Role of *IL6*, *IL12B* and *VDR* gene polymorphisms in *Plasmodium vivax* malaria severity, parasitemia and gametocytemia levels in an Amazonian Brazilian population

Vinicius A. Sortica, Maristela G. Cunhab, Maria D.O. Ohnishi, Jose M. Souza, Andréa K.C. Ribeiro-dos-Santos, Sidney E.B. Santos, Mara H. Hutz

*Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
*Programa de Ensaios Clínicos em Malária, Instituto Evandro Chagas, Sistema de Vigilância Sanitária, Ministério da Saúde, Ananindeua, PA, Brazil*
*Laboratório de Microbiologia e Imunologia, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil*
*Laboratório de Genética Humana e Médica, Universidade Federal do Pará, Belém, PA, Brazil*

**Abstract**

Objective: To investigate the influence of *IL6*, *IL12B* and *VDR* single nucleotide polymorphisms (SNPs) in uncomplicated *Plasmodium vivax* infection symptoms intensity, parasitemia and gametocytemia levels in a Brazilian Amazonian population.

Methods: A total of 167 malaria patients infected by *P. vivax* have parasitemia and gametocytemia levels estimated before treatment. Fourteen clinical symptoms were evaluated and included in a principal component analysis to derive a clinical symptom index. Patients were genotyped for *IL6*-174C > G, *IL12B* 735T > C, 458A > G, 159A > C, and *VDR* FokI, TaqI, BsmI SNPs by Taqman 5' nuclease assays. A General Linear Model analysis of covariance with age, gender, exposure period and infection history and genetic ancestry was performed to investigate the association of genotypes with parasitemia and gametocytemia levels and with a clinical symptom index.

Results: Higher parasitemia levels were observed in *IL6*-174C carriers (p = 0.02) whereas *IL12B* CGT haplotype carriers presented lower parasitemia levels (p = 0.008). VDR TaqI/BsmI haplotype carriers showed higher gametocyte levels than non-carriers (p = 0.013). Based on the clinical index values the *IL6*-174C > G polymorphism was associated with malaria severity. The *IL6*-174C carriers presented a more severe clinical index when compared to GC homozygotes (p = 0.001).

Conclusion: The present study suggests that *IL6*, *IL12* and *VDR* influence severity, parasitemia and gametocytemia clearance in *P. vivax* infections, and highlights their potential role in malaria immune response in an Amazonian population.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Malaria is a major cause of human mortality worldwide and is considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome [1]. Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Together with parasite virulence phenotypes and host’s immune response, the level of parasitemia, has been considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome [1]. Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Together with parasite virulence phenotypes and host’s immune response, the level of parasitemia, has been considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome [1]. Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Together with parasite virulence phenotypes and host’s immune response, the level of parasitemia, has been considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome [1].

*Plasmodium vivax* infections have peculiar biological features such a predilection of merozoites for reticulocytes as host cells, the early presence of round gametocytes in the peripheral blood, the circulation of all blood-stage developmental forms in the peripheral blood, and the most important, the development of dormant hypnozoite forms in the liver that cause subsequent relapses [5]. Traditionally treated as a milder infection, *P. vivax* malaria severe cases are becoming more common presenting symptoms as hepatic dysfunction, acute respiratory distress, renal failure, splenic rupture, severe anemia and cerebral malaria [6–10]. Mechanisms leading to malaria milder or severe symptoms are not fully understood, but immune system failure to control overwhelming parasite replication or immunopathology resulting from excessive inflammation are considered to be contributing factors [11].

Malaria parasites and parasite-infected red blood cells activate dendritic cells (DC) through a pattern of recognition receptors...
which present parasite antigens to T helper (Th) 1 cells and triggers a pro-inflammatory response. The inflammatory response that is required to remove parasites leads to considerable tissue damage, and the activation of phagocytes to kill intracellular or extracellular parasites requires the production of inflammatory cytokines, such as interleukin (IL)-1 and IL-6 [12]. It has been shown that the protective immunity in malaria is mediated by a cascade of events that also involves IL-12 [13]. It appears that early events in the cell-mediated immune response required for defense against malaria are initiated by the release of IL-12 from monocytes/macrophages, B cells and other cell types [13], and reveal a prognostic significance in malaria infection [13,14].

One of the most interesting findings is that infections by *P. vivax* and *Plasmodium falciparum* present similar characteristics, with similar levels of parasitemia, frequencies and intensities of anemia, and levels of different cytokines and antibody responses [15]. Clearing malaria parasites without inducing major host pathology requires a finely tuned balance between inflammatory and regulatory cytokine responses, whose timing and magnitude is crucial in determining malaria patient outcome [16]. Early production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-12 and other inflammatory cytokines allows fast *P. falciparum* clearance [11,17]. Cytokine responses reflect different host strategies for controlling infection with different malaria species [18]. Controlling virulent, fast-multiplying parasites such as *P. falciparum* blood stages may require a strong inflammatory response that can prevent fulminant infections but which may lead to severe disease whereas that tight control of parasite growth is not required in vivax malaria, since parasites find a limited supply of reticu-locytes to parasitize in peripheral blood and there is no risk of overwhelming parasitemias [18].

In *P. vivax* infections, asexual erythrocyte infective forms (parasitemia) and sexual mosquitoes infective forms observed in patients’ blood (gametocytemia) were related to fever symptoms and initial pro-inflammatory response [3,19,20]. Although gametocytes do not cause clinical disease when ingested by mosquitoes taking a blood meal, they can develop into ookinetaes, oocysts and ultimately sporozoites, thereby rendering the mosquito infectious to human beings. The development of a human immune response to gametocytes is expected because the vast majority of gametocytes are not taken up by mosquitoes but are cleared by the host immune system therefore a distinct human immune response may also reduce the infectiousness of gametocytes [3,19,20].

Interleukin (ILs) 6 and 12 are secreted by endothelial and anti-gen presenting cells in response to *P. vivax* infection. IL-6 levels are elevated in *P. vivax* infection and it is suggested to be involved in parasite clearance [21,22], however IL6 genetic variants were poorly investigated in malaria infections. IL-12 levels were inversely correlated with parasitemia in *P. falciparum* infections [23] and polymorphisms in IL12 gene promoter region were associated with malaria severity in *P. falciparum* infection [24,25].

The active form of vitamin D (1,25(OH)2D3), is an immune modu-lator and its interaction with the vitamin D receptor (VDR) influences both innate and adaptive immunity in response against intracellular pathogens. VDR gene polymorphisms were associated with mycobacteria and viral infections [26], but its relation to ma-laria has not been assessed so far.

The immune response mechanisms related to *P. vivax* infection clearance and severity are poorly understood and few studies investigate immune system gene polymorphisms in such infec-tions. The study of host immune system polymorphisms might be a valuable tool for risk assessment and progression of infectious diseases, therefore the present study investigates the role of IL6, IL12B and VDR single nucleotide polymorphisms (SNPs) in *P. vivax* parasitemia and gametocytemia levels and symptoms intensity in a Brazilian population from the Amazonian region.

2. Methods

2.1. Study population

The study was conducted at the Evandro Chagas Institute, Belém, Pará. A total of 167 unrelated cases were consecutively recruited between 2002 and 2009 as part of a larger study of malaria susceptibility [27]. Patients had their blood collected for parasite density estimates and DNA analyses at their first visit when they were clinically assessed and a malaria diagnosis was performed. After blood collection, all patients started the standard 1500 mg of chloroquine associated with 210 mg of primaquine treatment in seven days [28]. All subjects provided their written informed consent to participate in this study. The Ethics Commit-tees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

2.2. Malaria diagnoses and parasitemia estimates

Patients were clinical examined by a physician and symptoms as fever, headache, chills, myalgia, arthralgia, back pain, abdominal pain, asthenia, dizziness, dyspnea, cough, nausea, vomiting and diarrhea were evaluated as numerical scores from 0 to 4 (absent, mild, moderate, severe and very severe, respectively).

*P. vivax* malaria was diagnosed by thick blood smear, as recom-mended by the Brazilian Ministry of Health [29]. Asexual and sexual (gametocyte) forms density per μL of blood were estimated by counting the number of parasites per 100 fields and double-checked blindly by two expert microscopists.

2.3. Genotyping

Genomic DNA was extracted by standard procedures. The SNPs were determined by allelic discrimination with Taqman 5′–nuclease assays according to the manufacturer’s recommended protocol. The *IL12B* 735T > C (rs7709212), 458A > G (rs2546890), 159A > C (rs3212227) SNPs, and VDR FokI (rs10735810), TaqI (rs731236), BsmI (rs1544410) SNPs were genotyped with validated genotyping assays (Applied Biosystems, CA, USA). The *IL6*-174C > G (rs1800795) SNP, were genotyped by custom genotyping assay by design (Ap-plied Biosystems, CA, USA). All patients were also genotyped for ancestry informative markers as previously described [27].

2.4. Statistical analyses

Allele and genotype frequencies were estimated by gene count-ing. Deviation from Hardy–Weinberg equilibrium was assessed by Chi-square tests with Bonferroni correction. Haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1. The individual proportions of European, African and Amerindian ances-try were estimated using the STRUCTURE software 2.3.3 assuming three parental populations: Europeans, Africans and Amerindians [27]. A Principal Component Analysis was performed to aggregate symptom numerical scores into a clinical index that represents the overall intensity of malaria symptoms [18,30]. General Linear Model (GLM) analyses of covariance using the type III sums of squares were performed to assess the association of genotypes with parasite levels and also with the malaria severity clinical in-dex. The type III sums of squares apply to unbalanced study de-signs and quantifies the effect of an independent variable after adjustment for all other variables included in the model. Parasite-mia and gametocytemia levels were log-transformed before analy-sis because of their skewed distribution, but back-transformed values are presented in the results as geometric means. Age, gen-der, exposure time, infection history and genetic ancestry were in-
cluded in models based on conceptual analyses of the literature and/or by means of a statistical definition (association with the study factor and with the outcome at \( p < 0.15 \)). The GLM analysis and Principal Component Analysis were performed using the SPSS18.0 statistical package for Windows®. Statistical significance was defined as a two-tailed \( p \)-value \(< 0.05\).

3. Results

3.1. Study group characteristics

Malaria patients were aged between 12 and 88 years (36.0 ± 15.6 years), 67% of the individuals were males (Table 1). Patients presented 0.241 African, 0.417 European and 0.342 Native American mean genetic ancestry proportions (Table 1). Genetic ancestry estimates did not differ among genotype and haplotype distributions or parasitemia and gametocytemia levels (\( p > 0.05 \)), however African and Native American ancestry were positively correlated with the clinical severity index (\( p < 0.001 \) and \( p = 0.02 \), respectively).

In the study population, 68% of the individuals presented malaria for the first time, 12.2% were infected for the second time and 19.8% of the investigated subjects were infected for more than two times. The exposure period in malaria endemic regions was variable, 53.8% stayed up to 25 days, 26.1% more than 25 days and 20.1% were residents (Table 1).

Genotype distributions did not deviate significantly from Hardy–Weinberg equilibrium. Allele, genotype and haplotype frequencies are shown in Supplemental Tables 1 and 2 and did not differ from previously reported frequencies for healthy subjects from the same population [27].

3.2. Parasitemia and gametocytemia levels genetic association

Parasitemia levels ranged from 50 to 75,000 parasites/μL (8606.5 ± 10187.4). For the 75 patients who presented gametocytes in blood, gametocytemia levels ranged from 15 to 4500 gametocytes/μL (295.2 ± 622.3). In GLM analysis, after adjusting for age, gender, exposure time and infection history, \( IL6-174C > G \) and \( IL12B 159C/458G/735T \) haplotype were associated with parasitemia levels. \( IL6-174C \) carriers presented higher parasitemia levels when compared to \(-174G\) homozygotes (6254.1 vs. 3529.8 parasites/μL, \( p = 0.008 \)) (Table 2). Parasitemia levels were also associated with \( IL12B \) haplotypes (\( p = 0.008 \)) (Table 2). Pairwise comparisons showed that \( IL12B 159C/458G/735T \) haplotype carriers presented lower parasitemia levels when compared to \( CGC/CGC \) patients (1044.3 vs. 6004.3 \( p = 0.005 \)), \( CGC \) carriers (1044.3 vs. 5256.4 \( p = 0.008 \)) and \( AAT \) carriers 1044.3 vs. 4490.1 \( p = 0.01 \) (Table 2). \( IL6 \) and \( IL12 \) SNPs were not associated with gametocytemia densities (Table 3).

\( VDR \) TaqI/BsmI haplotypes were associated with gametocytemia levels in a GLM model including age, gender, exposure time and infection history as covariates. \( VDR \) TaqIC/BsmIA haplotype carriers showed higher gametocyte levels than non-carriers (225.5 vs. 113.3 gametocytes/μL \( p = 0.013 \)) (Table 3). This haplotype was not associated with parasitemia levels (Table 2). FokI SNP is not in linkage disequilibrium with the other two SNPs in the study population and was not associated with parasitemia or gametocytemia levels (Tables 2 and 3).

3.3. Malaria severity genetic association

The most frequent symptoms observed are shown in Supplemental Table 3. The first component obtained in the Principal Component Analysis showed higher weights for fever, headache, chills, myalgia, arthralgia, back pain, abdominal pain, asthenia, dizziness and nausea, and explained 32.1% of symptom variability. This component was used as a clinical malaria severity index for association analyses.

\( IL6-174C > G \) was associated with malaria severity in GLM analysis using age, gender, exposure period, infection history, parasitemia levels, African and Native American genetic ancestry as covariates. Higher clinical index values were observed for \( IL6-174C \) carriers as compared with \( GG \) homozygotes (0.475 vs. 0.048 \( p = 0.02 \)) (Table 4). \( IL12B \) and VDR polymorphisms were not associated with severity in this sample.

4. Discussion

Although there are several studies that reports genetic susceptibility to severe malaria in African populations, much less infor-
Gametocytemia levels were adjusted for age, gender, exposure period and infection history. 

\[ \text{Gametocytemia levels} = \frac{\text{Gametocyte counts}}{\text{Viable erythrocyte counts}} \]

These interleukins promote IFN-γ production by T and natural killer (NK) cells as part of the host immune response to invading pathogens. IL-12 is a heterodimer composed of IL-12p35 and IL-12p40 subunits, encoded by IL12A and IL12B genes located on chromosomes 3p12-q13.2 and 5q31-33, respectively, and exerts its biological function through binding to the heteromeric interleukin 12 receptor (IL-12R) α1 and β2 [31].

Previous examination of IL12B polymorphisms has shown conflicting results. Several studies reported association of the 159A > C with \( P. falciparum \) severity [23,24]. However, no association with severity was observed in costal Kenia and in Thailand [23,32.33]. In the present study carriers of IL12B 159C/458G/735T haplotype seems to have lower parasite density as compared to other haplotype carriers. The discrepancy among studies might be due to different study designs, ethnic background of the population and intrinsic differences between \( P. falciparum \) and \( P. vivax \) infections.

IL-6 is a pleiotropic cytokine which exerts pro- and anti-inflammatory activities by two different pathways. The IL-6 classic signaling through specificity-defining membrane IL-6 receptor (IL-6R) expressed in hepatocytes and immune system cells is correlated with regenerative and anti-inflammatory activities. Moreover, in a trans-signaling process IL-6 is able to use the soluble IL-6 receptor (sIL-6R) and binds to a ubiquitously expressed membrane GP130 protein exerting primarily pro-inflammatory activities [34].

The present study reports the association of \(-174C > G\) SNP in \( IL6 \)’ upstream region with \( P. vivax \) parasitemia density and symptom severity. This promoter SNP was related with impaired \( IL6 \) gene expression and IL-6 levels [35]. The \( IL6-174C \) homozygotes showed almost twice higher levels of circulating IL-6 than CC homozygotes suggesting a suppressive effect of the C allele on IL-6 production [36], but it has also been reported to be associated with higher IL-6 levels in neonates and adults with acute inflammations [35–38]. Higher IL-6 levels was also reported in \(-174C\) patients with schizophrenia and chronic inflammation [37,39,40], regarding malaria studies, circulating IL-6 levels are elevated in \( P. vivax \) and \( P. falciparum \) infections, and are associated with \( P. falciparum \) infection severity [36,41,42], hyperpyrexia and intensity of symptoms in \( P. vivax \) infection [18,21]. sIL-6R serum levels were also correlated with parasite clearance time in \( P. falciparum \) infection [36]. In the present study \(-174C\) carriers presented higher mean parasitemia counts and a higher clinical severity index as compared to GG homozygotes. Lower levels of circulating IL-6 due to the presence of the C allele could be potentially related to defective parasite clearance and symptoms severity. Taken together this data suggest an important role for this \( IL6 \) polymorphism in \( P. vivax \) malaria.

The 1.25 (OH) \(_2\)D\(_3\)/VDR interactions modulate immune functions as calcitriol and antimicrobial peptides expression in monocytes and macrophages. 1.25 (OH) \(_2\)D\(_3\) also reduce the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IFN-γ, IL-2 in immune cells, and can induce the expression of anti-inflammatory cytokines IL-4 and IL-10 [43]. Our study reports that TaqI/BsmI haplotype carriers presented higher gametocytemia densities compared to other haplotype carriers. Gametocytemia was found to correlate with high fever in \( P. vivax \) infection, but not in \( P. falciparum \) infection, in two studies [19,20] but, the effects of host’s immunity upon the intrahost gametocytes are less clear. Given that similar results were observed in individuals with tuberculosis [44,45], dengue virus [46], HIV virus [47]. This haplotype could be a marker of susceptibility to intracellular pathogens. Therefore the observation that VDR is potentially involved in gametocytemia clearance is an open question for future studies.

The exact mechanisms by which IL-6, IL-12 and Vitamin D influence and act in parasite clearance is difficult to determine, nevertheless, our result should be considered as positive preliminary.
evidence. IL-6 regulates the expression of diverse molecules and cytokines and also is regulated by the expression of different cytokines as IL-1, TNF-α, IL-4, and IL-10. Furthermore, IL-6 and IL-12 families share intra and intercytokines protein subunits and receptors [11,17]. Vitamin D also can inhibit Th1 and induce Th2 responses [43]. The extensive cytokine plasticity and “cross-talking” create a complex scenario to understand the biological function of these molecules in infection control. Genetic variants in immune modulator genes could point to new insights about different malaria infection aspects. Our results suggest an important role for IL6, IL12 and VDR gene polymorphism in pro inflammatory response in parasitemia and gametocytemia clearance and severity in P. vivax infection.

The overall results presented in this study should be viewed in the context of some limitations. The sample is of moderate size, thus we could not perform interaction analyses between gene polymorphisms. Since the blood was frozen after collection we could not perform functional studies or measure the cytokine levels in blood. Therefore future studies to correlate these polymorphisms and interleukins levels are warranted to better understand the role of these genes in P. vivax infections.

Cytokines are major players in immune response to malaria and to understand how genetic variants influence host defense mechanisms may be an important issue for infection prevention. The present investigation showed that parasitemia and gametocytemia densities in P. vivax malaria might be modulated by IL6, IL12 and VDR SNPs, and highlights the potential role of these cytokines in infection response.

Acknowledgments

The authors thank the financial support provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.09.014.

References


