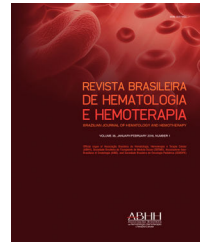




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Original article

Molecular typing of human platelet antigens in immune thrombocytopenia patients in northern Brazil



Julia Cavalcante do Carmo<sup>a</sup>, Prissyla de Souza Klippel<sup>a</sup>, Sabrina da Costa Cordeiro<sup>b</sup>,  
Ângela Maria dos Santos Fernandes<sup>b</sup>, Raquel Medeiros Pinto<sup>a</sup>,  
Simone Schneider Weber<sup>c,d,\*</sup>, Cleiton Fantin<sup>a</sup>

<sup>a</sup> Universidade do Estado do Amazonas (UEA), Manaus, AM, Brazil

<sup>b</sup> Fundação de Hematologia e Hemoterapia do Amazonas (Hemoam), Manaus, AM, Brazil

<sup>c</sup> Universidade Federal do Amazonas (UFAM), Itacoatiara, AM, Brazil

<sup>d</sup> Universidade Federal do Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil

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ABSTRACT

**Background:** Immune thrombocytopenia is an immune disease characterized by thrombocytopenia and bleeding due to platelet antibodies against platelet membrane glycoproteins. Human platelet antigens are derived from polymorphisms of these glycoproteins. The aim of this study was to investigate human platelet antigen frequencies in immune thrombocytopenia patients from the state of Amazonas, Brazil and investigate the potential association between specific antigens and risk for immune thrombocytopenia.

**Method:** Human platelet antigen typing was performed by BeadChip technology to determine allelic variants of 11 systems (HPA-1 to HPA-9, HPA-11 and HPA-15). Thirty-six patients (8 male and 28 female) with a median age of 34 years (range: 9–69 years) were evaluated and compared with data from Amazonas blood donors.

**Results:** Platelet counts varied from 3 to  $98 \times 10^9/L$ . The allele frequencies were 0.944 for HPA-1a, 0.056 for HPA-1b, 0.847 for HPA-2a, 0.153 for HPA-2b, 0.555 for HPA-3a, 0.444 for HPA-3b, 0.805 for HPA-5a, 0.222 for HPA-5b, 0.9975 for HPA-9a, 0.025 for HPA-9b, 0.486 for HPA-15a and 0.513 for HPA-15b. Among immune thrombocytopenia individuals, no b allele of the HPA-4, -6, -7, -8 and -11 were found.

**Conclusions:** The results suggest HPA-1a, HPA-3b and HPA-5b are immune thrombocytopenia-specific autoepitopes.

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\* Corresponding author at: Centro de Ciências Biológicas e da Saúde. Universidade Federal do Mato Grosso do Sul, Cidade Universitária S/N, Caixa Postal 549, 79070-900 Campo Grande, MS, Brazil.

E-mail address: [weberblood@gmail.com](mailto:weberblood@gmail.com) (S.S. Weber).

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## Introduction

Immune thrombocytopenia (ITP) is an immune-mediated acquired disease characterized by transient or persistent decrease affecting platelet numbers and, depending upon the degree of thrombocytopenia, increased risk of bleeding,<sup>1</sup> due to the presence of platelet autoantibodies. Platelet membrane glycoproteins (GPs) appear to be the principal binding sites of ITP serum antibodies.<sup>2</sup> The polymorphisms of the human platelet alloantigens occur due to single nucleotide substitutions that result in the substitution of an amino acid.<sup>3</sup>

The Immuno Polymorphism Database (IPD) of human platelet alloantigens (HPA) lists 35 platelet alloantigens, which are located in GPs (platelet receptors).<sup>4</sup> The three major platelet receptors are GPIIb-IIIa, GPIb-IX-V and GPIa-IIa.<sup>3,5,6</sup> GPIIb/IIIa is the most polymorphic complex and carries 19 antigens<sup>5</sup>: HPA-1 (176T>C); HPA-3 (2621T>G); HPA-4 (506G>A); HPA-6 (1544G>A); HPA-7w (1297C>G); HPA-8w (1984C>T); HPA-9w (2602G>A); HPA-10w (263G>A); HPA-11w (1976G>A); HPA-14w (1909\_1911delAAG); HPA-16w (497C>T); HPA-17w (662C>T); HPA-19 (487A>C); HPA-20w (1949C>T); HPA-21w (1960G>A); HPA-22w (584A>C); HPA-23w (1942C>T); HPA-24w (1508G>A) and HPA-26w (1818G>T). The von Willebrand factor (vWF) receptor GPIb/IX carries two antigens HPA-2 (482C>T) and HPA-12w (119G>A). In addition, the GPIa/IIa complex carries the HPA-5 (1600G>A), HPA-13w (2483C>T), HPA-18w (2235G>T) and HPA-25w (3347C>T) polymorphic systems.<sup>7,8</sup> Moreover, the HPA-15 (Gov) polymorphism is located in the CD109 molecule and its alleles differ at a single nucleotide polymorphism (C2108A) that causes a Tyr682Ser amino acid substitution.<sup>9,10</sup> These polymorphisms can be recognized as alloantigens or autoantigens and trigger the clearance of opsonized platelets by phagocytes in the reticuloendothelial system or inhibition of platelet production.<sup>11</sup> Several groups worldwide have tried to establish a possible association between HPA polymorphisms and ITP.<sup>12-15</sup> Some ITP-specific autoepitopes have been suggested, such as HPA-2a in German patients with chronic refractory ITP<sup>14</sup> and HPA-2b in Macedonian patients with ITP.<sup>15</sup> In addition, Castro et al.,<sup>12</sup> suggested the presence of HPA-5b in Brazilian patients with increased risk for acute ITP, while the HPA-5a has been implicated in Korean patients.<sup>13</sup> However, the results have been unclear, and studies exploring these hypotheses are still lacking.

The aim of this study was to analyze the frequencies of human platelet antigens, grouped as 11 biallelic HPA systems (HPA-1 to HPA-9, HPA-11 and HPA-15) in patients from Amazonas State with primary ITP, and to investigate the potential association between specific HPA polymorphisms and risk for ITP.

## Methods

### Study site

The Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) is a referral center for the diagnosis, treatment and monitoring of hematological diseases in the northern region of Brazil. The service receives approximately

100 new cases of ITP annually. This study was approved by the institution's Ethics Committee (CEP/HEMOAM #803.634/2014).

### Sample definitions

In total, 36 unrelated sequential ITP patients treated in HEMOAM participated in the study between October 2014 and April 2015. Informed consent was obtained from all enrolled patients. All patients in this study had chronic primary ITP, which by definition meant the disease lasted for more than 12 months after the initial treatment. The criterion for primary ITP was the presence of isolated thrombocytopenia (peripheral blood platelet count  $<100 \times 10^9/L$ ) in the absence of other causes or disorders that might be associated with this low platelet count, in accordance with the standardization of ITP diagnosis established by an international ITP working group.<sup>16</sup> Thus, the diagnosis of primary ITP was achieved by exclusion. The sample definition for HPA genotyping of blood donors excluded samples with platelet counts less than  $150 \times 10^9/L$ .

### Genomic DNA extraction

Genomic DNA samples were obtained from EDTA-preserved whole blood using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The automated epMotion 5075 system (Eppendorf, Hamburg, Germany) was adapted. The DNA concentration and quality were evaluated spectrophotometrically using NanoDrop technology (Thermo Fisher Scientific, Massachusetts, USA). The samples were stored at  $-80^\circ C$  until use.

### Platelet genotyping by BeadChip microarray technology

Platelet genotyping was performed using a BeadChip assay.<sup>17,18</sup> The BeadChip microarray method is capable of determining 22 allelic variants of 11 HPA systems (HPA-1 to HPA-9, HPA-11 and HPA-15). DNA amplification and post-polymerase chain reaction steps were performed according to the manufacturer's instructions. The BeadChip slides were analyzed in a fluorescent system using the Bioarray Solutions software (Immucor, Warren, NJ) in the HEMOAM genomic laboratory.

### Statistical analysis

The genotype and allele frequencies were estimated by direct counting, and the results were compared individually with the values published for healthy individuals from Amazonas.<sup>19</sup> The 95% confidence interval (CI), chi square ( $X^2$ ) test or Fisher's exact test were used for comparative analysis. The Hardy-Weinberg equilibrium of HPA system genotypes was evaluated using the Hardy-Weinberg calculator.<sup>20</sup> *p*-Values lower than 0.05 were considered significant in all statistical analyses.

## Results

The clinical data and allele frequencies obtained from the microarray for HPA-1 to HPA-9, HPA-11 and HPA-15 in chronic

**Table 1 – Clinical data and comparative analysis of allele frequencies between immune thrombocytopenia (ITP) patients and blood donors in the state of Amazonas.**

Clinical data		ITP patients			Blood donors <sup>b</sup>
n° patients		36			200
Gender (male:female)		8:28			140:60
Age (years)		34 (9–69)			36 (19–65)
Platelet count – ×10 <sup>9</sup> /L		41.5 (3–98) <sup>a</sup>			>150 <sup>c</sup>
System	a	b	a	b	p-value <sup>d</sup>
HPA-1	0.944	0.056	0.86	0.137	<0.0001
HPA-2	0.847	0.153	0.852	0.147	0.754
HPA-3	0.555	0.444	0.665	0.335	<0.0001
HPA-4	1.00	0.00	0.995	0.005	<0.0001
HPA-5	0.805	0.222	0.892	0.107	<0.0001
HPA-6	1.00	0.00	1.00	0.00	–
HPA-7	1.00	0.00	1.00	0.00	–
HPA-8	1.00	0.00	1.00	0.00	–
HPA-9	0.997	0.025	0.997	0.005	–
HPA-11	1.00	0.00	1.00	0.00	–
HPA-15	0.486	0.513	0.502	0.497	0.502

<sup>a</sup> Platelet count at diagnosis.  
<sup>b</sup> Control includes blood donor samples published by Portela et al.<sup>19</sup>  
<sup>c</sup> Platelet counts greater than 150 × 10<sup>9</sup>/L.  
<sup>d</sup> Chi-square with Yates' correction calculated Fisher's exact p-value for comparisons of allele frequencies between ITP patients and Amazonas blood donors.

**Table 2 – Genotype and allele frequencies for HPA-1 to HPA-9, HPA-11 and HPA-15 in chronic immune thrombocytopenia patients from the state of Amazonas.**

GP	SNP	Genotype frequency			Allele frequency		p-value Hardy-Weinberg	
		AA	AB	BB	a	b		
HPA-1	GPIIIa	T176C	0.888	0.111	0.00	0.944	0.056	0.724
HPA-2	GPIIa	T482C	0.694	0.305	0.00	0.847	0.153	0.279
HPA-3	GPIIb	T2621G	0.305	0.5	0.194	0.555	0.444	0.940
HPA-4	GPIIIa	G506A	1.00	0.00	0.00	1.0	0.00	–
HPA-5	GPIa	G1600G	0.611	0.388	0.027	0.805	0.222	0.479
HPA-6	GPIIIa	G1544A	1.00	0.00	0.00	1.0	0.00	–
HPA-7	GPIIIa	C1297G	1.00	0.00	0.00	1.0	0.00	–
HPA-8	GPIIIa	C1984T	1.00	0.00	0.00	1.0	0.00	–
HPA-9	GPIIb	G2602A	0.995	0.005	0.00	0.9975	0.025	0.97
HPA-11	GPIIIa	G1976A	1.00	0.00	0.00	1.0	0.00	–
HPA-15	CD109	C2108A	0.166	0.638	0.194	0.486	0.513	0.094

GP: glycoprotein; SNP: single nucleotide polymorphism.

ITP patients and comparative analysis are summarized in [Table 1](#). The study sample was comprised of eight (22%) male and 28 (78%) female individuals. The participants' ages ranged from 9 to 69 years (mean age: 34 years) and the platelet count at diagnosis varied from 3 to 98 × 10<sup>9</sup>/L (median: 41.5 × 10<sup>9</sup>/L). The disease severity ranged between mild or moderate when correlated with the degree of thrombocytopenia. The genotype and allele frequencies and p-values of the Hardy-Weinberg test of samples employed in this study are shown in [Table 2](#). The allele frequencies were 0.944 for HPA-1a, 0.056 for HPA-1b, 0.847 for HPA-2a, 0.153 for HPA-2b, 0.555 for HPA-3a, 0.444 for HPA-3b, 0.805 for HPA-5a, 0.222 for HPA-5b, 0.9975 for HPA-9a, 0.025 for HPA-9b, 0.486 for HPA-15a and 0.513 for HPA-15b. Of the ITP individuals, no b allele was identified for HPA-4, HPA-6, HPA-7, HPA-8 and HPA-11. Among these ITP individuals, the

allele frequencies of the HPA system were consistent with the Hardy-Weinberg equilibrium.

In the comparative analysis, the allele frequencies for HPA-2 (p-value=0.754) and HPA-15 systems (p-value=0.502) were not significantly different between analyzed groups (ITP patients and healthy individuals). On the other hand, the ITP Group had higher incidences of HPA-1a (0.944), HPA-3b and HPA-5b alleles when compared to the Control Group.

[Table 3](#) presents mismatch probabilities in homozygous chronic ITP patients (AA and BB) obtained considering the HPA genotype frequencies from Amazonas blood donors. Heterozygous patients, who present both alleles (a and b), have no post-transfusion mismatch development and this was not evaluated in this analysis.

**Table 3 – Probability of mismatch in homozygous patients.**

Systems	Gd <sub>AA</sub>	Gd <sub>AB</sub>	Gd <sub>BB</sub>	Mismatch in AA patients Gd <sub>AB</sub> + Gd <sub>BB</sub> (%)	95% confidence interval		Mismatch BB patients Gd <sub>AB</sub> + Gd <sub>AA</sub> (%)	95% confidence interval	
					Lower limit	Upper limit		Lower limit	Upper limit
HPA-1	0.740	0.245	0.015	0.26 (26)	0.227	0.292	0.98 (98)	0.915	1.00
HPA-2	0.720	0.265	0.015	0.28 (28)	0.244	0.315	0.98 (98)	0.921	1.00
HPA-3	0.430	0.470	0.100	0.57 (57)	0.518	0.621	0.90 (90)	0.894	0.905
HPA-4	0.990	0.010	0.000	0.01 (1)	0.008	0.011	1.00 (100)	0.862	1.00
HPA-5	0.800	0.185	0.015	0.20 (20)	0.176	0.223	0.98 (98)	0.898	1.00
HPA-9	0.995	0.005	0.000	0.005 (0.5)	0.004	0.005	1.00 (100)	0.861	1.00
HPA-15	0.252	0.495	0.250	0.745 (75)	0.710	0.779	0.747 (75)	0.712	0.781

Gd: genotype of Amazonas blood donor.

## Discussion

Studies have demonstrated relevant associations between GP polymorphisms and immune-mediated platelet disorders. The HPA allele frequencies were compared in 36 chronic ITP patients with published data of 200 healthy individuals from Amazonas.<sup>19</sup> The ITP group presented higher incidences of the HPA-1a, HPA-3b and HPA-5b alleles, which could suggest an association of these alleles with ITP in this population. In addition, Castro et al.<sup>12</sup> suggested that the presence of HPA-5b might be associated to increased risk for acute ITP in Brazilian patients. On the other hand, Thude et al.<sup>15</sup> showed that allele frequencies of the HPA-1, HPA-3 and HPA-5 were identical between patients with refractory autoimmune thrombocytopenia and blood donors in the German population. While, the HPA-2b was related to a higher risk for chronic ITP in Macedonian patients.<sup>14</sup> Therefore, we suppose that the diversity in the prevalence of autoepitopes among ITP individuals worldwide occurs in response to genetic inheritance of HPA polymorphisms.

There is evidence of clinical correlations between autoantibodies against extracellular GP epitopes and their pathogenic role in ITP.<sup>13</sup> ITP T cells recognize epitopes generated from GPIIb/IIIa and probably other platelet proteins.<sup>21</sup> Thus, the immune potentials of HPA-1, HPA-3 and HPA-5 alleles are very important, for example, GPIIb/IIIa, carrying HPA-1 and HPA-3 antigens, is the most abundant complex (50,000–80,000 copies per platelet). While, despite the low abundance of GPIa/IIa on the platelet membrane (800–2000 copies per platelet), HPA-5 has been considered an important immunogenic factor linked to immune syndromes in Caucasians<sup>22</sup> and has been associated with high predicted risk of inducing alloimmunization in the Amazonas population.<sup>19</sup> Ghevaert et al.<sup>22</sup> demonstrated that, most anti-platelet antibodies (95%) have specificity against HPA-1a or HPA-5b, while only 5% are specific to the other systems, such as HPA-2, HPA-3 and HPA-15. However, the pathogenesis of ITP is clearly heterogeneous due to the racial admixture among Brazilian individuals, a condition that can affect these findings. Antibody specificity was not evaluated in this study, making it impossible to speculate about this potential association.

Platelet membrane glycoproteins appear to be an important binding site for ITP serum antibodies. This study analyzed 36 patients with ITP from Amazonas, and described specific HPA antigens related to the occurrence of ITP. However, the

relationship between immunization and its potential clinical consequences is not straightforward. Hence, the findings of this study represent just an attempt to amplify the knowledge about HPA and ITP and suggest a potential association as a risk factor for the development of ITP.

Finally, studies have discussed the feasibility of applying molecular typing in the routine of hospital transfusion services.<sup>23,24</sup> Some authors have reported a correlation between the prevention of newly developed alloantibodies in previously immunized patients and reductions in transfusion rates. Thus, a simulation of platelet transfusion mismatch was performed considering the HPA genotype frequencies of Amazonas blood donors.<sup>19</sup> The results suggest a higher risk of alloimmunization in homozygous BB patients, due to the high frequency of AA and AB genotypes in the Amazonas blood donor registry. Therefore, when transfusions are necessary in previously immunized patients, a future strategy could involve the recruitment of homozygous BB donors by HPA molecular typing.

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## Authorship

JCC, PSK, RMP and SCC participated actively in all experiments and drafted the manuscript. CF and AMSF helped with data analysis and manuscript writing and provided suggestions during the course of the experiments. SSW designed the experiments, provided guidance during all parts of the work, including the preparation of the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

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All authors read and approved the final version of the manuscript.

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