



## Original article

# THPO gene variants in patients with acquired aplastic anemia



Pedro Henrique Padilha<sup>a</sup>, Gustavo Borges<sup>a</sup>, Barbara Amélia Santana<sup>a</sup>,  
Larissa Alessandra Medeiros<sup>b</sup>, Samir Kanaan Nabhan<sup>b</sup>, Ricardo Pasquini<sup>b</sup>,  
Flavia Sacilotto Donaires<sup>a</sup>, Rodrigo Tocantins Calado<sup>a,\*</sup>

<sup>a</sup> Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (FMRP USP), Ribeirão Preto, SP, Brazil

<sup>b</sup> Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil

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## ABSTRACT

**Background:** Human aplastic anemia is a hematologic disease characterized by low peripheral blood cell counts associated with reduced numbers of hematopoietic stem and progenitor cells and a hypocellular bone marrow. Thrombopoietin (THPO) regulates megakaryocytes, but it also stimulates hematopoietic stem and progenitor cells. Biallelic mutations in the THPO gene have been reported in a family with recessive inherited aplastic anemia.

**Methods:** This study screened 83 patients diagnosed with acquired aplastic anemia and 92 paired healthy controls for germline variants in the THPO gene using Sanger sequencing.

**Results:** Three common single nucleotide polymorphisms were identified in patients and controls at comparable allele frequencies. There was no correlation between the single nucleotide polymorphism carrier status and platelet counts at diagnosis.

**Conclusion:** The presence of THPO polymorphisms is comparable between patients with acquired aplastic anemia and healthy individuals.

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## Introduction

Aplastic anemia (AA) is a hematologic disorder caused by hematopoietic stem cell failure, which clinically results in a hypocellular bone marrow and reduced blood cell counts.<sup>1,2</sup> In humans, AA is rare, affecting approximately 1–2 patients per

million inhabitants<sup>3</sup> with the highest incidences in East Asian countries.<sup>1</sup> In acquired AA, hematopoiesis is suppressed by an immune-mediated mechanism in which cytotoxic T-cells target hematopoietic stem and progenitor cells in the marrow.<sup>1</sup> Other cases are inherited, and loss-of-function mutations in genes regulating DNA repair are etiologic.<sup>4</sup>

Thrombopoietin (THPO), a hormone produced in the liver, is a primary regulator of megakaryocyte expansion

\* Corresponding author at: Faculdade de Medicina de Ribeirão Preto da USP, Laboratório de Hematologia, bloco G, subsolo, HCRP, Av. Bandeirantes, 3900, Ribeirão Preto, SP CEP 14049-900, Brazil.

E-mail address: [rtcalado@usp.br](mailto:rtcalado@usp.br) (R.T. Calado).

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and differentiation and is vital for hematopoietic stem cell maintenance, working as a pan-hematopoietic cytokine.<sup>5</sup> Loss-of-function mutations in *THPO* and in *MPL*, which encodes the thrombopoietin receptor *MPL*, are associated with a spectrum of marrow failures, from amegakaryocytic thrombocytopenia to familial AA.<sup>6</sup> Biallelic mutations in the *THPO* gene were identified in a family with inherited AA.<sup>7</sup> Conversely, gain-of-function mutations in these genes are observed in myelofibrosis, further implicating the *THPO*-*MPL* pathway in the regulation of hematopoietic stem cells.<sup>8</sup>

This study investigated whether germline mutations in the *THPO* gene were associated with acquired AA.

## Methods

### Patients and controls

DNA was extracted from bone marrow or peripheral blood cells as previously described.<sup>9</sup> Samples were collected after informed consent, according to the protocol approved by the local Research Ethics Committee (CEP-HCRP, protocol number 518/2015). Patients diagnosed with AA at the hematology clinic of the Universidade Federal do Paraná (UFPR) for whom stored DNA was available were eligible for the study. No participant underwent hematopoietic stem cell transplantation prior to sample collection. AA was diagnosed based on standard criteria.<sup>10,11</sup> Patients with Fanconi anemia, dyskeratosis congenita, or Shwachman-Diamond syndrome were excluded. Age and gender-matched healthy blood donors were studied as healthy controls.

### Sanger sequencing

Primers were designed by the Primer 3 program (web version 4.0.0), using the sequence *Homo sapiens thrombopoietin (THPO) transcription variant 1*, mRNA of NCBI with reference number NM.000460.3. Exon 6 was divided into two amplification reactions. Primer sequences are detailed in Table S1. The final reagent concentration in each polymerase chain reaction (PCR) was 100 ng DNA, 0.2 mM of each dNTP, 1.5 mM of MgCl<sub>2</sub>, 1 U of Taq DNA Polymerase, buffer 1×, 150 nM of each primer (forward and reverse), in a final reaction volume of 25 µL. The PCR conditions used for the assays were 5 min at 95 °C for initial denaturing, and 35 cycles at 95 °C for 40 s, 55 to 62 °C (depending on the primer) for 45 s for annealing and 72 °C for 45 s for extension.

After the PCR, amplicons were purified and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), with initial denaturing at 96 °C for 2 min, and 35 cycles at 95 °C for 10 s, 51 °C for 10 s and 60 °C for 4 min. The sequences were processed by the ABI 3500 XL detection system (Applied Biosystems) and analyzed with the Sequencher software (Gene Codes Corporation).

**Table 1 – Demographic and clinical features of patients with acquired aplastic anemia (n = 83).**

Characteristic	Value
Age – years	
Mean	25
Range	2–74
Gender – n	
Male	49
Race/ethnicity – n	
White	57
Mixed race	13
Black	12
Asian	1
Peripheral blood counts – mean ± SD	
Hemoglobin (g/dL)	7.2 ± 0.4
Hematocrit (%)	20.8 ± 3.1
Neutrophils (/µL)	188 ± 158
Platelets (/µL)	2500 ± 707
Disease classification – n	
Moderate	14
Severe	20
Very severe	49
Treatment regimen <sup>a</sup> – n	
Cyclosporine	69
Anti-thymocyte globulin	20
Androgen	12
Erythropoietin	3
Immunoglobulin	3
No treatment	2
Death – n	
Yes	25
No	58

SD: standard deviation.

<sup>a</sup> Some patients underwent more than one treatment regimen.

### Statistical analysis

Pearson's chi-square test was used to compare the frequency of variants in patients with acquired AA (study group) and healthy individuals (control). A 5% level of statistical significance was considered for all the analyses. The IBM Statistical Package for Social Sciences (SPSS) version 19.0 was used.

## Results

### Clinical features

Fifty-nine percent of patients were male, and the mean age was 25 ± 17 years. Patients' demographics and blood counts at the time of diagnosis are provided in Table 1.

### Sequencing

Three synonymous single nucleotide polymorphisms (SNPs) in the *THPO* gene were identified in patients and controls (Table 2). The allele frequencies for the three SNPs were

**Table 2 – Single nucleotide polymorphisms in the patients with acquired aplastic anemia (study group) and healthy individuals (control group).**

Genotypes	Aplastic anemia patients (n = 83)	Healthy individuals (n = 92)	p-Value <sup>a</sup>
rs956732 T>C			
Homozygous	30	46	
Heterozygous	39	34	
Wild-type	14	12	
Allele frequency	0.6	0.7	0.2
rs6141 G>A			
Homozygous	42	52	
Heterozygous	34	34	
Wild-type	7	6	
Allele frequency	0.7	0.7	0.7
rs3804618 A>T			
Homozygous	0	0	
Heterozygous	8	7	
Wild-type	75	85	
Allele frequency	0.05	0.04	0.6

<sup>a</sup> Chi-square test.

comparable between patients and controls. No novel or rare variant was identified.

The three SNPs are located in untranslated regions (UTR) of the *THPO* exons and therefore not represented in the *THPO* translation. The SNP rs956732 is located in the 5' UTR of isoform 1 (NM.000460.3), and leads to a synonymous substitution in isoform 5 (NM.001290003.1). The SNPs rs6141 and rs3804618 are located in the 3' UTR of the *THPO* mRNA. According to the ClinVar database (of the NCBI), the rs956732, rs6141, and rs3804618 SNPs have a benign clinical significance and are observed in allele frequencies of 0.5, 0.4, and 0.07, respectively, in the population, which is similar to our findings (Table 2).

As the *THPO* gene regulates thrombopoiesis, we investigated whether the *THPO* SNPs correlated with platelet levels in patients; no association was found between SNP carrier status and platelet counts (data not shown).

## Discussion

This study screened 83 patients with acquired AA and 92 matched controls for variants in the *THPO* gene. Three common SNPs were identified, but their prevalences were comparable between patients and controls. SNPs did not correlate with platelet counts in patients.

The *THPO*-MPL pathway regulates hematopoietic stem cell maintenance, and loss-of-function mutations in the *THPO* gene have been previously described in patients with familial AA. More recently, *THPO* mutations were found in five AA patients who underwent hematopoietic stem cell transplantation but failed to show engraftment.<sup>12</sup> Two instances were treated with eltrombopag, an MPL agonist, resulting in effective engraftment. Together, these findings suggest that pathogenic mutations in the *THPO* gene may be etiologic in a minority of AA cases. Clinically, the addition of eltrombopag to standard immunosuppressive therapy as the first-line treatment of acquired AA resulted in a significantly better hematologic response in up to 90% of patients and patient survival in comparison to historic controls.<sup>13</sup> When eltrombopag

was added to horse antithymocyte globulin and cyclosporine, patients showed faster recovery of peripheral blood counts and normalization of the CD34<sup>+</sup> cell compartment in the bone marrow. In patients with refractory acquired AA, monotherapy with eltrombopag achieved hematologic response in 44% of cases.<sup>14</sup> In aggregate, these findings implicate the *THPO*-MPL pathway as a therapeutic target in acquired AA.

We asked the question whether genetic lesions would modulate *THPO* in acquired AA, since a previous study reported one mutation (*THPO* R17C) in familial AA.<sup>7</sup> However, this study did not identify pathogenic variants in patients, and the allele frequencies of three common SNPs were similar between patients and controls.

In conclusion, pathogenic variants in the *THPO* gene are not associated with acquired AA.

## Conflict of interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.htct.2018.01.009.

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