Association of NOD2 and IFNG single nucleotide polymorphisms with leprosy in the Amazon ethnic admixed population

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Abstract

Leprosy is a chronic infectious disease, caused by Mycobacterium leprae, which affects skin and peripheral nerves. Polymorphisms in genes associated with autophagy, metabolism, innate and adaptive immunity confer susceptibility to leprosy. However, these associations need to be confirmed through independent replication studies in different ethnicities. The population from Amazon state (northern Brazil) is admixed and it contains the highest proportion of Native American genetic ancestry in Brazil. We conducted a case-control study for leprosy in which we tested fourteen previously associated SNPs in key immune response regulating genes: TLR1 (rs4833095), NOD2 (rs751271, rs8057341), TNF (rs1800629), IL10 (rs1800871), CCDC122/LACC1 (rs4942254), PACRG/PRKN (rs9356058, rs1040079), IFNG (rs2430561), IL6 (rs2069845), LRRK2 (rs7298930, rs3761863), IL23R (rs76418789) and TYK2 (rs5582956). Genotyping was carried out by allelic discrimination in 967 controls and 412 leprosy patients. Association with susceptibility was assessed by logistic regression analyses adjusted for the following covariates: gender, age and ancestry. Genetic ancestry was similar in case and control groups. Statistically significant results were only found for IFNG and NOD2. The rs8057341 polymorphism within NOD2 was identified as significant for the AA genotype (OR = 0.56; 95% CI, 0.37–0.84; P = 0.005) and borderline for the A allele (OR = 0.76; 95% CI, 0.58–1.00; P = 0.053) and carrier (OR = 0.76; 95% CI, 0.58–1.00; P = 0.051). The rs2430561 SNP in IFNG was associated with disease susceptibility for the AT genotype (OR = 1.40; 95% CI, 1.06–1.85; P = 0.018) and carrier (OR = 1.44; 95% CI, 1.10–1.88; P = 0.008). We confirmed that NOD2 and IFNG are major players in immunity against M.leprae in the Amazon ethnic admixed population.
Author summary

Leprosy is chronic infectious diseases caused by Mycobacterium leprae that affect the skin and peripheral nerves. The incidence is still high where approximately 200,000 new cases are diagnosed each year. There is no clear sign for early diagnosis and transmission is likely to occur before treatment, which, irrespective of its success, has not hampered stationary incidence in the past 20 years. Thus, there is pressing need for markers that discriminate exposure, infection and disease in order to better detect leprosy progression, control transmission and prevent disabilities. Here, we investigated whether polymorphisms located in eleven genes are associated to leprosy in a population from Amazon state (northern Brazil) which is admixed and it contains the highest proportion of Native American genetic ancestry in Brazil. We validated NOD2 and IFNG associations with resistance and risk of leprosy, respectively, in the Amazon ethnic admixed population. Genetic patterns of leprosy susceptibility could have an impact on the prognosis of individuals that are more likely to develop the disease (among household contacts, for example). Therefore, this strategy could identify high-risk individuals prone for prophylactic measures such as treatment with single-dose rifampicin and BCG vaccination.

Introduction

Two hundred thousand new cases of Leprosy are continuously diagnosed every year. The disease is caused by Mycobacterium leprae that has a long incubation period, leads to nerve damage and development of physical disabilities [1]. There is no clear sign for early diagnosis and transmission is likely to occur before treatment, which, irrespective of its success, has not hampered stationary incidence in the past 20 years. Thus, there is pressing need for markers that discriminate exposure, infection and disease in order to better detect leprosy progression, control transmission and prevent disabilities. Genetic factors have proved to be key components for leprosy outcome. Studies with monzygotic/dizygotic twins, family, population-based designs, and more recently, genome-wide association studies (GWAS) and whole exome sequencing (WES) have pinpointed single nucleotide polymorphisms (SNPs) in genes that have been consistently replicated in different populations [2–6]. There is evidence that NOD2, LRRK2, TLR1, TNF, IFNG, IL10, IL23R, TYK2 and PACRG/PRKN (formerly PARK2), which are genes that participate in autophagy and recognition pathways, regulating the host innate immune response are associated with leprosy susceptibility, reaction or its clinical forms [6–18]. However, genetic association of SNPs in major genes that modulate the immune response need independent replication in different ethnic groups to confirm leprosy outcome [19]. As observed in other regions, the Brazilian population from Amazon state is admixed, having three main ancestral contributions: Native Americans (NAM), Europeans (EUR) and Africans (AFR). Of these, the Native American contribution is highest, even within the urban populations of the region [20].

Genetic patterns of leprosy susceptibility could have an impact on the prognosis of individuals that are more likely to develop the disease (among household contacts, for example). Therefore, this strategy could identify high-risk individuals prone for prophylactic measures such as treatment with single-dose rifampicin and BCG vaccination [21–23].

Here, we investigated whether SNPs located in eleven genes: CCDC122/LACC1, IFNG, IL6, IL10, IL23R, LRRK2, NOD2, PACRG/PRKN, TLR1, TNF and TYK2 are associated to leprosy susceptibility in a population in the North of Brazil. To avoid spurious associations due to
population stratification, as in the case of admixed populations such as Brazilians, we included genetic ancestry estimates, gender and age, as covariates in the logistic regression analysis.

Materials and methods

Ethical statement

This study was approved by the Research and Ethic Committee (N°555.620) of the Alfredo da Matta Foundation. All participants signed an informed consent before enrolment. Parents or legal guardians provided consent for participants under the age of 18. This study was performed in accordance with the guidelines strengthening the reporting of genetic association studies (STREGA) [24].

Design and study population

We performed a case-control study involving individuals from Manaus, a city in the Brazilian state of Amazonas, at the Alfredo da Matta Foundation (FUAM). Patients and controls were recruited from March 2014 to March 2017 by spontaneous demand at FUAM. Patients with leprosy were classified according to the clinical and laboratorial findings (slit skin smears and histopathological examination) and were treated as paucibacillary or multibacillary, according to World Health Organization guidelines. The control group was composed of individuals who lived in the same endemic area of the cases. They were subjected to a dermatoneurological examination, had no suspected leprosy lesions and declared not having contact with leprosy or tuberculosis patients.

Determination of SNP for genotypic analysis

Several genes have been tested associated in populations although few SNPs were already extensively evaluated and consensus estimates were calculated in meta-analysis (S1 Table). The present study was designed to investigate the association of TLR1, NOD2, TNF, IL10, CCDC122/LACC1, PACRG/PRKN, IFNG, IL6, LRRK2, IL23R and TYK2 genes with leprosy. The SNPs TLR1 (rs4833095), NOD2 (rs751271, rs8057341), TNF (rs1800629), IL10 (rs1800871), CCDC122/LACC1 (rs4942254), PACRG/PRKN (rs9356058, rs1040079), IFNG (rs2430561) and IL6 (rs2069845) were selected based on previously published data which showed their association in the Brazilian population to leprosy per se [3, 7, 10, 11, 13, 25] or reaction [14]. Based in the previous studies in the Chinese population, we also selected LRRK2 (rs7298930, rs3761863) [8], IL23 (rs76418789) and TYK2 (rs55882956) [6]. SNPs were included from the promoter, exonic, intronic and chosen based on their functional role, as reported in literature. We excluded SNPs with a call rate < 95% or deviation from Hardy–Weinberg equilibrium proportions (P<0.01) in the controls.

DNA extraction, genotyping and genetic markers

DNA was extracted from frozen blood samples using DNeasy Blood & Tissue kit according to manufacturer’s instructions (QIAGEN). DNA was quantified and diluted for genotyping by real-time PCR with TaqMan probe assays (Life Technologies, EUA) (S2 Table). Assays consisted of 5 μL reactions containing 2.5 μL of TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems), 1.375 μL of water, 0.125 μL of the TaqMan assay (primers and probes), and 1 μL of DNA template (10–40 ng). Genotyping was carried out in the StepOne Plus real-time PCR system (Applied Biosystems), by fluorescence-based allelic discrimination following standard cycling conditions.
We used a panel of 46 ancestry-informative autosomal Indels (AIM-Indels) that were genotyped in a single multiplex PCR followed by capillary electrophoresis, as described by Pereira and coauthors (2012) [26], using an ABI 3500 Genetic Analyzer (Life Technologies). Alleles were conferred in GeneMapper v.4.1 software (Life Technologies). Ancestry estimates for each of the three main population components (EUR, NAM and AFR) were determined using STRUCTURE v2.3.3 software [27] and the HGDP-CEPH reference sample panel [28].

Statistical analysis

Linkage disequilibrium (LD) estimates for two SNPs in NOD2 and two SNPs in LRRK2 and deviations from Hardy–Weinberg equilibrium in the control group were performed using Haploview software, version 4.2 [29]. The estimated haplotypes was based on genotypes using an Expectation Maximization (EM) algorithm. Cases and controls were compared according to genotype, allele, and carrier frequencies, with and without adjustment for the variables: gender, age and ancestry (NAM and EUR, as continuous variables). Statistical analysis was performed using R software version 3.4.3 for Windows using “genetics” and “rms” packages (https://www.r-project.org) [30].

Results

Patients and controls were ten years of age or older. A total of 412 leprosy patients (284 males, 128 females) and 967 controls (526 males, 441 females) were recruited (Table 1). Mean age was significantly lower in control subjects than in cases (mean ± SD, 29.8 ± 9.94 vs. 43.3 ± 18.14 years; P < 0.0001). Likewise, the proportion of males and females was different between the two groups (P < 0.001), with significantly more males among leprosy patients (Table 1).

The analyzed population demonstrated typical ancestral characteristics of admixed populations for European, Native American and African ancestry (S1 Fig).

All SNPs in the control group were in Hardy–Weinberg equilibrium (HWE). The PACRG/PRKN SNP rs1040079 was excluded from the analysis because the call rate was <95%. There were no statistically significant differences between cases and controls for SNPs PACRG/PRKN, IL10, TNF, TLR1, CCDC122/LACC1, IL6, LRRK2, TYK2 and IL23R in the three genetic models (genotypic, allelic and carriers) (Table 2).

The statistical power of the sample size from the present study was also evaluated considering the minor allele frequency (MAF) obtained from each of the SNPs and the Odds Ratio (OR) association effects ranging from 1.5 up to 2.5. For most of the SNPs the assessment of

Table 1. General characteristics of leprosy patients and controls.

| Variables         | Patients n = 412 | Controls n = 967 |
|-------------------|-----------------|-----------------|
| Age               | 43.3 ± 18.14    | 29.8 ± 9.94     |
| Gender            |                 |                 |
| Male              | 284 (68.9%)     | 526 (54.4%)     |
| Female            | 128 (31.1%)     | 441 (45.6%)     |
| WHO classification|                 |                 |
| PB                | 133             | -               |
| MB                | 279             | -               |

WHO, World Health Organization. Paucibacillary (PB); Multibacillary (MB)
Student’s t-test for age between patients and control subjects P < 0.0001; Chi-squared test for gender P < 0.0001;
Data shown as mean ± standard deviation.
*Student’s t-test
*Chi-squared test
https://doi.org/10.1371/journal.pntd.0008247.t001
Table 2. Association of Allele, Genotype and Carrier frequencies of candidate genes with Leprosy.

| SNP          | GenotypeN (%) | ControlsN (%) | LeprosyN (%) | OR(95% CI) | P-value | Adjusted OR*(95% CI) | P-value |
|--------------|---------------|---------------|--------------|------------|---------|----------------------|---------|
| rs8057341 NOD2 | AA 182 (19)   | 52 (13)       | 0.56 (0.39–0.80) | 0.0017     | 0.56 (0.37–0.84) | 0.0052 |
|              | GA 446 (47)   | 186 (46)      | 0.82 (0.64–1.05) | 0.1220     | 0.85 (0.63–1.13) | 0.2547 |
|              | GG^b 328 (34) | 167 (41)      |              |            |         |                      |         |
|              | Total         | 956           | 406          |            |         |                      |         |
| Allele       | A 0.42        | 0.36          | 0.76 (0.60–0.96) | 0.0243     | 0.76 (0.58–1.00) | 0.0529 |
|              | G^b 0.58      | 0.64          |              |            |         |                      |         |
| Carriers     | AA/GA 628 (66)| 239 (59)      | 0.74 (0.59–0.95) | 0.0153     | 0.76 (0.58–1.00) | 0.0512 |
| HWE          | 0.1886        |               |              |            |         |                      |         |
| rs751271 NOD2 | TT 203 (21.0)| 61 (15.0)     | 0.63 (0.44–0.89) | 0.0093     | 0.67 (0.44–1.01) | 0.0561 |
|              | GT 468 (49.0)| 208 (51.0)    | 0.93 (0.71–1.20) | 0.5738     | 0.93 (0.68–1.27) | 0.6690 |
|              | GG^b 286 (30.0)| 137 (34.0)|              |            |         |                      |         |
|              | Total         | 957           | 406          |            |         |                      |         |
| Allele       | T 0.46        | 0.41          | 0.81 (0.64–1.03) | 0.0879     | 0.84 (0.63–1.10) | 0.2092 |
|              | G^b 0.54      | 0.59          |              |            |         |                      |         |
| Carriers     | TT/GT 671 (70.0)| 269 (66.0)| 0.84 (0.65–1.07) | 0.1593     | 0.85 (0.64–1.15) | 0.2976 |
| HWE          | 0.7007        |               |              |            |         |                      |         |
| rs2430561 IFNG | TT 55 (6.0)  | 29 (7.0)     | 1.39 (0.87–2.25) | 0.1713     | 1.67 (0.97–2.89) | 0.0641 |
|              | AT 341 (35.0)| 164 (40.0)    | 1.27 (0.99–1.62) | 0.0531     | 1.40 (1.06–1.85) | 0.0184 |
|              | AA 566 (59.0)| 214 (53.0)    |              |            |         |                      |         |
|              | Total         | 962           | 407          |            |         |                      |         |
| Allele       | T 0.23        | 0.27          | 1.22 (0.94–1.59) | 0.1326     | 1.34 (0.99–1.81) | 0.0584 |
|              | A^b 0.77      | 0.73          |              |            |         |                      |         |
| Carriers     | TT/AT 396 (41)| 193 (47)      | 1.29 (1.02–1.63) | 0.0328     | 1.44 (1.10–1.88) | 0.0083 |
| HWE          | 0.7681        |               |              |            |         |                      |         |
| rs9356058 PACRG/ PRKN | CC 89 (9)  | 43 (11)     | 1.32 (0.88–1.98) | 0.1764     | 1.36 (0.86–2.15) | 0.1878 |
|              | TC 382 (41)  | 193 (48)     | 1.38 (1.08–1.77) | 0.0101     | 1.27 (0.96–1.69) | 0.0880 |
|              | TT^b 465 (50)| 170 (42)     |              |            |         |                      |         |
|              | Total         | 936           | 407          |            |         |                      |         |
| Allele       | T 0.70        | 0.66          | 0.81 (0.64–1.04) | 0.1069     | 0.83 (0.63–1.10) | 0.2011 |
|              | G^b 0.30      | 0.34          |              |            |         |                      |         |
| Carriers     | CC/TC 471 (50)| 236 (58)      | 1.37 (1.08–1.73) | 0.0086     | 1.29 (0.99–1.68) | 0.0603 |
| HWE          | 0.4600        |               |              |            |         |                      |         |
| rs1800871 IL10 | AA 143 (15.0)| 60 (15.0)    | 1.05 (0.73–1.50) | 0.7925     | 0.93 (0.62–1.39) | 0.7317 |
|              | GA 443 (46.0)| 196 (48.0)    | 1.11 (0.86–1.42) | 0.4351     | 0.94 (0.70–1.25) | 0.6551 |
|              | GG^b 375 (39.0)| 150 (37.0)|              |            |         |                      |         |
|              | Total         | 961           | 406          |            |         |                      |         |
| Allele       | A 0.38        | 0.39          | 1.04 (0.82–1.32) | 0.7315     | 0.96 (0.73–1.26) | 0.7599 |
|              | G^b 0.62      | 0.61          |              |            |         |                      |         |
| Carriers     | AA/AG 586 (61.0)| 256 (63.0)| 1.09 (0.86–1.39) | 0.4709     | 0.93 (0.71–1.23) | 0.6303 |
| HWE          | 0.5605        |               |              |            |         |                      |         |
| rs1800629 TNF | AA 7 (1.0)   | 1 (0.0)      | 0.33 (0.04–2.66) | 0.2954     | 0.14 (0.01–1.79) | 0.1291 |
|              | GA 135 (14.0)| 50 (12.0)    | 0.84 (0.60–1.20) | 0.3431     | 0.78 (0.53–1.16) | 0.2226 |
|              | GG^b 824 (85.0)| 361(88.0)|              |            |         |                      |         |
|              | Total         | 966           | 412          |            |         |                      |         |
| Allele       | A 0.08        | 0.06          | 0.81 (0.51–1.28) | 0.3604     | 0.74 (0.43–1.25) | 0.2559 |
|              | G^b 0.92      | 0.94          |              |            |         |                      |         |
| Carriers     | AA/GA 142 (15.0)| 51 (12.0)| 0.82 (0.58–1.15) | 0.2562     | 0.75 (0.51–1.11) | 0.1536 |

(Continued)
| SNP            | Genotype (%) | Controls (%) | Leprosy (%) | OR (95% CI) | P-value | Adjusted OR (95% CI) | P-value |
|----------------|--------------|--------------|-------------|-------------|---------|----------------------|---------|
|                | HWE          |              |             |             |         |                      |         |
| rs4833095 TLR1 |              |              |             |             |         |                      |         |
|                | HWE          | 0.7331       |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | T            | 0.47         | 0.50        | 1.10 (0.87–1.39) | 0.4173  | 1.06 (0.81–1.37)     | 0.6841  |
|                | C            | 0.53         | 0.50        |             |         |                      |         |
|                | Carriers     | TT/CT        | 695 (72.0)  | 30 (75.0)   | 1.13 (0.86–1.47) | 0.3770  | 1.04 (0.77–1.40)     | 0.8111  |
|                | HWE          | 0.7851       |             |             |         |                      |         |
| rs2069845 IL6  |              |              |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | G            | 0.30         | 0.32        | 1.06 (0.83–1.37) | 0.6208  | 1.04 (0.78–1.38)     | 0.7806  |
|                | A            | 0.70         | 0.68        |             |         |                      |         |
|                | Carriers     | GG/AG        | 494 (51.0)  | 220 (54.0)  | 1.13 (0.90–1.43) | 0.2985  | 1.09 (0.83–1.42)     | 0.5284  |
|                | HWE          | 0.5600       |             |             |         |                      |         |
| rs4942254 CCDC122/ LACC1 | |              |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | C            | 0.36         | 0.37        | 1.07 (0.84–1.36) | 0.6040  | 1.09 (0.82–1.44)     | 0.5486  |
|                | T            | 0.64         | 0.63        |             |         |                      |         |
|                | Carriers     | CC/CT        | 538 (57.0)  | 242 (61.0)  | 1.12 (0.88–1.42) | 0.3505  | 1.18 (0.90–1.56)     | 0.2276  |
|                | HWE          | 0.2849       |             |             |         |                      |         |
| rs7298930 LRRK2|              |              |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | A            | 0.36         | 0.37        | 1.03 (0.81–1.32) | 0.7807  | 1.06 (0.81–1.40)     | 0.6604  |
|                | C            | 0.64         | 0.63        |             |         |                      |         |
|                | Carriers     | AA/AC        | 543 (58.0)  | 247 (61.0)  | 1.10 (0.87–1.39) | 0.4399  | 1.13 (0.86–1.47)     | 0.3863  |
|                | HWE          | 0.471        |             |             |         |                      |         |
| rs3761863 LRRK2|              |              |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | T            | 0.47         | 0.44        | 0.88 (0.70–1.12) | 0.3012  | 0.95 (0.72–1.24)     | 0.7067  |
|                | C            | 0.53         | 0.56        |             |         |                      |         |
|                | Carriers     | TT/CT        | 675 (71.0)  | 265 (69.0)  | 0.87 (0.68–1.13) | 0.3044  | 0.95 (0.71–1.27)     | 0.7352  |
|                | HWE          | 0.3334       |             |             |         |                      |         |
| rs55882956 TYK2|              |              |             |             |         |                      |         |
|                | Allele       |              |             |             |         |                      |         |
|                | A            | 0.001        | 0.002       | 2.36 (0.15–37.8) | 0.5439  | 4.69 (0.26–83.5)     | 0.2932  |
|                | G            | 0.999        | 0.998       |             |         |                      |         |
|                | Carriers     | AA/AG        | 2 (0.21)    | 2 (0.49)    | 2.36 (0.33–6.84) | 0.3903  | 4.71 (0.61–36.3)     | 0.1366  |

(Continued)
power attained 0.80 for the tested values, except for TNF SNP that was lower (0.52). The TYK2 and IL23R SNPs had a rare frequency that resulted in power of less than 10%. Since no statistically significant associations were found for clinical forms comparison (PB vs MB and Controls vs MB) we did not include this power analysis.

The genotype, allele and carrier frequencies of NOD2 rs8057341 confirmed association with protection from leprosy. The rs8057341 polymorphism was identified as significant for the AA genotype (OR = 0.56; 95% CI, 0.37–0.84; P = 0.005) and borderline for the A allele (OR = 0.76; 95% CI, 0.58–1.00; P = 0.053) and AA/GA carriers (OR = 0.76; 95% CI, 0.58–1.00; P = 0.051). The rs751271 polymorphism was identified as borderline for the TT genotype (OR = 0.67; 95% CI, 0.44–1.01; P = 0.056) (Table 2). High LD (r² = 0.83) revealed dependent association for these two SNPs. Analysis of NOD2 SNPs haplotypes (rs751271-rs8057341) were statistically significant for the T-A combination (OR = 0.79; 95% CI, 0.64–0.97; P = 0.0226), T-G combination (OR = 1.91; 95% CI, 1.21–3.01; P = 0.0055) and G-A (OR = 3.88; 95% CI, 1.50–10.04; P = 0.0052) (S3 Table).

SNP rs2430561 in the IFNG gene was associated with disease susceptibility in AT heterozygotes (OR = 1.40; 95% CI, 1.06–1.85; P = 0.018) and TT/AT carriers (OR = 1.44; 95% CI, 1.10–1.88; P = 0.008) (Table 2).

SNPs in the LRRK2 gene showed a weak LD (r² = 0.18). Analysis of LRRK2 SNPs haplotypes was not statistically significant. The C-T combination (rs7298930—rs3761863) was shown to be borderline (P = 0.07), but lost association when adjusted for co-variates (OR = 0.78; 95% CI, 0.58–1.06; P = 0.1095) (S4 Table). Also, we tested rs9356058 in the PACRG/PRKN genes and observed association when TC/CC carriers were evaluated, however, this was not sustained following co-variante adjustment (OR = 1.29; 95% CI, 0.99–1.68; P = 0.0603) (Table 2).

**Discussion**

Genetic association studies in population designs, such as case-control, can lead to spurious associations, especially in admixed populations [31], which may confer false-positive results and the risk of developing a disease [32,33]. In our study, to reduce the chance of bias, we performed data adjustment by age, gender as well as ancestry. The current profile of the genetic ancestry of the Amazonas population began to form during the Portuguese colonization in the mid-16th century [34] and continued with the exploration of natural resources, primarily rubber [35]. Construction of the Transamazon Highway and the establishment of an Industrial Park (Manaus Duty Free Zone) in the 1960s also contributed to a huge migratory flow of
Brazilians from different regions of the country [36]. The populations analyzed in our study had similar ancestral contributions in both case and control groups, which is ideal in genetic association studies. Nevertheless, the data was highly impacted by age adjustment since cases and controls had very different mean ages.

We validated NOD2 and IFNG associations with resistance and risk of leprosy, respectively, in the Amazon ethnic admixed population. The NOD2 gene encodes an intracellular sensing molecule, which, along with NOD1, recruits ATG16L1 to the plasma membrane to initiate autophagy of bacteria entering the host cell [37]. Similarly, Sales-Marques and coauthors (2014) [7], in a meta-analysis with population samples from different Brazilian regions combining case-control and family-based studies, confirmed that the A allele of NOD2 (rs8057341) is a genetic resistance factor for leprosy. On the other hand, in the first GWAS in the Chinese population [4], the G allele (MAF = 0.22) of SNP rs8057341 was associated with disease risk. However, two other studies failed to validate this SNP in three populations: Indian, African and Vietnamese [5,38].

The SNP rs9356058 in PACRG/PRKN, for the T allele, points to protection, although statistical significance did not reach the threshold level. Although the specific function of the PACRG gene has not been elucidated [9], together with PRKN, they share a regulatory region and participate in the proteolytic system mediated by ubiquitin for the clearance of damaged biomolecules (lipids and proteins) and organelles [9,39]. The PRKN gene encodes the Parkin protein, an E3 Ubiquitin Ligase, the last sequential enzyme in the ubiquitination process [40]. Recently, the relationship of this protein with the innate immune response of the host, known as xenophagy, which is the degradation of intracellular pathogens [41], as described for M. tuberculosis [42], Chlamydia [43] and M. leprae [44] has been identified. Polymorphic variants in the PRKN gene were initially associated with the autosomal recessive form of Parkinson’s disease (AR-JP) [45]. In 2004, Mira and coauthors [3] identified polymorphisms in the promoter region shared by PACRG/PRKN genes associated with leprosy per se in Southern Brazilians and Vietnamese. In this same study, the common T allele of rs9356058 was associated with the risk of leprosy. Later, Alter and coauthors confirmed that this variant (T allele of rs9356058) is a risk factor for the disease in the Vietnamese and Indian populations [46]. In this study, PRKN analysis suggested that genetic association is higher in children/youths, when data is stratified for age. Since our population was older (40 years old) and the mean age of the control group varied, statistical adjustments might explain different results. The lack of consensus among ours and previous studies for the PRKN region is probably due to distinct patterns of LD in the analyzed populations, even within the same country, leading to differences in allele and haplotype frequencies of the studied SNPs, as previously observed for lymphotoxin-alpha [47]. It is likely that NAM ancestry could explain this pattern. Noteworthy, studies in Indian and Chinese populations failed to replicate the association of these PRKN polymorphisms with leprosy [48, 49].

Interferon gamma (IFNG) is one of the most significant cytokines involved in the protective immune response against mycobacterial infection and is secreted mainly by CD4+ Th1, CD8 + T cytotoxic and natural killer cells [50]. In synergy with TNF, it activates microbicide effector mechanisms in human macrophages [51]. IFNg expression may be influenced by the polymorphism present in the first intron of IFNG +874 T>A rs2430561, probably since this locus coincides with the binding site of the NF-kB transcription factor [52]. This polymorphism has been associated in meta-analysis studies with leprosy [24], tuberculosis [53] and Hepatitis [50]. Our results indicated T allele carriers have an increased risk of leprosy (P = 0.0083). A new meta-analysis would indicate a consensus estimate for this SNP. In studies that show an association, the presence of the T allele correlates with high IFNG expression and increased resistance to infection whereas the A allele correlates with low expression [54–56]. On the other
hand, in vitro clinical trials showed that interferon levels were not statistically different between T carriers and AA genotype, in the presence of M. leprae antigens [57].

We did not validate associations for SNPs in TLR1, TNF, PACRG/PRKN, IL10, CCDC122/LACC1, IL6, LRRK2, IL23R, and TYK2 genes. Nevertheless, some of these SNPs, such as rs3761863 in LRRK2 had been associated with a type-1 reaction, which is considered an endo-phenotype of leprosy [58]. These SNPs could possibly play a role in the uncontrolled inflammatory phenotype throughout the natural course of the disease. Recently, rare LRRK2 SNPs were confirmed as associated with either leprosy type-1 reactions or Parkinson´s Disease [59]. Hence, we also tested SNPs from TYK2 and IL23R, both low-frequency variants that were described in the Chinese population [6]. In the present Amazon population sample, these variants were rare leading to a low detection power of association with leprosy. Although significance was not found, TYK2 heterozygote frequency was two times higher among patients. However, we cannot rule out the possibility of other rare or common variants of IL23R or TYK2 being related to leprosy susceptibility in the Amazon population.

The few common SNPs that showed association with leprosy and the modest odds ratios values presented demonstrate the difficulty of unraveling the major genes involved in leprosy. Genome-wide association studies and exome analysis could possibly improve the ability to describe novel rare SNPs and call on a combination of different genotypes to explain one complex phenotype. It is likely that this can help define genetic variants and understand their role in the pathophysiology of leprosy, contributing to either diagnosis or treatment [39].

Supporting information

S1 Fig. Individual ancestry estimates obtained for the HGDP-CEPH reference samples and individuals tested from CASES (Leprosy) and CONTROLS (Endemic Control) STUDY using 46 AIM-Indels (AFR: African; EUR: European; NAM: Native American).

S1 Table. Summary of candidate genes of immune response in the case-controls studies in Leprosy.

S2 Table. Genotyping assays used for allelic discrimination.

S3 Table. Haplotypes of the intron region of NOD2 present in the study population.

S4 Table. Haplotypes of the LRRK2 present in the study population.

Acknowledgments

To all participants of this study, lab and clinical staff of Alfredo da Matta Foundation, a Reference Center for Diagnostics, Therapeutics and Research, Manaus, state of Amazonas, Brazil.

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References
1. Britton WJ, Lockwood DN. Leprosy. Lancet. 2004; 363:1209–1219. https://doi.org/10.1016/S0140-6736(04)15952-7 PMID: 15081655

2. Chakravartti MR, Vogel F. A twin study on leprosy. In: Becker PE, Lenz W, Vogel F, Wendt GG, editors. Topics in Human Genetics. Stuttgart: Thieme; 1973. pp. 1–29.

3. Mira MT, Alcaí A, Nguyen VT, Moraes MO, Di Flumeri C, et al. Susceptibility to leprosy is associated with PARK2 and PACRG. Nature 2004; 427:636–640. https://doi.org/10.1038/nature02326 PMID: 14737177

4. Zhang FR, Huang W, Chen SM, Sun LD, Liu H, Li Y, et al. Genomewide association study of leprosy. N Engl J Med. 2009; 361(27):2609–2618. https://doi.org/10.1056/NEJMoa0903753 PMID: 20018961

5. Wong SH, Hill AV, Vannberg FO, India–Africa–United Kingdom Leprosy Genetics Consortium. Genomewide association study of leprosy. N Engl J Med. 2010; 362(15):1446–1447. https://doi.org/10.1056/NEJMct1001451 PMID: 20393182

6. Liu H, Wang Z, Li Y, Yu G, Fu X, et al. Geno-Wide Analysis of Protein-Coding Variants in Leprosy. J Invest Dermatol. 2017; 137(12): 2544–2551; https://doi.org/10.1016/j.jid.2017.08.004 PMID: 28842327

7. Sales-Marques C, Salomão H, Fava VM, Alvarado-Arnez LE, Pinheiro EP, et al. NOD2 and CCDC122-LACC1 genes are associated with leprosy susceptibility in Brazilians. Hum Genet. 2014; 133(12):1525–1532. https://doi.org/10.1007/s00439-014-1502-9 PMID: 25367361

8. Wang D, Xu L, Lv L, Su LY, Fan Y, Zhang DF, et al. Association of the LRRK2 genetic polymorphisms with leprosy in Han Chinese from Southwest China. Genes Immun. 2015; 16(2):112–119. https://doi.org/10.1038/gene.2014.72 PMID: 25521227

9. Chopra R, Ali S, Srivastava AK, Aggarwal S, Kumar B, Manvati S, et al. Mapping of PARK2 and PACRG overlapping regulatory region reveals LD structure and functional variants in association with leprosy in unrelated Indian population groups. PLoS Genet. 2013; 9(7). Available from: http://www.journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1003578

10. De Sales Marques C, Brito-De-Souza VN, Guerreiro LTA, Martins JH, Amaral EP, Cardoso CC, et al. Toll-like receptor 1 N248s single-nucleotide polymorphism is associated with leprosy risk and regulates immune activation during mycobacterial infection. J Infect Dis. 2013; 208(1):120–129. https://doi.org/10.1093/infdis/jit133 PMID: 23547143

11. Areeshi MY, Mandal RK, Dar SA, Jawed A, Wahid M, Lohani M, et al. Impact of TNF -308 G>A (rs1800629) gene polymorphism in modulation of leprosy risk: a reappraise meta-analysis of 14 case-control studies. Biosci Rep. 2017; 37(5). Available from: http://www.bioscirep.org/content/37/5/BSR20178086

12. Wang D, Feng JQ, Li YY, Zhang DF, Li XA, Li QW, et al. Genetic variants of the MRC1 gene and the IFNG gene are associated with leprosy in Han Chinese from Southwest China. Hum Genet. 2012; 131(7):1251–1260. https://doi.org/10.1007/s00439-012-1153-7 PMID: 22932681

13. Alvarado-Arnez LE, Amaral EP, Sales-Marques C, Duães SM, Cardoso CC, Nunes Sarno E, et al. Association of IL10 Polymorphisms and Leprosy: A Meta-Analysis. PLoS One. 2015; 10(9). Available from: http://www.plosone.org/plosone/article?id=10.1371/journal.pone.0136282

14. Sales-Marques C, Cardoso CC, Alvarado-Arnez LE, Illarandi X, Sales AM, Hacker MdA, et al. Genetic polymorphisms of the IL6 and NOD2 genes are risk factors for inflammatory reactions in
15. Berrington WR, Macdonald M, Khadge S, Sapkota BR, Janer M, Hagge DA, Kaplan G, and Hawn TR. Common polymorphisms in the NOD2 gene region are associated with leprosy and its reactive states. J Infect Dis. 2010; 191(9): 1422–1435. https://doi.org/10.1086/615599 PMID: 20350193

16. Schuring RP, Hamann L, Faber WR, Pahan D, Richards JH, Schumann RR, et al. Polymorphism N248S in the human Toll-like receptor 1 gene is related to leprosy and leprosy reactions. J Infect Dis. 2009; 199(12): 1816–1819. https://doi.org/10.1086/599121 PMID: 19456232

17. Santos AR, Suffys PN, Vanderborght PR, Moraes MO, Vieira LM, Cabello PH, et al. Role of tumor necrosis factor-alpha and interleukin-10 promoter gene polymorphisms in leprosy. J Infect Dis. 2002; 186(11): 1687–1691. https://doi.org/10.1086/345366 PMID: 12447749

18. Silva GAV, Santos MP, Mota-Passos I, et al. IFN-gamma +875 microsatellite polymorphism as a potential protection marker for leprosy patients from Amazonas state, Brazil. Cytokine. 2012; 60(2): 493–497. https://doi.org/10.1016/j.cytog.2012.04.043 PMID: 22683002

19. Cardoso CC, Pereira AC, de Sales Marques C, Moraes MO. Leprosy susceptibility: genetic variations regulate innate and adaptive immunity, and disease outcome. Future Microbiol. 2011; 6(5): 533–549. https://doi.org/10.2217/fmb.11.39 PMID: 21585261

20. Manta FSN, Pereira R, Vianna R, Araújo ARB, Gitaí DLG, et al. Revisiting the genetic ancestry of Brazilians using autosomal AIM-Indels. PloS One. 2013; 8(9). Available from: http://www.journals.plos.org/plosone/article?id=10.1371/journal.pone.0071545

21. Moet FJ, Pahan D, Oskam L, Richardus JH, COLEP Study Group. Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ. 2008; 336(7647): 761–764. https://doi.org/10.1136/bmj.39500.885752.BE PMID: 18332051

22. Duthie MS, Balagon MF. Combination chemoprophylaxis and immunoprophylaxis in reducing the incidence of leprosy. Risk Manag Healthc Policy. 2016; 9: 43–53. https://doi.org/10.2147/RMHP.S76058 PMID: 27175099

23. Guidelines for the diagnosis, treatment and prevention of leprosy. New Delhi: World Health Organization, Regional Office for South-East Asia; 2017 https://www.who.int/lep/resources/9789290226383/en/

24. Little J, Higgins JP, Ioannidis JP, Moher D, Gagnon F, et al. STrengthening the REporting of Genetic Association Studies (STREGA): an extension of the STROBE Statement. PLoS Med. 2009; 6(2): 151–163. https://doi.org/10.1371/journal.pmed.1000022 PMID: 19192942

25. Silva GAV, Naveca FG, Ramosawmy R, Boechat AL. Association between the IFNG +874A/T gene polymorphism and leprosy resistance: A meta-analysis. Cyto. 2014; 65(2): 130–133. https://doi.org/10.1016/j.cyto.2013.12.002 PMID: 24389160

26. Pereira R, Philips C, Pinto N, Santos C, dos Santos SE, et al. Straightforward Inference of Ancestry and Individual Interethnic Admixture and Population Substructure Using a 48–Insertion-Deletion (INDEL) Ancestry-Informative Marker (AIM) Panel. Hum Mutat. 2010; (2): 184–190. https://doi.org/10.1002/humu.21159 PMID: 19953531

27. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155(2): 945–959. PMID: 10835412

28. Cann HM, de Toma C, Cazes L, Legrand MF, Morel V, et al. A human genome diversity cell line panel. Science. 2002; 296(5566): 261–262. https://doi.org/10.1126/science.296.5566.261b PMID: 11954565

29. Barrett JC, Fry B, Maller J, Daly MJ. Haploview : analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21: 263–265. https://doi.org/10.1093/bioinformatics/bth457 PMID: 15297300

30. R Core Team (2017). R: A language and environment for statistical computing. Vienna, Austria. https://www.r-project.org/.

31. Bhaskar A, Javanmard A, Courtade T, Ade D. Novel probabilistic models of spatial genetic ancestry with applications to stratification correction in genome-wide association studies. Bioinformatics. 2017; 33(6): 879–885. https://doi.org/10.1093/bioinformatics/btw720 PMID: 28025204

32. Kittles RA, Chen W, Panguluri RK, Abahgotu C, Jackson A, et al. CYP3A4-V and prostate cancer in African Americans: causal or confounding association because of population stratification? Hum Genet. 2002; 110(6): 553–560. https://doi.org/10.1007/s00439-002-0731-5 PMID: 12107441

33. Santos NP, Ribeiro-Rodrigues EM, Ribeiro do Santos AK, Pereira R, Gusmão L, et al. Assessing Individual Interethnic Admixture and Population Substructure Using a 48–Insertion-Deletion (INDEL) Ancestry-Informative Marker (AIM) Panel. Hum Mutat. 2010; (2): 184–190. https://doi.org/10.1002/humu.21159 PMID: 19953531

34. Mendes CMM. A questão da colonização do Brasil: Historiografia e documentos. Imagens Educ. 2012; 2(2): 1–13. https://doi.org/10.4025/imagenseduc.v2i2.172929
35. Afonso L. Panorama da cidade de Manaus: crise, progresso e cultura na década de 1960. SOMANLU. 2010; 10(2): 45–60. https://doi.org/10.17563/somanlu.v10i2.491

36. Murrie ZDF. História e Geografia: livro do estudante: ensino fundamental. In: Da Costa GP, editors. Mudanças no espaço geográfico do Brasil. Brasília: MEC: INEP; 2006. pp 33–40.

37. Travassos LH, Carneiro LAM, Ramjeet M, Husseym S, Kim Y-G, Magalhães JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol. 2010; (1): 55–62. https://doi.org/10.1038/ni.1823 PMID: 19989471

38. Grant AV, Alter A, Huong NT, et al. Crohn's disease susceptibility genes are associated with leprosy in the Vietnamese population. J Infect Dis. 2012; 206(11): 1763–1767. https://doi.org/10.1093/infdis/jis588 PMID: 22984114

39. Cambri G and Mira MT. Genetic Susceptibility to Leprosy—From Classic Immune-Related Candidate Genes to Hypothesis-Free, Whole Genome Approaches. Front Immunol. 2018; 9:1674. Available from: https://www.frontiersin.org/articles/10.3389/ffimmu.2018.01674/full PMID: 30079096

40. Seirafi M, Kozlov G, Gehring K. Parkin structure and function. FEBS J. 2015; 282(11): 2076–2088. https://doi.org/10.1111/febs.13249 PMID: 25712550

41. Huang J, Brumell JH. Bacteria-autophagy interplay: a battle for survival. Nat Rev Microbiol. 2014; 12(41–42). http://www.nature.com/articles/nrmicro3160 PMID: 24384599

42. Watson RO, Manznillo PS, Cox JS. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell. 2012; 150(4): 803–815. https://doi.org/10.1016/j.cell.2012.06.040 PMID: 22901810

43. Radomski N, Kagebein D, Liebler-Tenorio E, Karger A, Rufer E, Tews BA, et al. Mito-xenophagic killing by activating the host DNA-sensing pathway. Cell. 2012; 150(4): 803–815. https://doi.org/10.1016/j.cell.2012.06.040 PMID: 22901810

44. Li J, Liu H, Liu J, Fu X, Yu Y, et al. Association study of the single nucleotide polymorphisms of PARK2 and PACRG genes with leprosy in an Indian population. Eur J Hum Genet. 2006; 14(4): 438–442. https://doi.org/10.1038/sj.ejhg.5201563 PMID: 16391553

45. Sun Y, Lu Y, Li T, Xie L, Deng Y, Li S, et al. Interferon Gamma +874T/A Polymorphism Increases the Risk of Hepatitis Virus-Related Diseases: Evidence from a Meta-Analysis. PLoS One. 2015; 10(5). Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0121168

46. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. J Immunol. 2010; 185(1): 15–22. https://doi.org/10.4049/jimmunol.0903856 PMID: 20526268

47. Heinmeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Anako EA, Podkolenina OA, Kolpakov FA, Podkolodny NL, Kolchanov NA: Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. 1998; 26(1):362–367. https://doi.org/10.1093/nar/26.1.362 PMID: 9399875

48. Wei Z, Wenhao S, Yuanuyan M, Yang L, Daming Z, Jiangchun X, Jijun J. A single nucleotide polymorphism in the interferon-γ gene (IFNG +874 T/A) is associated with susceptibility to tuberculosis. Onco-target. 2017; 8(31): 50415–50429. https://doi.org/10.18632/oncotarget.17304 PMID: 28881572

49. Vallinoto ACR, Graca ES, Araujo MS, Azvedo VN, Cayres-Vallinoto I, Machado LFA, et al. IFNG +874T/A polymorphism and cytokine plasma levels are associated with susceptibility to mycobacterium tuberculosis infection and clinical manifestation of tuberculosis. Hum Immunol. 2010; 71(7):692–696. https://doi.org/10.1016/j.humimm.2010.03.008 PMID: 20353805
55. López-Maderuelo D, Arnalich F, Serantes R, González A, Codoceo R, Madero R, et al. Interferon-gamma and interleukin-10 gene polymorphisms in pulmonary tuberculosis. Am J Respir Crit Care Med. 2003; 167(7):970–975. https://doi.org/10.1164/rccm.200205-438BC PMID: 12531774

56. Sallakci N, Coskun M, Berber Z, Gurkan F, Kocamaz H, Uysal G, Bhuju S, Yavuzer U, Singh M, Yegin O. Interferon-gamma gene +874T-A polymorphism is associated with tuberculosis and gamma interferon response. Tuberculosis (Edinb). 2007; 87(3):225–230. https://doi.org/10.1016/j.tube.2006.10.002 PMID: 17276141

57. Cardoso CC, Pereira AC, Brito-de-Souza VN, Dias-Baptista IM, Maniero VC, Venturini J, et al. IFNG +874 T>A single nucleotide polymorphism is associated with leprosy among Brazilians. Hum Genet. 2010; 128(5):481–490. https://doi.org/10.1007/s00439-010-0872-x PMID: 20714752

58. Fava VM, Manry J, Cobat A, Orlova M, Van Thuc N, Ba NN, Thai VH, Abel L, Alcaïs A, Schurr E. A Missense LRRK2 Variant Is a Risk Factor for Excessive Inflammatory Responses in Leprosy. PLoS Negl Trop Dis. 2016; 10(2). Available from: https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0004412

59. Fava VM, Xu YZ, Lettre G, Van Thuc N, Orlova M, Thai VH, Tao S, Croteau N, Eldeeb MA, MacDougall EJ, Cambri G, Lahiri R, Adams L, Fon EA, Trempe JF, Cobat A, Alcaïs A, Abel L, Schurr E. Pleiotropic effects for Parkin and LRRK2 in leprosy type-1 reactions and Parkinson’s disease. Proc Natl Acad Sci U S A. 2019; 116(31):15616–15624. https://doi.org/10.1073/pnas.1901805116 PMID: 31308240