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Investigation of Host Candidate Malaria-Associated Risk/Protective SNPs in a Brazilian Amazonian Population

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Abstract

The Brazilian Amazon is a hypo-endemic malaria region with nearly 300,000 cases each year. A variety of genetic polymorphisms, particularly in erythrocyte receptors and immune response related genes, have been described to be associated with susceptibility and resistance to malaria. In order to identify polymorphisms that might be associated with malaria clinical outcomes in a Brazilian Amazonian population, sixty-four human single nucleotide polymorphisms in 37 genes were analyzed using a Sequenom massARRAY iPLEX platform. A total of 648 individuals from two malaria endemic areas were studied, including 535 malaria cases (113 individuals with clinical mild malaria, 122 individuals with asymptomatic infection and 300 individuals with history of previous mild malaria) and 113 health controls with no history of malaria. The data revealed significant associations (p<0.003) between one SNP in the IL10 gene (rs1800896) and one SNP in the TLR4 gene (rs4986790) with reduced risk for clinical malaria, one SNP in the IRF1 gene (rs2706384) with increased risk for clinical malaria, one SNP in the LTA gene (rs909253) with protection from clinical malaria and one SNP in the TNF gene (RS1800750) associated with susceptibility to clinical malaria. Also, a new association was found between a SNP in the CTL4 gene (rs2242665), located at the major histocompatibility complex III region, and reduced risk for clinical malaria. This study represents the first association study from an Amazonian population involving a large number of host genetic polymorphisms with susceptibility or resistance to Plasmodium infection and malaria outcomes. Further studies should include a larger number of individuals, refined parameters and a fine-scale map obtained through DNA sequencing to increase the knowledge of the Amazonian population genetic diversity.

Introduction

Malaria is a life-threatening parasitic disease transmitted by mosquitoes. Despite major efforts aimed at controlling the spread and impact of the disease, it still persists as a major health burden, being responsible for over a million deaths each year, mainly children in Sub-Saharan Africa. In Brazil, there were over 300,000 recorded cases of malaria in 2010, almost exclusively (99.8% of the cases) restricted to the Amazon Basin region [1].

Malaria is a complex disease with many genetic and environmental determinants influencing the observed variation in response to infection, progression and severity. Several factors important for these different phenotypes include the parasite genetic make-up and host age, state of immunity and genetic background [2]. Resistance involves genetically-based and cell-mediated immunological mechanisms, including the production of specific antibodies that are main actors in the acquired immune response [3], thereby reducing the severity of symptoms and mortality. Resistance mechanisms have been described for both the liver and blood stages of the parasite in the host [4].

Significant associations have been described between malaria and a variety of host genetic polymorphisms that occur in erythrocytes and cells of the immune system. The different geographic distributions of sickle-cell disease, α-thalassemia, glucose-6-phosphate dehydrogenase (G6PD), southeast asian ovalocytosis and the Duffy-negative blood group are examples of the general principle that different populations have selected different genetic variants to protect against Plasmodium infection (see [5,6,7] for reviews). The sickle-cell trait (HbS) [8], G6PD (reviewed in [9]), and ABO blood group [10], are amongst a number of host genes with polymorphisms found to reduce the risk of severe malaria. Some genes relevant to immunity and inflammation, such as the tumor necrosis factor (TNF) within the MHC class III region, (reviewed in [11]), Toll-like receptors (TLR-4, TLR-9) [12,13], CD40 ligand (CD40L) [14], interferon gamma (IFN-γ) (reviewed in [15]), and the nitric oxide synthase
type 2 (HbS2A) genes (reviewed in [16]) have also been associated with severe malaria.

Previous genetic studies in Brazilian Amazonian populations have demonstrated different malaria protective effects from blood-related polymorphisms (e.g., Duffy, ABO, Rh, MNSs and Kell systems [17,18,19,20]), erythrocyte enzymes (G6PD [18]), receptors (CR-1, complement receptor 1) [20,21], and polymorphisms which play a critical role in the early innate immune response to invading pathogens (e.g., TLRs [20,22]). Association of Duffy blood group gene polymorphisms and susceptibility to P. falciparum malaria has been observed in five endemic states of the Brazilian Amazon [17,18,19]. Variants in the TLRs associated with clinical outcomes of malaria have been reported [20,22]. In particular, a study across three areas of the Amazon basin found significant associations between TLR-1 and TLR-6 variants with mild malaria, whereas TLR-9 variants were associated with high parasitemia [22]. No association was found between TLR-4 polymorphisms and mild malaria [22]. These results are in agreement with previous studies that found no association between TLR-4 and protection to mild malaria in a community in the Baixo Amazonas region in the state of Pará [20]. Recent studies have also shown a possible association between CR1-polymorphisms and susceptibility to P. falciparum infection in individuals from an endemic area in the state of Amazonas [21]. However, in the state of Pará, no correlation was observed between CR1 with resistance to P. falciparum infections [20]. It has been reported that the regulation of IL-10 levels, an anti-inflammatory cytokine, in P. vivax infected patients may be unaltered by polymorphism in the promoter region of IL-10 gene [23].

Here, we investigated the association of a larger number of host candidate genes polymorphisms with susceptibility/resistance to Plasmodium infection with clinical (mild) malaria in a population of the Brazilian Amazon. We genotyped 64 single nucleotide polymorphisms (SNPs) in 37 human host genes, including loci related with erythrocyte receptors and immune response. Our study is the first to comprehensively survey important malaria candidate polymorphisms (including HbS and ABO) in a Brazilian population from the Medium Negro River Basin in the Amazon. This setting provided an excellent field to test hypotheses on the generalization of established malaria-genetic associations, particularly for low endemic areas. It also provided a mean to find other associations related to either different mechanisms or by linkage disequilibrium with putative markers associated with susceptibility/resistance to Plasmodium infection [18].

Materials and Methods

Ethics Statement

Approval for the recruitment of participants, collection of blood samples, DNA preparation and DNA genotyping was provided by the relevant research ethics committee (Oswaldo Cruz Foundation - protocol number 360/06) and an informed consent was obtained from each participant.

Study Participants

Potential participants were engaged between January 2002 and October 2006 from an ongoing epidemiological study of malaria in the cities of Barcelos (n = 596) and Santa Isabel do Rio Negro (n = 52), which are 350 km apart. The municipalities are located within the Negro river micro region in the state of Amazonas and display similar demographics. In 2006, the Annual Parasite Index (API) was 264.4 cases per 1000 inhabitants in Barcelos [24] and 127.2 cases per in Santa Isabel do Rio Negro [25]. The mean number of previous malaria episodes was 5.54±10.52 in Barcelos and 2.32±1.33 in Santa Isabel. These differences were not statistically significant (p = 0.053). Greater details of social, demographic and malaria data for the populations studied can be found elsewhere [25,26].

The individuals included in this study had lived in the study area for at least five year with similar social and genetic backgrounds. The population of both municipalities is predominantly of Amerindian descent from Tukano-Oriental speaking societies [25,27,28]. Individuals were recruited during consultations for malaria symptoms at the health service centers in both cities. For each malaria case identified, a field team was dispatched to perform an active search of the patient’s house and neighboring houses. Healthy controls, asymptomatic infected individuals and individuals with a previous malaria history, but not infected at the moment of the study, were recruited during these active searches. A previous history of malaria in uninfected individuals and healthy controls was verified using reviews of health services charts from each municipality. Those who had used anti-malarial drugs 30 days before the recruitment day were excluded. The participants had a median age of ~19 years (malaria cases: median 19.0, range 1.0–48.0; controls 19.0, 3.0–72.0), and 45.5% were males (malaria cases: 208, 53.7%; controls: 31, 28.4%) (see Table 1).

Cases Definitions and Parasite Identification

Three definitions of malaria cases were used for the association analysis: clinical malaria (mild, n = 113), asymptomatic infected individuals (n = 122) and individuals with a previous history of mild malaria (non-asymptomatic infection), but not infected at the moment of blood collection (n = 300). Clinical malaria was defined in accordance to the guidelines of the World Health Organization (WHO) for the American region and the Brazilian National Malaria Control Programme (PNCM) including symptoms associated to malaria (i.e., fever, chills or diaphoresis) and a positive thick smear or rapid diagnostic test (RDT). Asymptomatic Plasmodium-infected cases were defined as an individual without symptoms for malaria within 30 days before or after the blood collection, but with a positive thick smear and/or PCR, following the recommendations of the Brazilian consensus group for studies of asymptomatic individuals.

For each sample collected, a thick smear was prepared and stained with Giemsa using the National Guidelines that was examined by a certified expert using 200 microscope fields under immersion oil. All positive samples were confirmed by another individual blinded to the previous results and the infecting Plasmodium species was identified by PCR according to published protocols [17]. There was an even balance between P. falciparum and P. vivax (Table 1).

Sample Preparation and Genotyping

The sample collection consisted of 535 cases (Barcelos n = 494, Santa Isabel n = 41) and 113 healthy controls with no previous history of malaria (Barcelos n = 102, Santa Isabel n = 11). DNA samples were prepared by extraction from 300 µl of total blood using a commercial kit following the manufacturer's protocol (Promega®). Genomic DNA samples underwent whole genome amplification by Primer Extension Pre-amplification (PEP) before genotyping on a Sequenom® MassArray genotyping platform (http://www.sequenom.com) [29,30]. All samples underwent genotyping on the same instrument resulting in low rates of missing genotyping data. Sixty-four malaria candidate SNPs were genotyped, including: Haemoglobin variant S (HbS) (rs334), and an ABO blood group SNP that defines groups B and non-B (rs8176746). The full list can be found in Table S1.
Statistical Methods

Association studies were performed comparing different groups: a) any_malaria group (clinical malaria patients, asymptomatic infected individuals, and individuals with a previous history of malaria) with the never_malaria group (control); b) clinical_malaria group (current or previous mild malaria) with the never_malaria group; c) asymptomatic infection group with the never_malaria group; and d) asymptomatic_infection group (current or previous mild malaria) with asymptomatic_infection group.

Genotypic deviations from the Hardy-Weinberg equilibrium (HWE) were assessed using a chi-square statistical test. SNPs were excluded from the analysis if there was at least 10% of the genotype calls missing or a significant deviation from the HWE (p<0.0001). A case-control association analysis using SNP alleles was undertaken by logistic regression and included site (Barcelos or Santa Isabel do Rio Negro) and gender as covariates. In this approach, the SNP of interest was modeled assuming several related genotypic mechanisms (additive, dominant, recessive, heterozygous advantage and general models) and the minimum P-values from these correlated tests are reported. Haplotypes were estimated using an expectation-maximization algorithm [31], and score tests [32] were applied to assess the level of evidence for both global and individual haplotype associations with malaria. Linkage disequilibrium was estimated using the pairwise-SNP r² and D-prime metrics.

Population structure or differences in allele frequencies between sub-populations can lead to false positive associations. Differences in allele frequencies were estimated between Barcelos and Santa Isabel do Rio Negro using an Fst metric [33], where values close to zero imply no difference, and values close to one imply complete differentiation between locations.

Statistical analysis of SNPs on the X chromosome was performed using the R statistical package (http://www.r-project.org). Performing multiple statistical tests lead to an inflation in the occurrence of false positives and, by using a permutation approach that accounted for correlation between markers and tests, the estimated p-value cut-off of 0.003 was considered statistically significant.

Results

Overall, nine SNPs were excluded from the analysis because there was either a minor allele frequency of less than 1% (rs33953057, rs2227507, rs12720463, rs9282799, rs8306, rs5743089, hCD36_G1439C) or due to a high rate of missing genotype calls (rs795536, rs20541). Figure 1 shows the minimum p-values from the genotypic tests applied to the autosomal SNPs.

There were five significant results for those with any_malaria versus never_malaria group: rs1800896 - IL10-1082 (OR: 0.528, CI: 0.360–0.774; P = 0.0014), rs2706384 - IRF1 (OR: 1.881, 95% CI: 1.298–2.724; P = 0.0005), rs2242665 - CTLA (OR: 0.595, 95% CI: 0.434–0.816; P = 0.0012), rs4986790 - TLR4 (OR: 0.274, 95% CI: 0.124–0.604; P = 0.0014) and rs909253 - LTA+252 (OR: 0.343, 95% CI: 0.182–0.647; P = 0.0009) (see Table 2). Four of these SNPs were also significant when analyzing clinical_malaria versus never_malaria group: rs2706384 - IRF1 (OR: 2.023, 95% CI: 1.371–2.987; P = 0.0002), rs2242665 - CTLA (OR: 0.564, 95% CI: 0.406–0.784; P = 0.0006), rs4986790 - TLR4 (OR: 0.271, 95% CI: 0.116–0.633; P = 0.0002) and rs909253 - LTA+252 (OR: 0.366, 95% CI: 0.192–0.699; P = 0.0011). When analyzing clinical_malaria versus asymptomatic_infection group, associations were observed with the TNF-376 promoter SNP (rs1800750, OR:0.086, 95% CI: 0.016–0.473; P = 0.0026), previously associated in a number of other malaria studies. (see [11] for a review). The LTA and IL10 SNPs were also significant when analyzing asymptomatic_infection versus never_malaria group: rs909253 - LTA+252 (OR: 3.508, 95% CI: 1.641–7.502; P = 0.0007), rs1800896 - IL10-1082 (OR: 0.280, 95% CI: 0.142–0.552; P = 0.0001), rs3024500 (OR: 0.410, 95% CI:0.236–0.739; P = 0.0017) and rs1800090 - IL10-3533 (OR: 0.280, 95% CI:

Table 1. Baseline and clinical characteristics of the studied population.

<table>
<thead>
<tr>
<th>Controls (n = 113)</th>
<th>malaria cases (n = 535)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (median)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>(19.0)</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>31</td>
</tr>
<tr>
<td>Number of individuals</td>
<td></td>
</tr>
<tr>
<td>Barcelos</td>
<td>102</td>
</tr>
<tr>
<td>Santa Izabel do Rio Negro</td>
<td>11</td>
</tr>
<tr>
<td>Clinical phenotype of malaria cases</td>
<td></td>
</tr>
<tr>
<td>Previous history of mild malaria*</td>
<td>300</td>
</tr>
<tr>
<td>Clinical Malaria</td>
<td>–</td>
</tr>
<tr>
<td>Asymptomatic infection</td>
<td>–</td>
</tr>
<tr>
<td>Parasites in clinical malaria or asymptomatic infection</td>
<td></td>
</tr>
<tr>
<td>P. falciparum</td>
<td>–</td>
</tr>
<tr>
<td>P. vivax</td>
<td>–</td>
</tr>
<tr>
<td>both</td>
<td>–</td>
</tr>
</tbody>
</table>

*these individuals were not infected at the moment of blood collection but recorded as having previously mild malaria; Controls were healthy individuals with no history of previous malaria; For some statistical analysis we have grouped clinical malaria patients, asymptomatic infected individuals and individuals with previous history of malaria (any_malaria group) and clinical malaria patients previous history of mild malaria (clinical_malaria group).

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There was a high linkage disequilibrium (LD) between the three IL10 polymorphisms (minimum pairwise D' = 0.85). A haplotype analysis of these three polymorphisms (rs3024500, rs1800896, rs1800890) revealed that those with the GCT allelic combination (10% frequency in population) were at a lower risk of any form of malaria (OR: 0.40–0.63, 95% CI: 0.2–0.9) when compared to the common ATA combination (83% frequency in population; see Table 3).

Analysis of the four polymorphisms on the X-chromosome revealed no significant associations (Table S2). These included the G6PD+202A allele, also referred to as A2, which is a deficiency surrogate that occurs at a low frequency in our population (3%). Two other candidate polymorphisms with a strong presence in the malaria literature, type HbS and B blood groups, were present at low frequencies in our population (4%) and, therefore, lacked the power to detect an association (P > 0.03; see Table 4) in this study. It was interesting to note that in our population the null allele of the Duffy antigen was present at a frequency of 10% (Table 4).

There was no evidence of population structure effects from the two locations on the association analysis. First, the Fst values across all markers were close to zero (median 0.0009, range 0 to 0.0063; see Supplementary Table S3 for values). Second, by removing the data of Santa Isabel do Rio Negro from the analysis, the association hits were identical, but with a lower precision on the odds ratio estimates. Supplementary Tables S4, S5, S6, S7 display data on allele frequencies and tests of association between groups studied in this work.

Discussion

This genetic association study was designed to correlate the presence of various host gene polymorphisms within a Brazilian Amazonian population with the clinical presentation of malaria for the purpose of identifying candidate genes whose functions could impact disease progression. The results showed, for the first time, an association between alleles of CTL4 gene with malaria. Within the MHC class III region, the SNP (rs2242665) located in the CTL4 gene, displayed a significant association with reduced risk for clinical (mild) malaria. This gene encodes for a possible sodium-dependent transmembrane transport protein involved in the uptake of choline by cholinergic neurons. As the MHC class III region has a complex haplotype structure with long-range LD patterns, this finding could arise from a functional variant in high linkage with this SNP.

Polymorphic variability in the innate immune response gene IL-10 also showed a strong haplotype risk association (OR < 0.7 for the GCT haplotype, see Table 3) on both asymptomatic infection and clinical (mild) malaria. When analysed at the level of individual SNPs, an association was discovered for those individuals displaying IL10 - 1082 with a reduced risk for malaria symptoms. Previous studies in a Kenyan population...
Table 2. Allele frequencies and tests of association for the most significant SNPs.

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>SNP</th>
<th>Ancestral/ Derived Minor allele</th>
<th>MAF Controls</th>
<th>MAF Cases</th>
<th>Contrast</th>
<th>OR</th>
<th>LCL</th>
<th>UCL</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>rs909253 - LTA+252</td>
<td>A G A 0.476 0.519</td>
<td>3.508</td>
<td>1.641</td>
<td>7.502</td>
<td>0.0007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1800896 -IL10-1082</td>
<td>T C T 0.212 0.094</td>
<td>0.280</td>
<td>0.142</td>
<td>0.552</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1800890 -IL-10-3533</td>
<td>A T A 0.167 0.075</td>
<td>0.372</td>
<td>0.194</td>
<td>0.713</td>
<td>0.0014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never_malaria</td>
<td>rs1800896 -IL10-1082</td>
<td>G A A 0.207 0.107</td>
<td>0.418</td>
<td>0.236</td>
<td>0.739</td>
<td>0.0017</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1800890 -IL-10-3533</td>
<td>A T A 0.167 0.075</td>
<td>0.372</td>
<td>0.194</td>
<td>0.713</td>
<td>0.0014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any_malaria</td>
<td>rs909253 - LTA+252</td>
<td>A G G 0.524 0.468</td>
<td>0.343</td>
<td>0.182</td>
<td>0.647</td>
<td>0.0009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs2706384 -IRF1</td>
<td>G T T 0.273 0.397</td>
<td>1.881</td>
<td>1.298</td>
<td>2.724</td>
<td>0.0005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs2242665 - CTL4</td>
<td>C T C 0.449 0.383</td>
<td>0.595</td>
<td>0.434</td>
<td>0.816</td>
<td>0.0012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4986790 -TLR4</td>
<td>A G A 0.058 0.015</td>
<td>0.274</td>
<td>0.124</td>
<td>0.604</td>
<td>0.0014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical_Malaria</td>
<td>rs909253 - LTA+252</td>
<td>A G G 0.524 0.465</td>
<td>0.366</td>
<td>0.192</td>
<td>0.699</td>
<td>0.0010</td>
<td></td>
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<tr>
<td></td>
<td>rs2706384 -IRF1</td>
<td>G T T 0.273 0.397</td>
<td>2.023</td>
<td>1.371</td>
<td>2.987</td>
<td>0.0002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs2242665 - CTL4</td>
<td>C T T 0.449 0.372</td>
<td>0.564</td>
<td>0.406</td>
<td>0.784</td>
<td>0.0006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4986790 -TLR4</td>
<td>A G A 0.058 0.015</td>
<td>0.271</td>
<td>0.116</td>
<td>0.633</td>
<td>0.0020</td>
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<tr>
<td>Asymptomatic</td>
<td>rs1800750 -TNF-376</td>
<td>G A A 0.080 0.022</td>
<td>0.086</td>
<td>0.016</td>
<td>0.473</td>
<td>0.0026</td>
<td></td>
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</tbody>
</table>

Any_malaria group consisted of: clinical malaria, asymptomatic infection and previous history of malaria; Asymptomatic group: asymptomatic infection; Clinical_Malaria group: clinical mild malaria (current or previous mild malaria); Never_malaria group: no history of malaria; Ref: reference; MinA: minor allele; MajA: major allele; MAF: minor allele frequency; HWEP: Hardy-Weinberg p-value; OR: odds ratio; 95% Confidence interval (LCL - UCL).

doi:10.1371/journal.pone.0036692.t002
Table 3. Haplotype analysis of IL10 gene.

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>Haplotype</th>
<th>Hap-score</th>
<th>p-value</th>
<th>Controls Cases</th>
<th>OR</th>
<th>LCL</th>
<th>UCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any_malaria</td>
<td>GCT</td>
<td>-2.697</td>
<td>0.007</td>
<td>0.162</td>
<td>0.097</td>
<td>0.573</td>
<td>0.385</td>
</tr>
<tr>
<td></td>
<td>GCA</td>
<td>-1.582</td>
<td>0.114</td>
<td>0.041</td>
<td>0.022</td>
<td>0.457</td>
<td>0.202</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>1.299</td>
<td>0.194</td>
<td>0.005</td>
<td>0.017</td>
<td>3.337</td>
<td>0.449</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>2.363</td>
<td>0.018</td>
<td>0.784</td>
<td>0.849</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>global-stat = 12.495, df = 4, P = 0.0142</td>
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<td></td>
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<tr>
<td>Asymptomatic</td>
<td>GCT</td>
<td>-2.894</td>
<td>0.004</td>
<td>0.162</td>
<td>0.069</td>
<td>0.406</td>
<td>0.221</td>
</tr>
<tr>
<td></td>
<td>GCA</td>
<td>-1.468</td>
<td>0.142</td>
<td>0.041</td>
<td>0.017</td>
<td>0.342</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>2.846</td>
<td>0.004</td>
<td>0.784</td>
<td>0.887</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>global-stat = 12.127, df = 3, P = 0.0070</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical_Malaria</td>
<td>GCT</td>
<td>-2.225</td>
<td>0.026</td>
<td>0.162</td>
<td>0.105</td>
<td>0.625</td>
<td>0.416</td>
</tr>
<tr>
<td></td>
<td>GCA</td>
<td>-1.380</td>
<td>0.168</td>
<td>0.041</td>
<td>0.024</td>
<td>0.492</td>
<td>0.212</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>1.428</td>
<td>0.153</td>
<td>0.005</td>
<td>0.018</td>
<td>3.887</td>
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<td></td>
<td>ATA</td>
<td>1.903</td>
<td>0.057</td>
<td>0.784</td>
<td>0.839</td>
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<td>global-stat = 9.7201, df = 4, P = 0.0454</td>
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<tr>
<td>Asymptomatic</td>
<td>GCT</td>
<td>1.599</td>
<td>0.110</td>
<td>0.069</td>
<td>0.105</td>
<td>1.579</td>
<td>0.914</td>
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<td>1.465</td>
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<tr>
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<td>GTC</td>
<td>0.394</td>
<td>0.694</td>
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<td>0.018</td>
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<td>0.887</td>
<td>0.839</td>
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<td>global-stat = 3.2488, df = 4, P = 0.5171</td>
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Two other associations with cytokines were identified, both within MHC class III region, that included TNF and the lymphotoxin alpha (LT-α/LTA) and beta (LT-β/LTB) genes, which are closely related. The TNF and LTA genes are implicated in the host defense and pathogeneses of severe malaria [5,35]. An intronic SNP in LTA (rs909253) was associated with protection from clinical (mild) malaria. Previous studies showed no significant risk associated with LTA in a Sri Lankan population [36] or cohorts in Kenya and Malawi [11]. These disparities of association could be due to differences in the genetic background between the Brazilian population with those of the Sri Lankan and African populations.

In addition, the TNF-376 promoter SNP (rs1800750) was identified to have an association with clinical malaria (Table 2). An association was previously found for this SNP with an increased risk for cerebral malaria in Kenya and Gambia, conferring an allele-specific binding of the transcription factor OCT-1[37,38]; see [11] for a review). This observation adds support to the current dogma that the TNF locus is important for malaria pathogenesis. However, it is necessary to corroborate these findings with a large scale epidemiological and immunological functional study.

Other cytokine-related findings include one SNP (rs2706384) in the Interferon Regulatory Factor 1 gene (IRF1), which was associated with an increased risk for clinical malaria. IRF1 is a critical mediator of IFN-γ activity and is crucial in both innate and adaptative immune responses against *P. falciparum* [39]. Our finding are consistent with a previously reported association between polymorphisms in the IRF1 and the control of *P. falciparum* infection both in healthy adults and children displaying severe and uncomplicated malaria [40]. Moreover, this gene is located in the 5q31 region, which has been shown to be associated with *P. falciparum* infection levels [32,33].

Finally, a polymorphism was seen in the TLR4 gene (rs4986790) that was associated with reduced risk for clinical malaria. Previous studies in an Amazonian population reported no association between TLR4 and mild malaria [20,22]. However, the absence of an apparent association may be due to the lower minor allele frequencies and smaller samples sizes of those studies. Unlike the results reported here, two studies in Ghana have revealed frequent polymorphisms at TLR4 that conferred an increased risk of severe malaria [12] and clinical manifestations of malaria during pregnancy [13]. The absence of severe malaria in Brazil could explain the differences in association results. Together, the genetic data support a role for TLR4 in modulating the presentation of malaria symptoms.

Overall, this study represents the first association study from an Amazonian population involving a large number of host genetic polymorphisms with susceptibility or resistance to *Plasmodium* infection and malaria outcomes. To understand which are the real causal variants, re-sequencing of LTA, TNF and CTL4 genes and the surrounding MHC class III in a range of populations will be necessary to assist the design of large scale epidemiological studies. Previous candidate polymorphisms have arisen mostly from studies...
Table 4. Odds ratios for the Duffy antigen, HbS (rs334), ABO, and G6PD-202.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>SNP</th>
<th>Ancestral/ Derived allele</th>
<th>Minor allele</th>
<th>MAF Controls</th>
<th>MAF Cases</th>
<th>Contrast</th>
<th>OR</th>
<th>LCL</th>
<th>UCL</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Any_malaria</td>
<td>rs2814778 - DARC</td>
<td>T</td>
<td>C</td>
<td>0.106</td>
<td>0.098</td>
<td>CC vs TT/TC</td>
<td>0.434</td>
<td>0.082</td>
<td>2.276</td>
<td>0.357</td>
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<td></td>
<td>rs334–HbS (HBB)</td>
<td>T</td>
<td>A</td>
<td>0.014</td>
<td>0.017</td>
<td>TA vs TT</td>
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<td>3.723</td>
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<tr>
<td></td>
<td>rs1876746 - ABO</td>
<td>G</td>
<td>T</td>
<td>0.037</td>
<td>0.027</td>
<td>TG vs other</td>
<td>0.532</td>
<td>0.225</td>
<td>1.260</td>
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<tr>
<td></td>
<td>rs1050828-G6PD+202</td>
<td>C</td>
<td>T</td>
<td>0.005</td>
<td>0.021</td>
<td>Additive T</td>
<td>5.235</td>
<td>0.758</td>
<td>36.179</td>
<td>0.093</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>rs2814778 - DARC</td>
<td>T</td>
<td>C</td>
<td>0.106</td>
<td>0.087</td>
<td>CC vs TT/TC</td>
<td>0.216</td>
<td>0.018</td>
<td>2.525</td>
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<td>rs334–HbS (HBB)</td>
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<td>A</td>
<td>0.014</td>
<td>0.023</td>
<td>TA vs TT</td>
<td>1.526</td>
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<td>6.964</td>
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<td>T</td>
<td>0.037</td>
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<td>0.923</td>
<td>0.328</td>
<td>2.600</td>
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<td>rs1050828-G6PD+202</td>
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<td>T</td>
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<td>Additive T</td>
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<tr>
<td>Clinical_Malaria</td>
<td>rs2814778 - DARC</td>
<td>T</td>
<td>C</td>
<td>0.106</td>
<td>0.101</td>
<td>CC vs TT/TC</td>
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<td>0.090</td>
<td>2.667</td>
<td>0.434</td>
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<tr>
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<td>rs334–HbS (HBB)</td>
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<td>A</td>
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<td>0.015</td>
<td>TA vs TT</td>
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<td>3.300</td>
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<td>rs1876746 - ABO</td>
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<td>T</td>
<td>0.037</td>
<td>0.024</td>
<td>TG vs other</td>
<td>0.398</td>
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<td>1.010</td>
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<td>rs1050828-G6PD+202</td>
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<td>T</td>
<td>0.005</td>
<td>0.015</td>
<td>Additive T</td>
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<td>0.586</td>
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<td>Any_malaria</td>
<td>rs2814778 - DARC</td>
<td>T</td>
<td>C</td>
<td>0.087</td>
<td>0.101</td>
<td>CC vs TT/TC</td>
<td>1.194</td>
<td>0.674</td>
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<td>0.539</td>
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<td>rs334–HbS (HBB)</td>
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<td>0.023</td>
<td>0.015</td>
<td>TA vs TT</td>
<td>0.595</td>
<td>0.201</td>
<td>1.763</td>
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<tr>
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<td>rs1876746 - ABO</td>
<td>G</td>
<td>T</td>
<td>0.040</td>
<td>0.024</td>
<td>TG vs other</td>
<td>0.515</td>
<td>0.220</td>
<td>1.206</td>
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<td></td>
<td>rs1050828-G6PD+202</td>
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<td>0.043</td>
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<td>Additive T</td>
<td>0.359</td>
<td>0.143</td>
<td>0.905</td>
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</table>

Any_malaria group consisted of: clinical malaria, asymptomatic infection and previous history of malaria; Asymptomatic group: asymptomatic infection; Clinical_Malaria group: clinical mild malaria (current or previous history of mild malaria); Never_malaria group: no history of malaria; Ref: reference; MAF: minor allele frequency; OR = odds ratio; 95% Confidence interval (LCL, UCL).

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in an African setting, where the linkage disequilibrium or correlation structure between SNPs, the absence of *Plasmodium vivax*, endemicity, and disease severity complicate their relevancy to Amazonian populations. For example, the data analysis from our study revealed no significant associations for gene polymorphisms of the sickle-cell trait, blood-related polymorphisms and G6PD A′ surrogate, which display inherited innate resistance to malaria. These results were not unexpected, given that the low frequencies of these alleles in the Brazilian population together with the current sample size that restricted the resolving power of the analysis to detect associations.

The issue of malaria disease association mapping and its implications for disease management in multiple geographic locations remains a major challenge confronting the field. The differences highlighted here and the association of specific SNP polymorphisms with clinical malaria in the Amazon basin support the need for additional studies. The results suggest a need for associations studies with more dense mapping of candidate genes, especially CTL4 and IL-10, along with other genes from the major histocompatibility complex region to identify additional functional variants most relevant for malaria in Amazonian populations.

**Supporting Information**

**Table S1** List of all polymorphisms genotyped in this study.  
(XLS)

**Table S2** X chromosome analysis.  
(XLS)

**Table S3** Allele frequencies differences between Santa Isabel do Rio Negro and Barcelos.  
(XLS)

**References**


23. Medina TS, Costa SP, Oliveira MD, Ventura AM, Souza JM, et al. (2011) Increased interleukin-10 and interferon-gamma levels in Plasmodium vivax malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism. Malar J 10: 264.


**Table S4** Allele frequencies and test of association - *Any malaria versus never malaria* group.  
(XLS)

**Table S5** Allele frequencies and test of association - *Asymptomatic versus never malaria* group.  
(XLS)

**Table S6** Allele frequencies and test of association - *Clinical malaria versus never malaria* group.  
(XLS)

**Table S7** Allele frequencies and test of association - *Clinical malaria versus asymptomatic* group.  
(XLS)

**Acknowledgments**

We thank the participants from Barcelos and Santa Isabel do Rio Negro, as well as the many healthcare workers involved in the original and ongoing studies in these populations for their contributions. We would also like to thank Anna Jeffrey, Angela Green, Kate Rowlands and Christina Hubbard for their support in sample handling and Sequenom genotyping at the Wellcome Trust centre for Human Genetics in Oxford, UK, as well as Dr. Filipe Amilval Costa-Carvalho and Dr. Márcio Neves Bóia for sample collections from Santa Isabel do Rio Negro.

**Author Contributions**

Conceived and designed the experiments: SSS TGC KAR. Performed the experiments: SSS TGC. Analyzed the data: SSS TGC SC KAR. Contributed reagents/materials/analysis tools: TGC SC MCSM KAR DPK OF. Wrote the paper: SSS TGC. Discussion of results: SSS TGC SC KAR DPK OF. Acknowledgments

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