,e}	1 (4,00%) ^{a,d,e}	2 (4,00%) ^b	17 (13,93%)
16-31	16 (22,22%) ^{a,b,c,d}	2 (8,00%) ^{a,c,e}	3 (12,00%) ^{a,d,e}	5 (10,00%) ^b	21 (17,21%)
32-46	14 (19,44%) ^{a,b,c,d}	6 (24,00%) ^{a,c,e}	13 (52,00%) ^{a,d,e}	19 (38,00%) ^b	33 (27,05%)
>46	27 (37,50%) ^{a,b,c,d}	16 (64,00%) ^{a,c,e}	8 (32,00%) ^{a,d,e}	24 (48,00%) ^b	51 (41,80%)
BCG					
0	18 (25,00%) ^{f,g,h,i}	12 (48,00%) ^{f,h,j}	13 (52,00%) ^{f,i,j}	25 (50,00%) ^g	43 (35,25%)
1	40 (55,56%) ^{f,g,h,i}	12 (48,00%) ^{f,h,j}	11 (44,00%) ^{f,i,j}	23 (46,00%) ^g	63 (51,64%)
≥2	14 (19,44%) ^{f,g,h,i}	1 (4,00%) ^{f,h,j}	1 (4,00%) ^{f,i,j}	2 (4,00%) ^g	16 (13,11%)

133 $P>0.05$; ^a $P=0.003$; ^b $P=0.004$; ^c $P=0.001$; ^d $P=0.003$; ^e $P=0.002$; ^f $P=0.024$; ^g $P=0.004$; ^h $P=0.025$; ⁱ $P=0.015$; ^j $P=0.036$, Fisher's
134 Exact Test

135 The genotypic frequencies in the study population agreed with the Hardy-Weinberg equilibrium ($P=0.053$). The genotype
136 distribution for the SNP in TLR2 revealed that 117 individuals presented the T/T genotype (95,90%), followed by 5
137 presenting the T/C genotype. (4,10%). As for the alleles, 239 were T (97,95%) and 5 were C (2,05%) (Graph 1).

138 **Graph 1. Distribution of the T/T, T/C, C/C genotypes and of the T and C alleles for the SNP in TLR2.**



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140 Considering the distribution of SNP rs3804100 genotypes of the TLR-2 gene among the evaluated groups, the majority of
141 household contacts presented T/T genotype (68 – 94,4%) and only four the T/C genotype (5,56%). Among the LL group,
142 one individual presented the T/C genotype (4,00%), while the T/T genotype was detected among all TT individuals (Table
143 3).

144 **Table 3. Distribution of genotypes in the contact, LL, TT and patient groups for SNP rs3804100.**

GENOTYPE	CONTACT N (%)	LL N (%)	TT N (%)	PATIENT N (%)
T/C	4 (5,56%)	1 (4,00%)	0 (0,00%)	1 (2,00%)
T/T	68 (94,44%)	24 (96,00%)	25 (100,00%)	49 (98,00%)
TOTAL	72 (100,00%)	25 (100,00%)	25 (100,00%)	50 (100,00%)

146 $P>0.05$, Fisher's Exact Test
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Allele distribution for SNP rs3804100 among the groups demonstrated that all TT individuals had only the T allele (100%), while in LL there was only 1 C allele, mutant (2%). In the contact group there were only 4 C alleles (2,78%). In the patient group, the only C allele found represented 1% (Table 4).

Table 4. Allele distribution for the contact, LL, TT and patient groups for SNP rs3804100.

ALLELE	CONTACT N (%)	LL N (%)	TT N (%)	PATIENT N (%)
T	140 (97,22%)	49 (98,00%)	50 (100,00%)	99 (99,00%)
C	4 (2,78%)	1 (2,00%)	0 (0,00%)	1 (1,00%)
TOTAL	144 (100,00%)	50 (100,00%)	50 (100,00%)	100 (100,00%)

$P > 0.05$, Fisher's Exact Test

4. DISCUSSION

Even though not fatal, and despite the advances in therapy, diagnosis and research, leprosy remains as a global public health problem, being one of the most frequent causes of non-traumatic peripheral neuropathy worldwide. Leprosy reactions, as a result of acute episodes of clinical inflammation during the chronic course of the disease are a challenging issue as nerve damage increases morbidity even after treatment. In addition, its endemicity in tropical and developing countries is delimited by epidemiological, immunogenetic, socioeconomic and environmental risk factors [21].

Immunogenetic studies in relation with leprosy are essential to identify new markers (genes) in the genetic profile which may be associated with susceptibility and/or protection against the disease [22]. The innate immune response mediated by PRRs, such as TLR2, is the first line of host defense, which takes into account pathogen recognition. TLR2 is also required for the induction of IL-12, as the IL-12-dependent IFN- γ pathway is of paramount importance in cellular immunity, promoting Th1 response. The human TLR2 gene is located on chromosome 4 (4q32), consists of 3 exons, two coding and one non-coding [23]. TLR2 SNP rs3804100 has been described to affect and reduce exonic splicing sites, but still with uncertain role in TLR2 function [11].

Mycobacterium tuberculosis and *M. leprae* are mycobacteria that have common surface elements detected by the host. Krutzik et al., 2003 demonstrated that TLR1/2 heterodimers mediate monocyte activation promoted by *M. leprae*, indicating the presence of mycobacterial lipoproteins in addition to lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM), characterizing examples of TLR2 binding to mycobacterial components [24,25]. Thus, SNPs in the TLR2 gene may impair binding of the TLR1/2 heterodimer to mycobacterial lipoproteins, as in the case of SNP rs121917864, which promoted susceptibility to tuberculosis and leprosy in studies by Ben-Ali et al., 2004 and Kang and Chae, 2001 [10,18], respectively. The 19 kDa adhesion lipoprotein from *M. tuberculosis* shares 47% amino acid sequence similarity with the lipoprotein from *M. leprae* [26].

Genotyping studies have allowed associations between SNPs in the TLR gene and infectious diseases, such as those caused by mycobacteria. A study by Chen Y-C et al., 2010 in a Taiwanese population correlated the SNP rs3804100 and susceptibility to latent tuberculosis conferred by the CC genotype (in 4,3% of patients and in 4,3% of controls), which triggered elevation in the NK cell counts in the blood and, consequently, lead to disease progression [27]. Human NK cells have been reported to directly recognize mycobacteria through TLR2 and release TNF- α and IFN- γ [28]. However, these results are divergent in different populations due to distinction in the genetic makeup [29,30]. This genetic makeup corresponds to the genetic basis of the individual, which undergoes variations over time and due to environmental exposure [31].

There were no statistically significant associations between sex and leprosy, which was also observed among a Indian population in study by De Vries and Perry, 1985 [32]. However, most of the recent studies portray a discrepancy between the sexes: in Asian countries, leprosy affects men more than women, likewise in Brazilian studies, while in African countries women are more affected. Interestingly, among LL groups, African and Brazilian men were more affected [33–37]. The detection of leprosy among African women is probably explained by an aesthetics (body care) compared to men, as well as easier access to health services such as prenatal care [33]. The predominance of leprosy cases among men globally is probably due to the higher environmental exposure to *M. leprae* and/or the lack of women undergoing a complete physical examination in some cultures, leading to delayed diagnosis and the generation of physical disabilities [38].

In Brazil, studies by Rocha et al., 2020 and Romão and Mazzoni, 2013 revealed that higher detection of new cases are observed among those older than 46 years, likewise in the present study [39,40], which is probably linked e to the lower exposure of younger people to the bacillus and longer incubation period for LL cases [38]. Analysis regarding age highlight the involvement of the economically active population and low transmission of the disease, in addition to

203 contributing factors related to age-related immunological changes and higher susceptibility to infectious diseases among
204 elderly people. Despite this concerning fact, most of cases among elderly are excluded from the Global Health Strategies
205 for leprosy, making it difficult to conduct surveillance and control programs for the disease [41,42].

206 Since the 1960s, studies conducted in six different countries support the results obtained in the present study regarding
207 the protection conferred by the second dose of BCG vaccine against leprosy among the groups, as 12,5% of the group of
208 patients who took the 2nd dose in our study population [43,44]. In the present study, within the group of patients, about
209 50% had no BCG vaccination scar, revealing the lack of protective factor against the disease. The presence of a
210 vaccination scar was the most common finding among all the groups analyzed. The calculation of BCG vaccine efficacy
211 ranged from 0 to 50%, respectively, in Brazil and Malawi [45,46]. In India, the protection afforded by the first dose of BCG
212 vaccine was 14% in the general population and 80% in contacts with leprosy. Several case-control studies have been
213 conducted to evaluate the protective effect of BCG vaccine in leprosy, 12 of which included individuals with the first dose
214 [44,47–50]. Most of these studies were conducted with people in tuberculosis control programs. The overall protection
215 observed in these studies was between 36% and 90%, which is in line with the present study [48,50–53].

216 As similar in study by **Bochud P-Y** et al., 2008, the TLR2 SNPs rs3804099; rs3804100 were associated with leprosy
217 reverse reaction, involving Ethiopian population, and for rs3804100 no significant associations were observed ($P= 0.73$),
218 with CC genotype present in 0,2% of patients and 0% among controls, with C allele being found rarely [19], corroborating
219 the information in the present study about the lack of association between the studied SNP and leprosy. The T/C
220 genotype was also more detected in both studies in the LL group compared to the TT group, which means similarity
221 between contacts and TT due to lack of mutation in these groups, which could indicate that the T allele is related to
222 protection from the disease and/or its more severe form.

223 Furthermore, in vivo recognition of mycobacteria seems to depend on multiple TLRs, since mice lacking a single type of
224 receptor (such as TLR2) are only slightly more susceptible when exposed to aerosolized *M. tuberculosis* [54]. Another
225 study by Bafica et al., 2005 demonstrated that concomitant recognition of mycobacteria through TLR2 and TLR9
226 produced most or all of the stimuli required to induce protective antimycobacterial immune responses that depend on
227 TLRs [55].

228 The results of the present study have not shown associations between SNP rs3804100 and susceptibility or protection to
229 leprosy, also demonstrating that this genomic region is well preserved in the Brazilian Amazon population. Thus, due to
230 the low frequency of SNPs evaluated in the population investigated, further in-depth studies are required, in addition to the
231 investigation of other polymorphisms, in order to elucidate the relationship between SNP rs3804100 in the TLRs gene and
232 the immune response developed by the pathogen-host interaction.

233 5. CONCLUSION

234 In conclusion, no significant association between sex and leprosy was observed in this study. An association between age
235 group and leprosy was observed, which was significant among those older than 46 years. Correlation of 2nd dose of BCG
236 was found to be more protective among the groups analyzed. The allelic and genotypic frequencies of the SNPs found
237 were undifferentiated among the three study groups. No association was observed between the **SNP** rs3804100 of TLR2
238 and leprosy. A predominance of T/T genotype and T alleles was observed in all PB group individuals. The mutant
239 genotype, T/C was observed only in contacts and TT.
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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. MJAS was responsible for the conceptualization, formal analysis, investigation, methodology, statistical analysis, validation, visualization, writing, drafting and editing. ECS managed the analyses of the study. YCR performed the supervision, validation, visualization, writing, reviewing and editing. KVBL performed the supervision, validation, visualization, writing, reviewing and editing. LNGCL managed the conceptualization, investigation, methodology, protocol writing, project administration, supervision, visualization, writing, reviewing and editing. All authors read and approved the final manuscript.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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