Duffy Blood System and G6PD Genetic Variants In *P. Vivax* Malaria Patients From Manaus, Amazonas, Brazil

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Research

Keywords: malaria severe, G6PD, System Duffy, P. vivax

Posted Date: December 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-41862/v3

Abstract

Over a third of the world's population lives at risk of potentially severe *Plasmodium vivax* induced malaria. The unique aspect of the parasite's biology and interactions with the human host make it harder to control and eliminate the disease. Glucose-6-phosphate dehydrogenase (G6PD) deficiency and Duffy-negative blood groups are two red blood cell variations that confer protection against malaria. Molecular genotyping of G6PD and Duffy was performed in 225 patients with severe and non-severe malaria. Of the 225 patients, 29 (12.94%) and 43 (19.19%) were carriers of the G6PD c.202G>A and c.376A>G, respectively. For the Duffy genotype (c.-67T>C in the GATA promoter region), 70 (31.11%) were phenotyped as Fy(a+b-), 98 (43.55%) Fy(a+b+), 56 (24.9%) Fy(a-b+) and 1 (0.44%) Fy(a-b-). The *FY*01/FY*02* genotype was prevalent in both non-severe and severe malaria. However, the frequency increased when SNP c.376A>G was also present. In women, the *FY*01/FY*02* allele occurred concomitantly with c.376A>G more frequently in non-severe malaria, while in men, this combination is revealed predominantly in severe malaria. G202A and A376G G6PD variants were higher in severe malaria, with c.202G>A (RR= 4.76 – p=.009) and c.376A>G (RR: 6.47 – p<0.001) strongly associated with the trials malaria (p<0.001). Duffy phenotype Fy(a-b+) (p=0.003) and genotype FY*02/ FY*02 (p=0.007) presented the highest values parasitemia density of the vivax malaria. Research on G6PD and Duffy antigen deficiencies has been valuable, particularly when focused on densely populated areas. Altogether, c.202G>A and c.376A>G SNPs seem to be risk factors for the development of severe vivax malaria. Molecular diagnosis before treatment may be necessary in the Amazonian population and uncomplicated malaria showed a greater frequency of variation for GATA and G6PD variants than severe malaria.

Introduction

Malaria is one of the most serious public health problems worldwide. In the tropical and subtropical regions, this parasitic disease is the leading cause of social and economic problems (1,2). In Brazil, approximately 90% of malaria cases result from *P. vivax* infection, and in 2018, over 33,000 cases were reported from the state of Amazonas (3).

Susceptibility to malarial infection includes individuals with repeated occurrence of the disease. The severity depends on the relationship between the host, its immunological vulnerability, the *Plasmodium* ssp. responsible for the infection, and the parasitic density. The clinical manifestation of malaria may include severe anemia, coagulation disorders, prominent thrombocytopenia, and numerical or functional changes in leukocytes with spleen involvement (4-6). In endemic regions of *P. vivax* infection, clinical complications and mortality have been reported and has led to the characterization of *P. vivax* malaria as a serious or even fatal disease; however, these findings are uncommon characteristics of previous infections (7,8).

Although little is known of the pathophysiology, the progression and aggravation of *P. vivax* malaria is mainly associated with anemia, occasionally due to severe hemolysis (9-11). Other pathophysiological events, such as oxidative stress, may influence the development of clinical conditions (12,13).

Some studies have cited that the Duffy glycoprotein acts as a possible facilitator in the process of erythrocyte invasion by *P. vivax*. Blood system antigens act as receptors for *P. vivax* merozoite ligands that contain Duffy-Binding-Like Domain (DBL) domains across alleles. These Duffy blood group system antigens (*Fy^a, Fy^b, Fy3, Fy5*, and *Fy6*) are encoded by two co-dominant allelic forms *FY*01* and *FY*02* that differ by the single nucleotide polymorphism (SNP) in position c.125A>G of exon (14). The SNP located at position c.-67T>C in the GATA promoter region is characterized by the allele *FY*02N.01* that silences the *Fy^b* expression in erythroid cells and homozygous individuals (*FY*02N.01 / FY*02N.01*) bear the Fy(a-b-) phenotype that is known to be a protective factor against vivax malaria infection (15).

Another phenotype associated with weak *Fy^b* antigen expression is determined by SNP c.256C> T and c.298G>A. These polymorphisms occur within the first intracellular loop of the Duffy protein resulting in reduced expression of the *Fy^b* antigen. The frequency of *$Fy^{b\ weak}$* (*FY*02W.01)* is approximately 2% in Caucasians. Some polymorphisms resulting in $Fy^{a\ weak}$ (*FY*01W.01)* allele have not been observed (16,17).

Glucose 6-phosphate dehydrogenase (G6PD) is an enzyme involved in the pentose monophosphate pathway. Deficiency of this enzyme leads to free radical-mediated oxidative damage to erythrocytes, causing hemolysis. G6PD deficiency, linked to the inheritance of X chromosome(s) with disease causing variant(s), is mostly prevalent in people of African, Asian, and Mediterranean descent (18,19). In females, there are selective advantages with G6PD A-, which is characterized by the combination of variants A376G (c.376A>G) with variants G202A (c.202G>A), A542T, G680T or T968C. The heterozygous state is suggested to offer women a selective advantage against severe malaria (20-22). G6PD deficiency is prevalent (8%) in populations where malaria is endemic (23).

The clinical severity between malaria endemic areas can be complex with multifactorial influence and genetic factors is one. In endemic areas of *P. vivax* malaria, comorbidities such as inherited hemoglobinopathies and G6PD deficiency are relevant to investigate as they may also be factors affecting phenotypic heterogeneity (24).

Vivax malaria is a challenge for control strategies and elimination (25). The unique parasite biology that involves the formation and subsequent reactivation of latent forms in the liver, and with the ability to infect vector before symptoms occur, favors the perpetuation of the parasitic life cycle (26). In this regard, there is added difficulty in tracking infected individuals owing to subpatent infections (27). This study aimed to determine the frequencies of Duffy alleles and G6PD c.202G>A and c.376A>G variants in malaria patients, to correlate with clinical signs, susceptibility, and resistance to acquired *P. vivax* infections.

Methodology

This study was based on a cross-sectional model. Blood samples and data from medical records were included from March 2013 to April 2016 period. Inclusion criteria were patients (> 18 years old) of any sexes and skin color with severe (hospitalized) or uncomplicated (outpatient) malaria without any associated disease. Patients were randomly admitted at the Tropical Medicine Foundation Dr. Heitor Vieira Dourado (FMT-HDV), a reference center for infectious disease in Manaus, capital of the Amazonas state, Brazil. All patients were treated at the Clinical Research Ward (PES CLIN) of the hospital. All the patients included in the study are unrelated individuals. Patients with comorbidities, hemoglobinopathies, mixed *Plasmodium* infections, and viral infections were excluded. Patients were classified as uncomplicated or severe malaria as described previously (28,29), according to the World Health Organization (WHO) recommendations.

This research was initiated after approval by the Research Ethics Committee (CEP) of the FMT-HVD. To ensure patient welfare, all the patients included in the study signed the Free and Clarified Commitment Term (FICF), in compliance with CNS Resolutions 196/96.

The samples were from a larger study entitled “Clinical characterization of malaria complicated by *Plasmodium vivax*”, approved by the National Commission for Ethics in Research (CONEP), in June 2009, opinion nº. 343/2009, protocol nº 25.000.011.792 / 2009-15. This new project was approved by the Research Ethics Committee of (FMT-HVD) (study number 343/2009) entitled “Study of DUFFY Polymorphisms in patients infected with *Plasmodium vivax*” with opinion no. CAAE-0004.0.112.000-11 on 12/29/2011.

Approximately 0.5 mL of peripheral blood were collected in tube with an anticoagulant such as EDTA (ethylenediaminetetraacetic acid disodium salt) at a concentration of 1.5 mg / mL for blood counts. An aliquot of blood in 1.5 mL tubes were kept for extraction of nuclear DNA. An addition 0.6 mL was collected in a tube without anticoagulants for biochemical analyses. Immediately after blood collection, hematological determinations were performed on the automated counter - ABX Pentra 80 (Horiba Diagnostics, Montpellier, FR) and biochemistry was performed on a Beckman Coulter (Inc, CA, US).

The determination of parasitaemia was based on the count of asexual parasites per 200 white blood cells. The total number of leukocytes of each patient was used for the determination of the parasite density using the following formula (30):

$$\text{Parasite density}/\mu L = \frac{\text{Number of parasites} \times \text{Total white blood cells (WBC) counts}}{\text{Number of white blood cells (WBC) counted}}$$

DNA Analysis

DNA was extracted from 200 µL of whole blood according to the QIAamp DNA Mini Kit (Qiagen, Hilden, DE) manufacturer's protocol (Cat No./ID 51304). After extraction, the DNA was quantified with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, EUA), and stored at -20ºC.

Duffy Genotyping

The DNA amplification step was divided into two: a conventional PCR for amplification of the sequence of interest, followed by restriction enzyme digestion by PCR-RFLP, one for Duffy blood group (Duffy PCR) and one for the GATA Box variant (GATA PCR).

Genotyping for Duffy blood system groups was performed with synthetic oligonucleotides FYAB1 (5 'TCC CCC TCA ACT GAG AAC TC 3') and FAB2 (5 'AAG GCT GAG CCA TAC CAG AC 3'). Amplified products were visualized on a 1.5% agarose gel stained with ethidium bromide. After PCR amplification was confirmed, the products were processed with BanI restriction enzyme digestion and incubated for at least 4 hours at 37ºC. The enzyme digestion product was electrophoresed on a 1.5% agarose gel stained with ethidium bromide for alleles discrimination.

Duffy genotypes as well as gene phenotypes were followed according to the FY (ISBT 008) Blood Group Alleles (31).

For verification of c.-67T>C SNP in the GATA Box promoter region, *FY*01/FY*02* and *FY*02/FY*02* genotype samples were used. Synthetic oligonucleotides FYN1 (5 'CAA GGC TGA CCC TA 3') and FYN2 (5 'CAT GGC ACC GTT TGG TTC AG 3') were used for the GATA PCR.

The GATA PCR product was treated with a *StyI* restriction enzyme and incubated for at least 4 hours at 37°C. The enzyme digestion product was observed on a 2.5% agarose gel. GATA normal genotypes appeared as 108 and 81bp bands, while GATA mutated showed an additional 61 bp band.

Samples with genotypes *FY*01/FY*01* and *FY*01/FY*02* were used to verify the SNPs c.265C >T and c.298G>A in the *FY*01W.02* coding region and for Fy^x.

The PCR product was treated with the restriction enzyme *MspAI* to verify the SNP c.265C>T and incubated for 4 hours at 60°C. For the SNP c.298G>A, the *MwoI* restriction enzyme was used and incubated for 4 hours at 37°C. Both were discriminated on an 8% polyacrylamide gel. The mutated genotypes for the SNP c.265C>T had an additional band of 161 bp and c.298G>A had an additional band of 343 bp.

G6PD Genotyping

For characterization of the variants, Real Time PCR (qPCR) was performed with the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific®) using TaqMan® probes specific for each polymorphism. The amplification reaction was performed for a final volume of 12uL/reaction, which contained 5uL of 2x TaqMan Universal Master Mix, 0.3uL of 20x SNP Genotyping Assay, 4.8uL of sterile water and 2.0uL of DNA (~ 100ng) of the sample. The G6PD variants analyzed in this project were chosen based on globally observed frequencies and their clinical importance according to WHO classifications. For qPCR Technique, we used the (A-) VAL68MET (202G>A) (rs1050828) and (A+) ASN126ASP (376A>G) (rs1050829) probes. To confirm the mutations found, amplification of the relevant DNA segments by PCR followed by DNA sequencing (ABI 3100, Applied Biosystems, Foster City, CA) (32) were performed.

Statistical Analysis

Data were entered into a database using Graphpad-Prism 5.0 software (Graphpad Software, San Diego, CA-US) and IBM SPSS Statistics, version 19 (IBM Corp., Armonk, NY, US), organized by variable type. The analysis of qualitative or categorical variables of three or more groups was performed by the non-parametric Chi-square test (x2), corrected by the Mantel-Haenszel and Yates tests. The analysis of groups with only two categorical variables were performed with the Fisher's exact test. Confidence intervals of 95% and prevalence ratios were calculated for these variables. The Hardy-Weinberg equilibrium (HWE) was tested by comparing observed genotypes to expected genotypes frequencies by chi-square test.

Results

A total of 225 patients diagnosed with *P. vivax* malaria were included in the study. 97 patients (43.1%) had uncomplicated and 128 (56,9%) severe malaria. Table 1 showed the epidemiological and physical parameters for uncomplicated and severe *P. vivax* malaria stratified by gender. Most severe malaria male cases had Pallor (82.5%), Choluria (77.8), Headache (76.2%), Anorexia (74.6%) and Hepatosplenomegaly (71.4%). Among the female patients, Headache (92.9%), Anorexia (85.7%), Pallor (83.3%), Vomiting (83.3%) and Choluria (73.8%) were the common symptoms.

The biochemical and hematological parameters in the *P. vivax* infected patients are shown in Table 2. As expected, significance decrease of RBC, Hemoglobin and hematocrit and high bilirubin levels was observed. Male patients with severe malaria presented lowest Hematological values ($P<0.001$).

Genotyping of c.202G>A resulted in 29 (11 women and 18 men) positive patients. Ten (4.44%) and one (0.44%) women were heterozygous and homozygous, respectively. 18 (8.0%) were hemizygous males. The c.376A>G was identified in 43 (19.11%) patients, with 18 (8.0%) heterozygous and 02 (0.88%) homozygous females, while 23 (10.22%) were hemizygous males. Both SNPs were in HWE as calculated from the frequencies of the alleles among the females as the G6PD gene is located on the X chromosome. The G6PD mutations in the clinical perspective, however, is better explain in male patients as male individual is either deficient or normal (33).

Analysis of the Duffy system showed a phenotypic distribution of 70 (31.10% - Fy(a+b-), 96 (42.70%) - Fy(a+b+), 56 (24.89%) - Fy(a-b+) and 1 (0.44%) - Fy(a-b-) among the 225 malaria patients. Table 3 shows the genotypic distribution for the Duffy system. The genotype *FY*01/FY*02* was predominant in the population.

The *FY*01/FY*02* genotype was present at higher frequencies in both uncomplicated and severe malaria, 45.3% and 39.2%, respectively (Figure 1). Males individuals with the *FY*01/FY*02* genotype bearing the c.376A>G variant of the G6PD gene seem to have a severe clinical course of malaria compared with female individuals.

Furthermore, when the clinical severity of malaria ($p=0.003$) was compared with G6PD genotypes, the c.202G>A/c.376A>G genotypes were strongly associated with previous malaria episodes ($p=0.004$) (Table 4). It is worth mentioning that G6PD deficiency in male patient are considered deficient as G6PD is located on the X-chromosome (male can be either deficient or normal). G6PD heterozygous females may exhibit an intermediate G6PD activity. Because of this, these (202/376 heterozygous female) were excluded from that analysis.

For c.202G>A, the highest frequency was among females with the Fy(a+b+) and Fy(a+b-) phenotype in severe malaria cases compared with the Fy(a-b+) phenotype, that was frequent among cases of uncomplicated malaria. In males, all Duffy phenotypes were more frequent in severe malaria, with Fy(a+b-) representing the largest number of cases. For c.376A>G, we observed a higher number of cases with the polymorphism for the Fy(a+b) phenotype in patients with severe malaria, with 47.36% of cases reported in males (data not shown).

The *FY*02N.01/FY*02N.01*, responsible for the Fy(a-b-) phenotype, was found in only one patient (female) with uncomplicated malaria and was is not carrier of the G6PD variants. Among patients with uncomplicated malaria, the frequency of c.202G>A and c.376A>G variants decreased, particularly when these polymorphisms were concomitant. Severe malaria patients showed no variations in frequency for the c.202G>A variant. However, in the presence of A376G there was a slight decrease in frequency. When combined with the GATA variant, the c.202G>A and c.376A>G variants were observed at a lower frequency in uncomplicated malaria (Table 5). For severe malaria, the presence of the combined polymorphisms showed a moderate variation in frequency (Figure 2).

No significant association was observed between the mutated Duffy alleles and *P. vivax* infection among the females. The participants' race was difficult to assess, as a strong regional ancestral mix is found among the Amazonian Caboclos that originated with the arrival of Caucasians and African-Brazilian to indigenous lands.

The Duffy phenotype Fy(a-b+) (p=0.003) and genotype FY*02/FY*02 (p=0.007) correlated with high parasitemia density (Figures 3, 4).

Discussion

Hypoglycemia (<70 mg/dL) has been detected in approximately 16% of patients and is considered a clinical and laboratory features of severe malaria (34). Careful glucose monitoring should be targeted to these complications, especially in those patients with G6PD deficiency. However, none of the G6PD alleles segregated with low level glucose among the severe malaria patients.

In this study, we found in uncomplicated malaria patients a three times higher frequency of genotype *FY*01/FY*02* (45.3%) when compared to the *FY*01/FY*01* (15.4%) and *FY*02/FY*02* (14.8%). In severe malaria patients, the frequency of genotype *FY*01/FY*02* (39.2%) was approximately two times higher with regards to the genotypes *FY*01/FY*02N.01* (20.6%) and *FY*02/FY*02* (17.5%). These findings corroborate to study reported by Cavasini et al. (2007), that correlated the high frequency of the *FY*01* and *FY*02* alleles among *P. vivax* malaria patients. The *FY*01/FY*02* and *FY*01/FY*01* genotypes were associated with a high frequency of *P. vivax* infection suggesting that these individuals have a higher risk of developing disease (35). The *FY*01/FY*01* and *FY*01/FY*02* genotypes are associated with increased frequency of *P. vivax* infection, while *FY*01/ FY*02W.01* and *FY*02/ FY*02W.01* were associated with low parasitic density levels (17).

The presence of a single case with null Duffy variants in this study supports a possible advantageous selection, driven by defense mechanisms against *P. vivax*. In endemic areas, the Duffy negative blood group is reported as a protective factor against *P. vivax* malaria infection (36). In a study conducted in São Paulo, the phenotypic frequencies found in blood donors for Duffy blood system antigens were 19.8% for the phenotype Fy(a+b-) in Caucasians and 14.0% in African-Brazilian, Fy(a+b+) in 41.4% of Caucasians and 1.6% of African-Brazilian, Fy(a-b+) in 37.8% of Caucasians and 17.5% of African-Brazilian and Fy(a-b-) in 1.1% of Caucasians and 66.9% of African-Brazilian (37). Our results indicate that the Manaus-Amazonas region has individuals who express three Duffy phenotypes Fy(a+b+), Fy(a+b-) and Fy(a-b+) more frequently with expression of *FY*01* and *FY*02* antigens.

The results and clinical presentation of patients in this study support the hypothesis that G6PD deficiency does not confer a decreased risk of severe malaria infection (Table 5). Controversies continue as hemizygous males and heterozygous females failed to have changes in frequency distribution in uncomplicated malaria. However, the results showed that both male or female sex is independent and provides no protection against severe malaria (33,38,39).

For the process of parasite invasion into red blood cells, Duffy protein is functionally important. In regions of high malaria transmission rates, as in the inhabitants of the Amazon, Duffy protein is naturally immunogenic. Woolley et al. demonstrated (in *vitro*) that the expression level of the *Fy6* epitopes was significantly lower in reticulocytes and red blood cells from individuals carriers of the FYB/FYB genotypes compared with individuals of the FYA/FYA or FYA/FYB genotypes. Another similar study showed that the presence of the *FY*02N.01* allele resulted in a 50% reduction of that protein on invasion by *P. vivax* (40,41).

In a study with *P. vivax* malaria patients, the *FY*01* and *FY*02* alleles were found to have low, medium, and high parasitic density. However, in the presence of the GATA variant, genotypes with alleles *FY*02N.01* and *FY*02W.01* were found only in patients with low parasitic density and low symptomatology (17).

A study performed in the state of Pará with a population of African descent demonstrated the presence of the c.202G>A variant was 0.060, the Duffy blood group included 24.3% Duffy negative and 41.3% individuals heterozygous for $FY*02^{W}$. The frequency of the $FY*02^{W}$ allele was 41.0%. These findings support the monitoring of individuals with G6PD deficiency for use of primaquine during the routine care of Afro-descendant communities of the Trombetas, Erepecuru, and Cumná rivers, to assess risks of hemolytic crisis in recurrent cases of malaria in the region (42).

Several studies have revealed that the phenotypic and genotypic variation complexity of the Duffy system and the variants of G6PD (A-) can have a significant impact on the distribution of human populations in areas where malaria is endemic. The Duffy system and G6PD are polymorphic systems that offer great challenges to researchers not only due to their academic importance, but also due to their potential applications in the treatment of vivax malaria (43). Whenever natural selection occurs within a population in an area of endemicity for malaria, natural adaptations may result from genetic variation that provides a partial defense mechanism against *P. vivax* infections (40).

In this study, we were careful to include hemoglobinopathies status like sickle cell anemia (HbSS) or Sickle cell diseases (HbSC, HbSD, HbSE) of the patients before antimalarial treatment intervention.

One of the major confounders of our analysis and limitations in our study, is the inherent disadvantage of G6PD deficiency associate acute hemolysis as of one factor that influence no accord of consensus on the G6PD potentially protect against malaria. Despite most G6PD-deficient persons are asymptomatic, a hemolytic anemia, main clinical sign, can occur 1-3 days after exposure, eating fava beans, triggered by infections or by certain drugs, such as those used to treat malaria (44,45). In many cases, this acute hemolytic anemia is usually self-limiting and resolve on their own (46). In addition, we acknowledge that an important limitation of our analysis is the small sample size and we understand that additional studies of larger samples will be required to confirm our results.

Conclusion

This study reports the frequency of G202A and A376G variants and Duffy alleles in patients with severe and uncomplicated malaria in Manaus, Amazonas, Brazil.

The presence of the G202A and A376G variants is a risk factor for the development of severe *P. vivax* malaria. Before treatment, molecular diagnosis for the G202A and A376G variants in patients diagnosed with malaria may be necessary in the Amazonian population.

The *FY*01/FY*02* Duffy genotypes was more frequent than the null expression genotype in the vivax malaria patients and the *FY*01/FY*02* genotype demonstrated greater association with severe malaria cases.

We found one only of the uncomplicated vivax malaria patient with Duffy phenotype Fy(a-b-).

Heterozygous GATA variants do not confer protection against malaria infection.

Duffy phenotype Fy(a-b+) and genotype *FY*02/ FY*02* presented the highest values parasitemia density of the vivax malaria.

Uncomplicated malaria showed a greater frequency of variation for GATA and G6PD variants than severe malaria.

Declarations

AUTHORS' CONTRIBUTIONS

- Natália Santos Ferreira and Jéssica Lorena dos Santos Mathias performed the collection of samples and performed the practical and laboratorial parts of the entire project.
- Natalia Santos Ferreira, Jéssica Lorena dos Santos Mathias and Anne Cristine Gomes Almeida performed the molecular tests for the genotyping of G6PD and Duffy genes polymorphisms. It should be noted that this project was the dissertation for his Master's Degree of Natalia Santos Ferreira and Jéssica Lorena dos Santos Mathias.
- Ana Carla Dantas and Fernanda Cozendey Anselmo assisted in the collection of samples and digitization of results in data analysis programs.
- Sérgio Roberto Lopes Albuquerque, Marcus Vinicius Guimarães Lacerda and Emerson Silva Lima assisted wrote the manuscript with support from Jose Pereira de Moura Neto.
- Marilda de Souza Gonçalves, Paulo Afonso Nogueira, Sérgio Roberto Lopes Albuquerque, Rajendranath Ramasawmy and Marcus Vinicius Guimarães Lacerda assisted in the development of the project and contributed to the final version of the manuscript.
- José Pereira de Moura Neto conceived the study, was the idealizer of the project and advised Natalia Santos Ferreira and Jéssica Lorena dos Santos Mathias. He helped supervise the project and assisted in sample collection and performed the analytical calculations and results simulations.
- The authors declare no conflict of interest and have no have financial relations with the industry. All donors were volunteers and provided (or a legal guardian provided) informed, written consent.

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Tables

Table 1. Epidemiological clinical parameters for uncomplicated and severe *P. vivax* malaria patients from Manaus, Amazon.

Clinical Signs	FEMALE			MALE		
	Severe (n=60) %	Uncomplicated (n=47) %	p-value	Severe (n=68) %	Uncomplicated (n=50) %	p-value
Abdomen Pain	43	35	.461	44	33	.521
Severe Anemia	13	01	.002	11	01	<.001
Anorexia	51	35	.132	51	37	.533
Choluria	44	23	.008	53	21	<.001
Cough	24	16	.334	21	16	.526
Diarrhoea	17	12	.460	28	18	.353
Dyspnoea	20	23	.075	24	18	.544
Epistaxis	03	05	.231	03	10	.008
Headache	56	39	.084	51	46	.013
Haemoglobinuria	11	06	.305	14	03	.021
Hepatosplenomegaly	39	20	.016	49	14	<.001
Jaundice	43	09	<.001	43	12	<.001
Leukocyturia	20	18	.371	30	08	<.001
Obesity	05	05	.467	09	01	.028
Oliguria	09	04	.237	10	04	.206
Pallor	50	33	.083	56	43	.393
Petechia	03	11	.005	03	05	.204
Vomiting	50	40	.509	44	36	.262
Whole Blood Transfusion	10	04	.170	18	03	<.001

Independent – Samples T-tests Post Hoc/ Bonferroni test ¯: Increase or decrease of frequency N: cases
N: Cases [a] Fischer Test [b] c2 test (Yates's corrected)

Table 2. Hematological and biochemical parameters for uncomplicated and severe *P. vivax* malaria patients from Manaus, Amazon.

	FEMALE			MALE		
	Severe (n=60)	Uncomplicated (n=47)	p-value	Severe (n=68)	Uncomplicated (n=50)	p-value
Age, median	37.07 ± 12.45	35.36 ± 13.76	.402	39.43 ± 13.45	25.01 ± 19.39	.770
Temperature, median	37.25 ± 0.79	37.13 ± 0.89	.908	36.95 ± .74	36.62 ± 0,91	.623
Weight (Kg)	56.47 ± 17.91	62.45 ± 16.09	.561	51.89 ± 24.07	58.30 ± 15.90	.537
Numbers of days in hospital	2.80 ± 1.56	3.68 ± 3.43	.363	3.51 ± 2.40	5.93 ± 7.61	.277
Asexual Parasite Density	15735.4 ± 13276.2	8597.3 ± 9176.5	.032	5264.4 ± 9994.7	8760.1 ± 11474.1	.318
Parasitaemia	344.53 ± 207.06	302.91 ± 201.67	.404	169.80 ± 209.01	229.63 ± 181.54	.591
Platelet Count $x10^9$/L	59.94 ± 46.06	87.36 ± 77.78	.079	125.42 ± 113.01	109.19 ± 73.95	.428
WBC $x10^3$ ul	5.27 ± 2.01	5.56 ± 2.21	.542	7.67 ± 4.03	5.78 ± 2.35	.010
RBC $x10^6$ ul	3.51 ± 0.89	3.98 ± 0.75	.008	3.48 ± 1.12	4.46 ± 0.74	<.001
Hemoglobin (g/dl)	99.72 ± 2.42	11.33 ± 2.15	.042	10.06 ± 2.94	12.71 ± 2.01	<.001
Hematocrit (%)	30.45 ± 7.75	35.95 ± 6.95	.029	30.22 ± 8.97	39.62 ± 6.17	<.001
MCV (fL)	87.72 ± 5.57	86.13 ± 5.64	.240	88.68 ± 8.83	89.05 ± 4.34	.790
MCH (pg)	28.94 ± 2.84	27.94 ± 2.05	.086	29.56 ± 3.14	28.57 ± 1.94	.081
MCHC (g/dl)	32.99 ± 1.77	32.46 ± 1.43	.166	33.65 ± 1.59	32.09 ± 1.72	<.001
Reticulocytes (%)	1.71 ± 2.16	1.75 ± 1.41	.951	4.48 ± 4.84	3.18 ± 4.14	.371
Direct bilirubin (mg/dL)	7.02 ± 7.68	3.76 ± 8.29	.127	3.61 ± 4.78	6.84 ± 7.26	.023
Indirect bilirubin (mg/dL)	4.90 ± 5.58	2.62 ± 5.79	.133	6.41 ± 8.73	11.86 ± 9.91	.012
Total bilirubin (mg/dL)	12.50 ± 13.46	7.81 ± 16.58	.239	10.36 ± 12.56	19.97 ± 17.94	.007
Urea (mg/dL)	33.06 ± 14.20	30.63 ± 16.84	.829	61.45 ± 35.63	36.86 ± 13.75	<.001
Creatinine (mg/dL)	0.81 ± 0.34	0.88 ± 0.38	.426	1.40 ± 0.94	1.02 ± 0.34	.022
Lactate dehydrogenase (U/L)	855.86 ± 339.73	548.95 ± 173.95	<.001	921.06 ± 524.01	650.07 ± 235.41	.072
Alkaline Phosphatase (IU/L)	260.41 ± 169.77	251.24 ± 148.09	.823	232.53 ± 132.78	127.85 ± 114.66	<.001
Ca^{++} (mM)	8.64 ± 0.80	8.85 ± 0.99	.466	8.73 ± 1.21	9.33 ± 0.89	.130
K^+ (mM)	3.66 ± 0.49	3.92 ± 0.41	.079	3.99 ± 0.59	3.97 ± 0.45	.938
Glucose mg/dL	88.82 ± 32.71	97.58 ± 28.06	.255	99.20 ± 35.03	101.44 ± 33.65	.773

Independent – Samples T-tests Post Hoc/ Bonferroni test Continuous variables are presented as mean ± SD N: Cases

WBC: White Blood Cells RBC: Red Blood Cells MCV: Mean corpuscular volume

MCH: Mean corpuscular hemoglobin MCHC: Mean corpuscular hemoglobin concentration

Table 3. Allele frequency of the Duffy blood group from *vivax* malaria patients.

Phenotypes	Genotypes	Percentage (%)
Fy(a+b+)	*FY * 01 / FY * 02*	42,70
Fy(a+b-)	*FY * 01 / FY * 02N.01*	16,00
	*FY * 01 / FY * 01*	15,10
Fy(a-b+)	*FY * 02 / FY * 02*	16,00
	*FY * 02 / FY * 02N.01*	8,88
Fy($a+^w$)	*FY*01 / FY * 01W.02*	0,88
Fy(a-b-)	*FY * 02N.01 / FY * 02N.01*	0,44

Table 4. Genotypic frequency of G6PD variants among patients with uncomplicated and severe *P. vivax* malaria.

G6PD Genotypes	MALARIA N (%)		RR (CI)	p-value	PREVIOUS MALARIA EPISODES N (%)		RR (CI)	p-value
	Severe	Uncomplicated			Yes	No		
c.202G>A	11 (15.7)	03 (3.3)	4.76 (1.31-	.009**	11 (20.0)	03 (3.3)	6.73 (1.85-	<.001**
202wt	59 (84.3)	88 (96.7)	21.22)		44 (80.0)	98 (97.0)	29.91)	
c.376A>G	16 (21.3)	03 (3.3)	6.47	<.001**	14 (24.1)	04 (3.9)	6.15	<.001**
376wt	59 (78.7)	88 (96.7)	(1.89- 2753)		44 (75.9)	98 (96.1)	(2.02- 21.64)	
c.202G>A / c.376A>G	11 (15.7)	02 (2.2)	6.99 (1.54-	.003**	09 (17.0)	03 (3.0)	5.71 (1.49-	.004**
202wt / 376wt	59 (84.3)	87 (97.8)	45.34)		44 (83.0)	98 (97.0)	26.18)	

wt: Wild Type N: cases RR: Relative Risk (CI): Interval of Confidence * * Fisher's exact test

Table 5. Genotypic frequency distribution of G6PD variants among uncomplicated and severe *P. vivax* malaria patients by the presence of Duffy GATA normal or mutated variants.

Malaria	Duffy GATA	G202A N (%)		p-value	A376G N (%)		p-value	G202A / A376G N (%)		p-value
		202wt	c.202G>A		376wt	c.376A>G		202wt/376wt	c.202G>A/c.376A>G	
Uncomplicated	Normal	86 (94.5)	05 (5.5)	.487**	86 (88.7)	11 (11.3)	.452**	86 (97.7)	02 (2.3)	.531**
	Mutated	24 (92.3)	02 (7.7)		24 (92.3)	02 (7.7)		24 (96.0)	01 (4.0)	
Severe	Normal	45 (77.6)	13 (22.4)	.181*	45 (70.3)	19 (29.7)	.252*	45 (83.3)	09 (16.7)	.107*
	Mutated	17 (65.4)	09 (34.6)		17 (60.7)	11 (39.3)		17 (68.0)	08 (32.0)	
Total	Normal	131 (87.9)	18 (12.1)	.087*	131 (81.4)	30 (18.6)	.249*	131 (92.3)	11 (7.7)	.043*
	Mutated	41 (78.8)	11 (21.2)		41 (75.9)	13 (24.1)		41 (82.0)	09 (18.0)	

wt: Wild Type N: cases * c2 test (Yates's corrected) * * Fisher's exact test

Figures

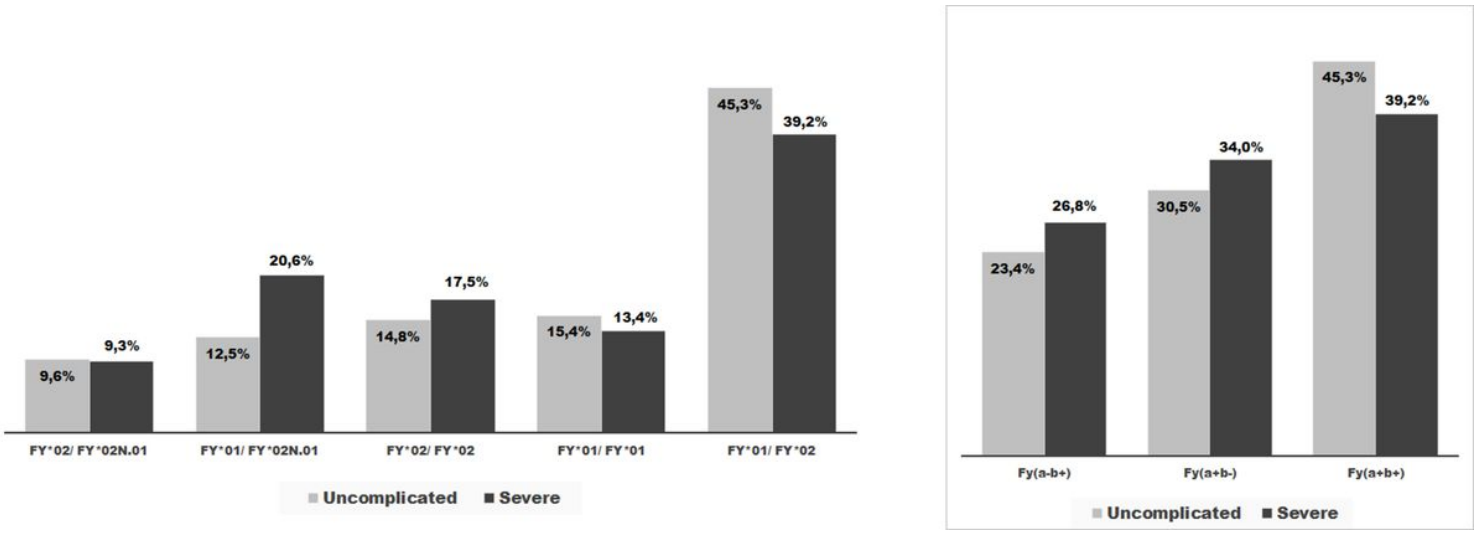

Figure 1

Genotypic and phenotypic distribution of Duffy antigens among uncomplicated and severe P. vivax malaria patients. The genotypes with null and weak expression of Duffy System were found in patients with uncomplicated malaria and the FY*01/FY*02 genotype demonstrated greater association with severe malaria cases.

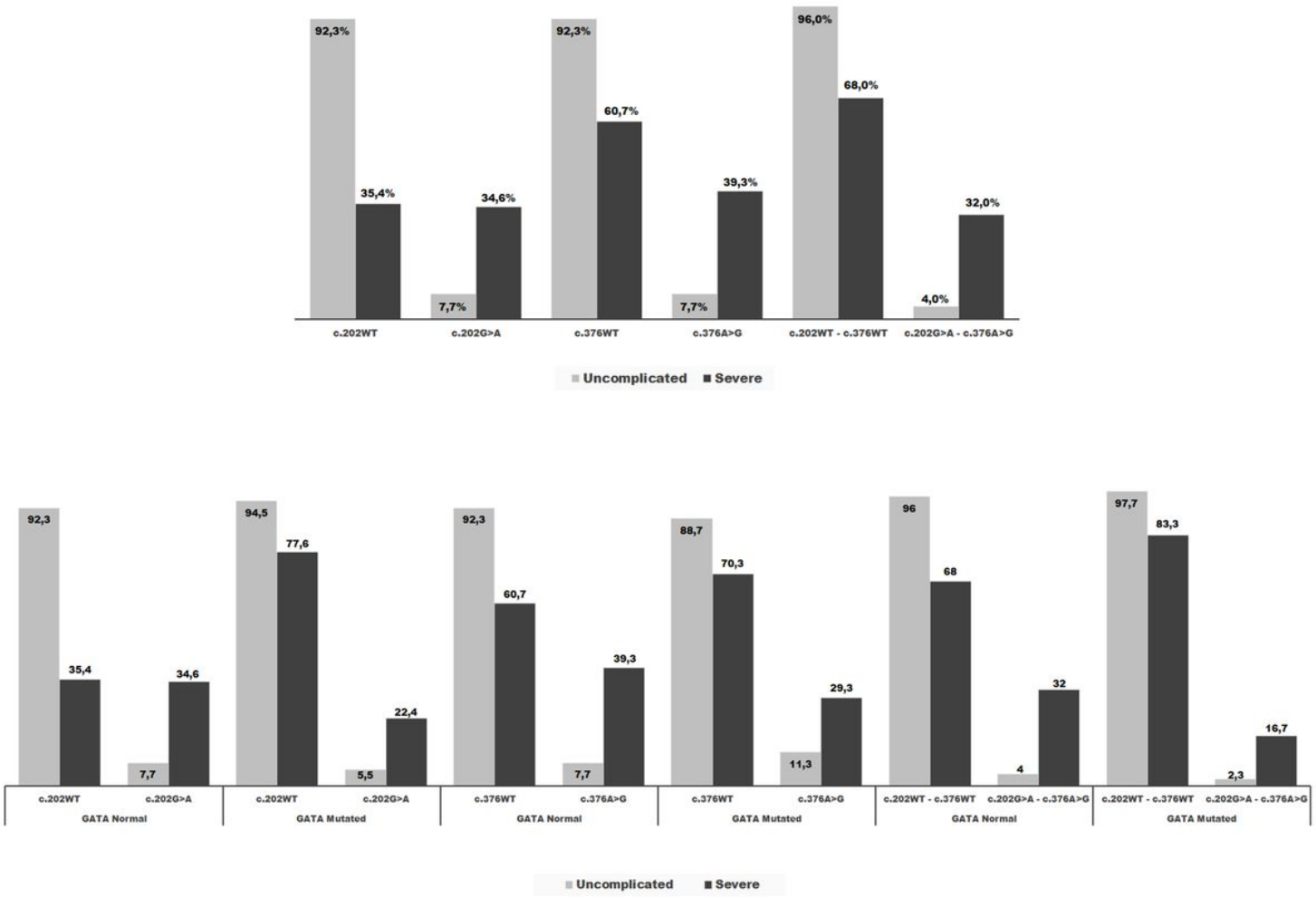

Figure 2

Distribution of c.202G>A and c.376A>G variants in the presence and absence of the Duffy variant among P. vivax malaria patients. The top panel shows that the presence of G202A and A376G variants were more frequent in cases of severe vivax malaria, with the lowest frequencies in uncomplicated malaria cases concomitant with the presence of other variants. On the lower panel, the GATA variant has been associated with virtually all records of GATA / G6PD variants in severe vivax malaria cases.

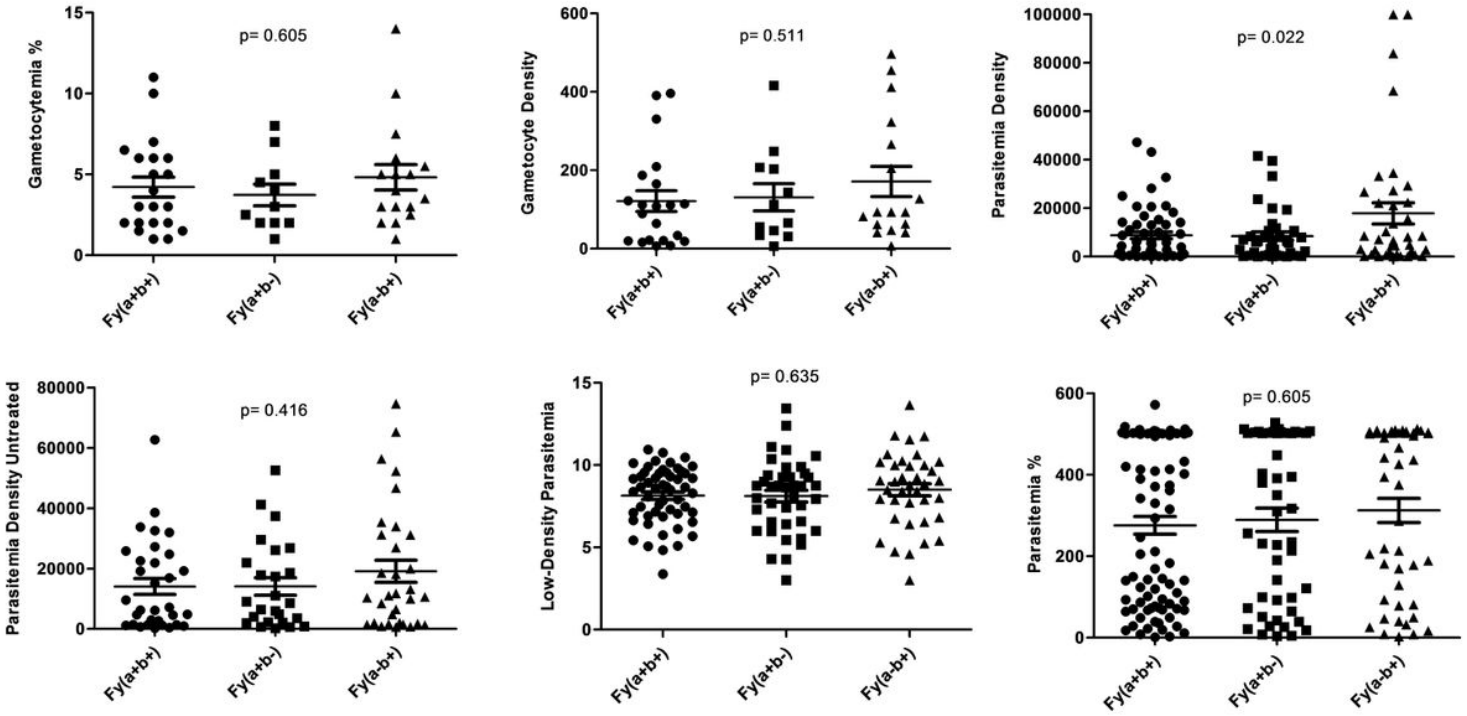

Figure 3

Parasitemia in P. vivax malaria patients and Duffy phenotypes. There was statistical significance (p = 0.003) for parasitic density, mainly in the phenotype Fy(a-b+).

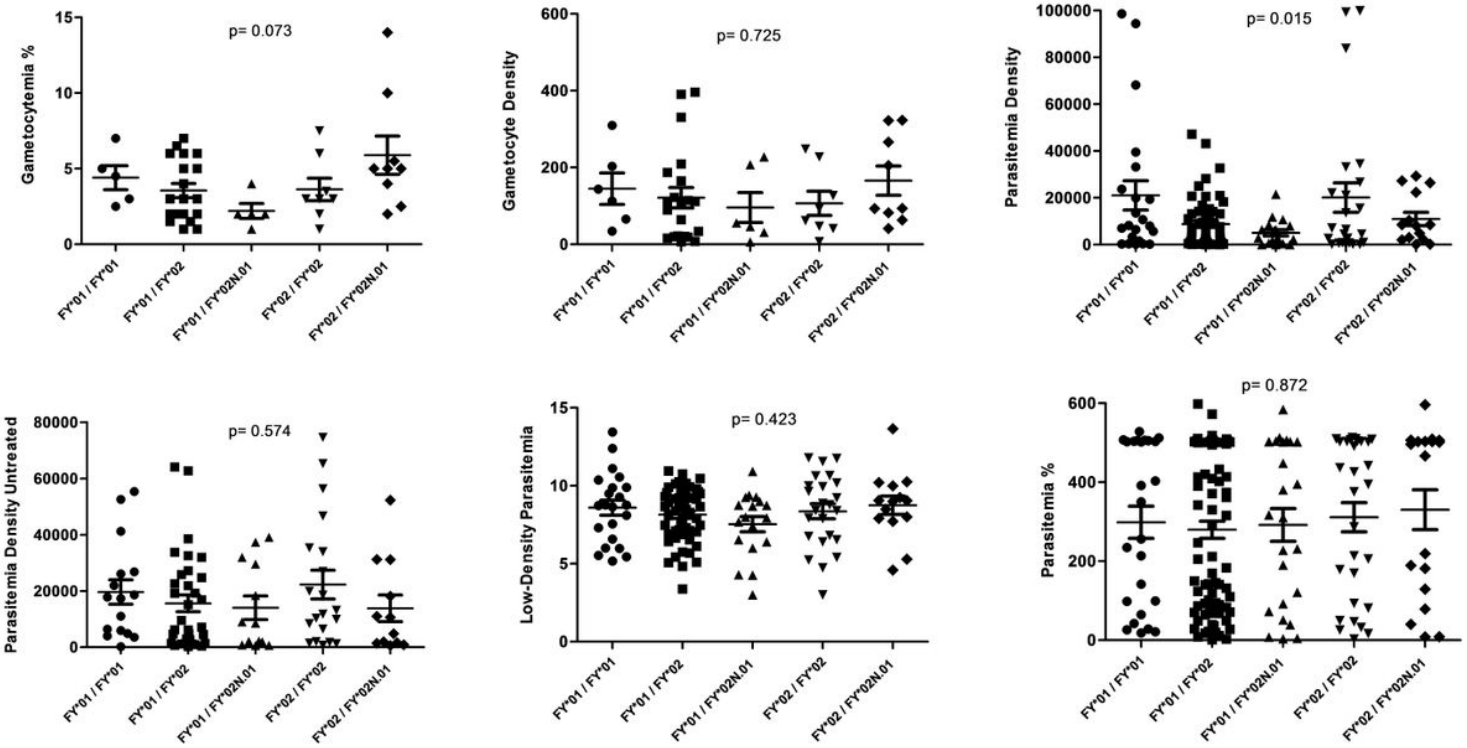

Figure 4

Parasitemia in P. vivax malaria patients and Duffy genotypes. The FY*02/ FY*02 genotype had the highest parasitic values, and when an FY*02 allele was present and expressed, these values were higher. The parasitic density was lower in the presence of the GATA variant.