REGULAR ARTICLE

blood VTH
vessels, thrombosis & hemostasis

# Refractoriness to eltrombopag in adult primary immune thrombocytopenia: utility of next-generation sequencing techniques

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**Key Points**

- One hundred and ten genes of the most important cell-signaling pathways involved in the mechanism of action of eltrombopag were studied.
- Thirteen DNA variants associated with eltrombopag refractoriness in ITP were observed.

Thrombopoietin receptor agonists, for example eltrombopag, are standard second-line treatment for immune thrombocytopenia (ITP). Eltrombopag has demonstrated high response rates, both in clinical trials and in routine practice studies. However, some patients with ITP are refractory to this drug. Next-generation sequencing (NGS) may help us identify underlying molecular biology variants that may be involved in eltrombopag refractoriness. Our multicenter national NGS study investigated 110 genes of the most important cell-signaling pathways involved in the mechanism of action of eltrombopag in 35 refractory cases and 35 eltrombopag-responsive controls. Our refractory population comprised 51.4% men with a median age at diagnosis of 48 (range, 38-69) years and a median platelet count of $7 \times 10^9/\mu L$ (range, $4 \times 10^9/\mu L$ to $16 \times 10^9/\mu L$). At eltrombopag initiation, 78.3% had chronic ITP with a median platelet count of $8 \times 10^9/\mu L$ (range, $5\times 10^9/\mu L$ to $30 \times 10^9/\mu L$). Treatment with eltrombopag was maintained for a median of 3 (range, 1-9) months before discontinuation. No major grade 3-4 side effects were observed. Several statistical differences were observed in relation to the control responders. Of the total sum of the NGS variants found, 13 variants with statistical significance ($P \leq .05$) between case and controls were observed. Two of these have been shown to be associated with cancer. Seven variants are considered benign. Four variants are not previously described, and their significance is unknown. To our knowledge, none of the 13 variants described here has ever been correlated with ITP or eltrombopag refractoriness. Further studies are required to establish their role in this setting.

Submitted 20 May 2024; accepted 13 January 2025; prepublished online 17 February 2025; final version published online 6 June 2025. https://doi.org/10.1016/j.bvth.2025.100061.

Data are available on request from the corresponding author, Tomás José González-López (tjgonzalez@saludcastillayleon.es).

The full-text version of this article contains a data supplement.

## Introduction

Primary immune thrombocytopenia (ITP) is an acquired autoimmune hemorrhagic disease caused by antibodies directed against platelets. The disease is characterized by accelerated destruction of platelets in the reticuloendothelial system and inhibition of proliferation and maturation of bone marrow megakaryocytes, resulting in impaired platelet production.[1] Despite advances in pathology, the diagnosis of ITP remains a diagnosis of exclusion.

The incidence of ITP in adults has been estimated to be between 1.6 and 3.95 per 100 000 persons per year, depending on the diagnostic cutoff point chosen, which can range from $<150 \times 10^9$/L to $<50 \times 10^9$/L.[2,3] The signs and symptoms of ITP and its clinical course may present great individual variability.[4] The severity of bleeding does not clearly correlate with the established bleeding risk for the patient.[2,5]

The therapeutic goal in ITP is to control and prevent bleeding, and to maintain a safe platelet count.[6,7] Corticosteroids remain the first line of treatment. Thrombopoietin receptor agonists (TPO-RAs), that is, romiplostim, avatrombopag, and eltrombopag, are currently used to treat patients with ITP at risk of bleeding or who have failed at least 1 line of ITP therapy.[7] Eltrombopag is an oral nonpeptide TPO analog that activates the TPO receptor and thus stimulates normal human megakaryopoiesis.[8,9] Several clinical trials have shown that eltrombopag is effective and safe in adult patients with chronic ITP with a very high duration of response to long-term treatment (up to 3 years).[10-12]

Molecular studies in ITP were anecdotal until a few years ago because of the absence of precise diagnostic techniques for the study of the etiopathogenesis of ITP. One of the current objectives in ITP research is to try to discover genetic markers that help in the diagnosis of the disease and/or predictors of drug response. A pioneering study in this field, published in 2013, demonstrated the potential usefulness of genetic studies in ITP. Basciano et al reported the finding of a single-nucleotide polymorphism found in the β-tubulin gene that was shown to be related to refractoriness to various treatments in ITP.[13] Since then, new and cheaper molecular biology tools have been developed. Currently, next-generation sequencing (NGS) techniques allow us to implement this technology, as seen in other hematologic diseases, for the investigation of the molecular pathophysiology of ITP.[14]

The utility of NGS in hematology is a topical issue at the present time. However, the use of genetics in ITP is still emerging.[15-18] At the 2015 American Society of Hematology meeting, Despotovic et al presented data on several genes related to the development and degrees of severity of chronic ITP observed in the pediatric population.[19]

However, there are few studies examining the molecular characteristics of adult patients with ITP at diagnosis that can differentiate ITP from other related diseases such as myelodysplastic syndromes (MDS) or thrombopathies.[20] In addition, we know little about possible single-nucleotide polymorphisms and/or mutations involved in refractoriness to TPO-RAs.[21]

Some years ago, the Spanish REVOES registry was developed to obtain efficacy and safety data of eltrombopag in real-world clinical practice conditions.[22-28] The REVOGEN study combined NGS technology with our data from the REVOES study. Here, we analyzed >100 genes in 35 patients with ITP that were refractory to eltrombopag and compared these with 35 ITP controls that responded to eltrombopag. These genes involve eltrombopag downstream signaling, cytokine signaling and cytotoxic pathways, growth factors, adhesion molecules, apoptosis, and physiological mechanisms of platelet production. This study aimed to report DNA variants that may be involved in the refractoriness to treatment with eltrombopag in our Spanish patients with ITP.

## Methods

### Patients and database study design

In this study, 70 adult primary patients with ITP (aged ≥18 years) from 17 Spanish centers who had been treated with eltrombopag for ITP and previously included in our Spanish REVOES registry, were recruited. Thirty-five patients with ITP who were refractory to eltrombopag were selected, and, in contrast, another 35 patients with ITP who responded satisfactorily to eltrombopag were considered controls. Data from these patients who received eltrombopag were analyzed in a retrospective, observational, and noninterventional manner.

Each center collected clinical and biological data from all patients with primary ITP who met the inclusion and exclusion criteria, by conducting a review of the patient's medical history. Researchers reviewed the main characteristics of each patient in the study by means of a predetermined case report form. Patient and disease characteristics were recorded.

Eltrombopag was administered at doses approved by the European Medicines Agency. Primary ITP was defined as a platelet count of $<100 \times 10^9$/L in the absence of other causes or disorders that might be associated with thrombocytopenia.[29] The stages of primary ITP (newly diagnosed, persistent, and chronic ITP) were also defined according to International Consensus Definitions.[29]

Before their inclusion in the study, frequent causes of refractoriness to eltrombopag were ruled out, such as poor compliance with the dietary restrictions associated with its use (taking the drug at least 2 hours before, or 4 hours after, any of the following products: antacids, dairy products [or other foods containing calcium], or mineral supplements containing polyvalent cations [eg, iron, calcium, magnesium, aluminum, selenium, and zinc]).

***End points.*** Eltrombopag efficacy was evaluated based on platelet response, bleeding resolution, and the percentage of patients able to reduce or discontinue their doses of concurrent ITP treatment. Response (R) to eltrombopag was defined as a platelet count $>50 \times 10^9$/L. Treatment failure (refractoriness to eltrombopag) was defined as no response to eltrombopag after at least 6 weeks of treatment with eltrombopag (4 weeks of the maximum approved dose of 75 mg per day and 2 additional weeks with 50 mg per day of eltrombopag without response).[30] Adverse events from eltrombopag were evaluated and classified according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

***Ethical aspects.*** The study was performed in accordance with the guidelines of the institutional review boards of the participating

**Table 1. Panel of monoclonal antibodies for the characterization of PB platelets by NGF cytometry techniques**

| Panel of monoclonal antibodies for the characterization of PB platelets | | | | | |
|---|---|---|---|---|---|
| | PacB | FITC | PE | PercPCy5.5 | APC |
| Tube 1 | CD9 | CD42a | CD31 | CD61 | - |
| Tube 2 | CD9 | CD36 | CD41 | - | CD42b |
| Tube 3 | CD9 | - | CD34 | CD47 | CD84 |

APC, allophycocyanin; FITC, fluorescein Isothiocyanate; PacB, phycocyanin allophycocyanin B; PE, phycoerythrin; PercPCy5.5, peridinin-chlorophyll protein-cyanine 5.5.

centers and the standards of the Declaration of Helsinki. It was approved by the Hospital Universitario de Burgos Ethics Committee (protocol code: REVOGEN) as a postauthorization observational study by the Spanish Medicines and Health Products Agency.

***Statistical analysis.*** Descriptive statistical analysis was carried out using Excel (Microsoft Corp, Redmond, WA). Nonnormally distributed continuous variables were summarized as the median and interquartile range (IQR). Discrete variables were summarized as percentages. Quantitative and qualitative data were compared using the Mann-Whitney *U* and $\chi^2$ tests, respectively. Statistical significance was concluded for *P* values of <.05. All statistical analyses were performed using SPSS version 19.0 for Windows (SPSS, Chicago, IL).

## NGF cytometry methods

Simultaneous to the enrollment of the 70 patients in the study, biological samples were collected from these patients at the National DNA Bank in Salamanca, Spain, for DNA extraction and subsequent NGS studies at the molecular biology laboratories of the Doce de Octubre University Hospital in Madrid. The investigators also sent EDTA-anticoagulated peripheral blood (PB) samples (7-10 mL) for next-generation flow (NGF) cytometry studies. These studies allowed MDS to be excluded before performing NGS studies.

At the Flow Cytometry Service (NUCLEUS) of the University of Salamanca, the immunophenotypic characterization of PB platelets was carried out immediately after obtaining the samples using a panel of monoclonal antibodies (Table 1). The immunophenotypic profiles of PB platelets from patients with ITP were compared with the immunophenotypic profiles of PB platelets from previously known patients with MDS, and from healthy donors.

## Molecular biology studies: NGS techniques

Refractory and responder patients to eltrombopag and their biological samples were verified at the National DNA Bank in Salamanca. After exclusion of MDS by NGF at the Flow Cytometry Service (NUCLEUS) of the University of Salamanca, the DNA samples were sent to the 12 de Octubre University Hospital in Madrid for NGS studies.

***Preparation of libraries.*** For the generation of libraries for each sample, 75 ng of DNA from PB leukocytes extracted with automatic methods based on magnetic bead technology was used. A customized panel of amplicons was designed including 1979 amplicons distributed in 110 genes, with a total size of 376.1 kb covering 99.9% of the coding regions and splicing sites of all genes included. The complete list of analyzed genes and their mechanisms of action is described in Table 2.

**Table 2. Complete list of analyzed genes and their mechanisms of action**

| Mechanism of action | Implicated gene(s) |
|---|---|
| JAK/STAT signaling | TPO, JAK1/2/3, TYK2, PI3K, KRAS, NRAS, RAF1, STAT1/3/5, SOCS1, LNK, PRMT5, MEP50, SHC, GRB2, SOS1, MAPK1/3 |
| Treg, TGF-β signaling | IFNA17, SMAD2 |
| IL-17 induction | DGCR14 |
| Th17/Treg balance | CD83 |
| Class II cytokine receptor | IFNLR1 |
| T- and B-cell function, inflammation | REL |
| Fcγ receptors | FCGR2A, FCGR2B, FCGR2C, FCGR3A, FCGR3B |
| Treg, cytokine related immune responses | CXCL5, CCL5, EGFR, CD40L, CNR2 |
| T-cell immune regulation | CTLA4, CD28, ICOS, PD1, BTLA, TIGIT, CD80, CD86 |
| Actin regulators | VAV1, CFL1, RLTPR, RCSD1 |
| Apoptosis | TNFA, PRKACA, PRKACB, PRKACG, LTA, IFNG, DNMT3B, TBX21 |
| B-cell receptor | TNFRSF13B, SOX13, PTPN22 |
| Transcriptional factors | MPL, RUNX1, TAL1, MLL, GFI1, TEL, BMI1, GATA1, GATA2, LMO2, NFE2, FOG1, NFKB1, NFKB2, FOS, JUN, SMAD7, TNFAIP3, VEGFR, FLI1, SDF1 |
| Early acting hematopoietic growth factors | SCF, IL1A, IL2, IL3, IL4, IL6, IL10, IL11, IL17A, IL17B, IL17C, IL18, IL21, IL22, MTOR, PRKAA2 |
| Cytoskeleton and structural proteins | ACTN1, TUBB1, WAS, MYH9, FLNA, ANKRD26, ABCA1, FERMT3, MASTL, PRKACG, ABCG5 |
| Complement system | C1QBP, CPB2 |

GFI1, growth factor independence 1; IL-17, interleukin-17; JAK/STAT, Janus kinase signal transducer and activator of transcription; TGF-β, transforming growth factor β; Treg, regulatory T cells.

***Analysis procedures.*** The search for variants was performed by aligning the data of each sequence with the reference sequence "human genome build 38" using the software Torrent Suite and Ion Reporter (Thermo Fisher, Waltham, MA). For variant analysis, proprietary scripts aligning to the human genome version "human genome build 38" were used. The coverage of the regions included in the panel is 99.39% and the average depth of coverage is 1168×. A minimum of 100 reads at ≥85% of the detected bases is set as a minimum coverage criterion and a Phred score of ≥20 as quality criterion. All the data can be observed in the file "Stats_Cases_Controls.xlsx."

From the total sum of variants found, all artifacts were first eliminated. All variants with a bias of variant reads between the positive and negative strand of >0.7 and <0.3 were considered artifacts. Only those mutations that showed statistically significant differences with respect to the normal allele were considered in the clinical report. The clinical value of variants meeting these requirements was confirmed in the ClinVar, Cosmic, OMIM, Varsome, and Franklin databases. If necessary, confirmation of the results obtained by NGS was performed using Sanger sequencing.

## Results

### Patient characteristics

The main demographic and hematologic features of the 70 patients (35 refractory and 35 responders) who received eltrombopag, are presented in Table 3.

The median age of the eltrombopag-refractory population (cases) was 48 (IQR, 38-69) years. This cohort comprised 18 males and 17 females. The cohort that successfully responded to eltrombopag (controls) was an older population with a median age of 55 (IQR, 43-67) years and most were female (24, 68.6%). Interestingly, the percentage of the population aged ≥65 years was similar in both cohorts (31.4% vs 28.6%, respectively).

At the time of diagnosis, only 2 cases (5.7%) and 5 controls (14.3%) had a Charlson Comorbidity Index (CCI) score of ≥1; their platelet counts were 7 × $10^9$/L (IQR, 4-16 × $10^9$/L) and 16 × $10^9$/L (IQR, 6-30 × $10^9$/L), respectively; 19 cases and 26 controls were asymptomatic; and 24 refractory vs 21 responders experienced hemorrhages. Twenty-four of the nonresponders and 19 of our controls were patients with chronic ITP.

At eltrombopag initiation, statistically significant differences were observed when the platelet count or bleeding were compared in the refractory and responder cohorts with, 8 × $10^9$/L (IQR, 5-30 × $10^9$/L) and 54.3% of hemorrhages in the former cohort vs 25 × $10^9$/L (IQR, 10-43 × $10^9$/L) and 20.0% of bleeding in the latter group.

**Table 3. Patient characteristics**

| Variable | Refractory to eltrombopag (n = 35) | Responders to eltrombopag (n = 35) | Statistical significance (*P*) |
|---|---|---|---|
| No. of DNA variants present in the patient, median [$Q_1$-$Q_3$] | 4 [3-5] | 2 [1-2] | <.001 |
| Age at diagnosis, y, median [$Q_1$-$Q_3$] | 48 [38-69] | 55 [43-67] | .447 |
| Age at diagnosis ≥ 65 y, n (%) | 11 (31.4) | 10 (28.6) | .794 |
| Male/female, n | 18/17 | 11/24 | .089 |
| CCI > 1, n (%) | 2 (5.7) | 5 (14.3) | .232 |
| Months with ITP, median [$Q_1$-$Q_3$] | 14 [8-114] | 12 [5-55] | .039 |
| Platelet count at diagnosis (×$10^9$/L), median [$Q_1$-$Q_3$] | 7 [4-16] | 16 [6-30] | .202 |
| Asymptomatic at diagnosis, n (%) | 19 (59.4) | 26 (74.3) | .194 |
| Bleeding at diagnosis, n (%) | 24 (75.0) | 21 (60.0) | .192 |
| **Type of primary ITP,** n (%) | 35 (100) | 35 (100) | |
| Newly diagnosed | 5 (14.3) | 8 (22.9) | .356 |
| Persistent | 6 (17.1) | 8 (22.9) | .550 |
| Chronic | 24 (68.6) | 19 (54.3) | .220 |
| Eltrombopag treatment duration (mo), median [$Q_1$;$Q_3$] | 3 [1;9] | 44 [27;75] | < .001 |
| **Past ITP treatments**, median [$Q_1$-$Q_3$] | 2 [2-4] | 1 [1-3] | .005 |
| Romiplostim, n (%) | 14 (40.0) | 4 (11.4) | .006 |
| IV immunoglobulins, n (%) | 16 (45.7) | 8 (22.9) | .044 |
| Rituximab, n (%) | 12 (34.3) | 2 (5.7) | .003 |
| Splenectomy, n (%) | 14 (40.0) | 2 (5.7) | .001 |
| Vincristine, n (%) | 1 (2.9) | 2 (5.7) | .555 |
| Three or more previous treatment lines, n (%) | 17 (48.6) | 9 (25.7) | .048 |
| Platelet count at eltrombopag treatment initiation (×$10^9$/L), median [$Q_1$-$Q_3$] | 8 [5-30] | 25 [10-43] | .005 |
| Bleeding at eltrombopag treatment initiation, n (%) | 19 (54.3) | 7 (20.0) | .003 |
| Platelet count at last visit under eltrombopag treatment (×$10^9$/L), median [Q1-Q3] | 15 [8-26] | 125 [105-174] | <.001 |
| Platelet count at last visit follow-up under any ITP drug (×$10^9$/L), median [Q1-Q3] | 65 [22-115] | 126 [110-182] | .003 |
| Platelet count at last visit follow-up (×$10^9$/L), median [Q1-Q3] | 62 [29-167] | 126 [107-179] | .029 |
| Concomitant treatment, n (%) | 19 (54.3) | 7 (20.0) | .003 |
| Adverse events under eltrombopag, n (%) | 2 (5.7) | 4 (11.4) | .393 |
| Any treatment(s) after eltrombopag discontinuation, n (%) | 33 (94.3) | 4 (11.4) | <.001 |
| No. of treatments after eltrombopag discontinuation, median [$Q_1$-$Q_3$] | 1 [1-3] | 0 [0-0] | <.001 |

When large statistical differences were seen it was with respect to the duration of eltrombopag treatment; in patients in whom eltrombopag was not effective, this treatment was shorter. Thus, the cases refractory to eltrombopag showed a treatment duration of 3 months (IQR, 1-9) as a median vs 44 months (IQR, 27-75) in responder controls ($P < .001$). Similarly, the number of prior treatments before starting eltrombopag was much higher in refractory nonresponders especially if we consider the fact of having received multiple (≥3) previous treatment lines ($P = .048$) or different classical ITP treatments, for example previous use of romiplostim ($P = .006$), IV immunoglobulins ($P = .044$), rituximab ($P = .003$), or splenectomy ($P = .001$). A 24-month follow-up of these patients with ITP was performed, also showing differences between both the refractory and responder cohorts ($P = .029$).

Among patients refractory to eltrombopag, 12 had been previously treated with romiplostim. Only 3 (25%) had responded to romiplostim, with a median response time of 2 months (IQR, 2-3). In contrast, only 5 patients who responded to eltrombopag had previously been treated with romiplostim. Of 5 patients, 2 (40%) had responded to romiplostim, with a median treatment response of 5 months (IQR, 3-6).

Only 2 mild adverse events were observed in our refractory population, with 4 seen in the responder cohort. Thus, 2 refractory patients developed hepatotoxicity with transaminase levels 3- and 5-times the upper limit of normal with eltrombopag doses of 75 mg per day, respectively. When eltrombopag was discontinued, liver function normalized. Among the responders, 3 patients (with a prior history of migraine) developed episodes of mild headache that responded to their regular antimigraine therapy. A fourth patient developed dry mouth for the first 2 weeks of treatment and needed only to drink a little more than usual during that time.

### NGS findings

We performed the NGS analysis of PB DNA samples from 35 eltrombopag-refractory cases and 35 eltrombopag-responding controls. We started working with a file that listed the 53 variants found in at least a difference of 4 between cases and controls and, in addition, the allele frequency of the variant is at least 20%. Finally, 13 DNA variants were found with statistically significant differences between refractory and responder populations ($P < .001$). Of 110 genes analyzed, we only found statistically significant differences between patients refractory to eltrombopag and responders in 12 genes. Those genes were as follows: MAPK1, RAF1, GFI1, FCGR2A, ETV6, STAT5A, MAPK3, ABCA1, ACD, THPO, ACTN1, and SHC1 genes. Interestingly, our DNA variant 8 is an intronic variant in the *MAPK3* gene that we have observed in 7 refractory cases but in no responder controls. The number of DNA variants present in the refractory and control cohorts showed statistically significant differences between them. Thus, in our refractory cohort we observed 4 DNA variants in each patient as a median (IQR, 3-5), with only 2 (IQR, 1-2) in our responder controls.

Confirmation of the results obtained by NGS was performed using Sanger sequencing in only 3 patients who had 3 new variants with an allele frequency of >15% that were detected in any of the genes in the panel and were not previously described in the usual databases. These DNA variants were NM_014160.5 chr3:12585017, NM_005263.5:c.951T>G,(p.Lys317Asn), and NM_024307.3 chr16:30116567.

The final list of DNA variants observed is summarized in Table 4. What follows is the description of the 13 different DNA variants observed. The synonymous variant 1 in the *MAPK1* gene has an odds ratio (OR) of 20.091 ($P < .001$). This OR indicates that there is a 20.091-times greater risk of being a refractory to eltrombopag if this variant is present. The intronic polymorphism 2 in the RAF1 gene presents an OR of 2.909 ($P = .030$). The variant 3 involves the GFI1 gene with an OR of 5.565 ($P = .009$). The variants 4 and 5 are both found in the *FCGR2A* gene. Both variants are found in the same number of cases and controls. So, their OR is 4.580 ($P = .012$) for both. With regard to the variant 6, we observe an OR of 3.130 ($P = .048$). The variant 7 is a synonymous variant in the *STAT5A* gene that has an OR of 3.000 ($P = .039$). The variant 8 is an intronic variant in the *MAPK3* gene. It is found in 7 of 35 cases and it is absent in the whole control population. Here, the OR could not be calculated because there are no controls presenting the DNA variant 8 although statistically significant differences were observed ($P = .005$). Variant 9 is a synonymous variant in the *ABCA1* gene that has an OR of 3.222 ($P = .034$). Variant 10 is a synonymous variant of the *ACD* gene, with an OR of 4.267 ($P = .031$). The variant 11 is found in the gene that encodes for TPO; its OR is 4.889 ($P = .040$). Variant 12 is a missense variant in the *ACTN1* gene that encodes for actin, with an OR of 7.034 ($P = .046$). We found this in 5 heterozygous cases and 1 heterozygous control. Finally, variant 13 was found in the *SHC1* gene; its OR is 7.034 ($P = .046$). A full description of the 13 statistically significant DNA variants observed and their correlation with the most important epidemiological variables are shown in supplemental Tables 1-12 (supplemental Material).

## Discussion

Eltrombopag is a TPO-RA drug that has demonstrated very high response rates (>80%) in clinical trials and/or real life clinical practice studies.[10-12,24-28] Although refractoriness to eltrombopag is not common, this topic is of great interest because patients who fail to respond to this drug often did not, or will not, respond to other drugs, and these patients will very often present an unfavorable clinical course.[31-33] The fact that ITP continues to be a disease with a diagnosis of exclusion and in which no reliable predictors of response have been found so far, makes a study such as ours of great interest when approaching the treatment of refractory ITP.

Our series of refractory patients, although younger and with fewer comorbidities than the cohort of responders (48 years with only 2 patients with a CCI score of ≥1 vs 55 years and 5 patients with CCI score of ≥1), had lower platelet counts, higher number of hemorrhages, elevated symptoms associated with ITP, and many of them received concomitant ITP treatments at the time of initiation of treatment with eltrombopag. This worse clinical situation of our refractory patients may be attributed to the fact that many of them had demonstrated previous refractoriness to several other treatments for ITP.

Furthermore, most of the refractory patients started eltrombopag in case of chronic ITP in which the underlying immunological disorder associated with the disease is higher, or after having received multiple ITP treatment lines. On the contrary, many patients in response to eltrombopag started this drug just after corticosteroid failure and/or intolerance. We believe that our objective of

**Table 4. Summary of the DNA variants and their percentages observed in refractory cases and responsive controls**

| Variant no. | Gene | Protein | GMAF | Locus no. (hg38) | Coding | Transcript | Incidence in the general population, % | Refractory cases, % | Responsive controls, % | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *MAPK1* | P.L116L | 0.001 | chr22:21805996 | c.346T>C | NM_002745.5 | 0.001 | 37 | 3 | <.001 |
| 2 | *RAF1* | - | 0.365 | chr3:12585017 | - | NM_014160.5 | 0 | 69 | 43 | .030 |
| 3 | *GFI1* | p.C317W | 0.000 | chr1:92478727 | c.951T>G | NM_005263.5 | 0 | 34 | 9 | .009 |
| 4 | *FCGR2A* | p.P215P | 0.055 | chr1:161510859 | c.645A>G | NM_001136219.3 | 10 | 37 | 11 | .012 |
| 5 | *FCGR2A* | p.Q63* | 0.056 | chr1:161506414 | c.187C>T | NM_001136219.3 | 12-15 | 37 | 11 | .012 |
| 6 | *ETV6* | p.T86T | 0.213 | chr12:11839234 | c.258G>A | NM_001987.5 | 15-22 | 34 | 14 | .048 |
| 7 | *STAT5A* | p.D634D | 0.241 | chr17:42307719 | c.1902C>T | NM_001288718.2 | 19-24 | 43 | 20 | .039 |
| 8 | *MAPK3* | - | 0.008 | chr16:30116567 | - | NM_024307.3 | 3.5 | 20 | 0 | .005 |
| 9 | *ABCA1* | p.I680I | 0.339 | chr9:104828991 | c.2040C>A | NM_005502.4 | 20-34 | 40 | 17 | .034 |
| 10 | *ACD* | p.P457P | 0.019 | chr16:67657612 | c.1371G>T | NM_001082486.2 | 3.5 | 29 | 9 | .031 |
| 11 | *THPO* | - | 0.062 | chr3:184372454 | c.*59G>A | NM_000460.4 | 0 | 23 | 6 | .040 |
| 12 | ACTN1 | p.T890S | 0.006 | chr14:68874936 | c.2668A>T | NM_001130004.2 | 1 | 17 | 3 | .046 |
| 13 | SHC1 | p.M410V | 0.061 | chr1:154966186 | c.1228A>G | NM_001130040.2 | 4 | 17 | 3 | .046 |

GMAF, global minor allele frequency; hg38, human genome build 38.

selecting a population as refractory as possible to eltrombopag, with/without other drugs, has been achieved.

It is also interesting that, at the time of ITP diagnosis, our refractory population had lower platelet counts, greater asthenia, and more frequent and severe bleeding than patients who respond to eltrombopag months or even years later, which suggests a possible predisposition (genetic or not) to develop refractory ITP. In this sense, recent publications advocating a certain genetic predisposition to develop ITP are of great interest.[34-36] Moreover, Zhang et al[37] published their transcriptome study with 19 patients evaluating the impact of eltrombopag on gene expression. Surprisingly, although platelet counts increased steadily, only 5 genes remained overexpressed at the 1-month time point in responders.

To our knowledge, this is the first and probably the only study to attempt to correlate the presence of several genetic DNA variants and the occurrence of ITP, refractory to second-line treatment of this disease. Although some studies have reported a genetic basis for refractoriness to corticosteroid treatment for ITP, this is, to our knowledge, the only study in which NGS tools and a large panel of genes have been used to try to elucidate the genetic basis for the refractoriness to ITP treatment.[38,39]

Our genomic study used NGS techniques to evaluate variants that could potentially explain the refractoriness to eltrombopag treatment in ITP. After discarding artifacts, we were able to obtain 13 DNA variants (with 12 genes involved) with statistically significant differences between refractory and responder populations ($P \leq .05$).

Here, we proceed to describe their frequency and eventual pathogenic role. The synonymous variant 1 in the *MAPK1* gene is classified as benign in predictors of pathogenicity. The gene belongs to the mitogen-activated protein (MAP) kinase pathway. The intronic variant 2 in the *RAF1* gene is associated with gastrointestinal cancer.[40] Variant 3 in the GFI1 gene is not described in the main cancer or population databases. This variant has been classified as likely pathogenic. Its GFI-1 protein causes a blockage of hematopoiesis, and its inactivation could cause an uncontrolled hematopoiesis. Variants 4 and 5 are both found in the *FCGR2A* gene. The first (p.P215P) is an undescribed synonymous variant, classified as benign, and the p.Q63* variant is a benign mutation that produces a truncation of the protein at amino acid 63. In most cases, this mutation presents in heterozygosity with which it is haplosufficient, thus the protein is functional with a single, healthy allele.[41] Variant 6 is a synonymous variant in the *ETV6* gene and the ETV6 protein is a transcription factor that is a member of the E-twenty-six family. This protein interacts with FLI1, regulating the differentiation of megakaryocytes toward platelets. There are no publications that point to this variant. Variant 7 is a synonymous variant in the *STAT5A* gene that is classified as benign but has already been described in lung cancer in exome sequencing studies in a 100-patient Chinese population.[42] The STAT5A protein is a transcription factor that acts as a dimer and has an antiapoptotic function. Variant 8 is an intronic polymorphism in the *MAPK3* gene not previously described. It is found in 7 of 35 cases in our cohort and it is absent in the control population. Variant 9 is a synonymous variant in the *ABCA1* gene that has been described in 2 publications of massive whole-genome sequencing studies, and it is classified as benign.[43,44] This gene encodes for an ABC transporter protein (adenosine triphosphate dependent) and it is involved in cholesterol homeostasis, allowing the synthesis of high-density lipoprotein fraction of cholesterol and membrane phospholipids. Variant 10 has not been previously described synonymous variant of the *ACD* gene. This gene encodes for a protein involved in telomeric function, being 1 of the central proteins of the telosomal complex, maintaining telomere length, and it is classified as benign. Variant 11 is found in the gene that encodes for TPO. It is found in the noncoding region after the stop codon. It is classified as benign and has been described twice previously.[45,46] Variant 12 is a previously undescribed missense variant in the *ACTN1* gene that encodes the protein α-actinin-1, a protein responsible for the structure of the cell cytoskeleton. Finally, variant 13 can be found in the *SHC1* gene that encodes for the SHC-transforming protein important in the regulation of apoptosis and drug resistance in mammalian cells. It has previously been described as benign.[47-49]

The role of genetics in refractory ITP remains poorly understood. Both germ line (inherited) and somatic (acquired) mutations in pathways regulating the immune response can be associated with this condition. STAT1/3 and FoxP3 mutations may cause an associated ITP regulatory T-cell impairment.[33] Interestingly, in our NGS studies, we have observed 1 STAT5A gene mutation not previously described in ITP. This is, to our knowledge, the first time that a mutation in the Janus kinase signal transducer and activator of transcription pathway has been postulated as a cause of TPO-RA refractoriness. Fc receptor polymorphisms are also known to play a role in ITP. Here, we observed 2 variants in the *FCGR2A* gene that were not previously described in ITP.

To our knowledge, none of the 13 statistically significant variants reported here have ever been correlated with eltrombopag refractoriness although some of them have been previously associated with different cancers. Thus, RAF1 and STAT5A variants are described in gastric and lung cancer, respectively. In contrast, MAPK1, ETV6, ABCA1, THPO, SHC1 and both FCGFR2A variants are considered benign. Here, we describe 4 previously unpublished DNA variants (GFI1, MAPK3, *ACD, and ACTN1 variants) in which their significance is unknown and 9 others not previously described in ITP, whose role in megakaryopoiesis and ITP drug refractoriness needs to be clarified.

Limitations of our epidemiological data are the retrospective multicenter analytical approach to the patients and the possibility of significant selection bias. Most of the limitations of our NGS study are based on the fact that this test may not detect all variants in the noncoding regions that could affect gene expression or the copy number change of the gene or part of it.

The identification of variants that differ between cases and controls may allow us to identify potential predictors of nonresponse to eltrombopag, especially the MAPK3 variant that we only observed in refractory patients. Although these findings are quite promising, more studies are needed to establish a more robust correlation between these variants and eltrombopag refractoriness.

## Acknowledgment

This research was funded by a Novartis Research grant (CETB115BES06T).

Novartis did not have any role in the design, execution, analysis, or interpretation of the study.

## Authorship

Contribution: T.J.G.-L. conceptualized and designed the study; R.S. and J.M.L. performed molecular biology studies; S. Matarraz performed flow cytometry studies; T.J.G.-L. performed statistical analyses; T.J.G.-L. wrote the manuscript (text and tables); and all authors analyzed and interpreted the results, collected data, and provided final approval of the manuscript.

Conflict-of-interest disclosure: T.J.G.-L. has received research grants from Amgen and Novartis and speaker honoraria from Amgen, Novartis, Sobi, Grifols, Alpine, and Argenx. R.S. has received a research grant from Novartis. I.J. has received speaker honoraria from Amgen, Novartis, Sobi, Grifols, and Shionogi. D.P. acknowledges the following competing interests: research support from Amgen and Novartis; honoraria from Amgen, Novartis, Sobi, UCB, and Argenx; and consultancies with UCB Pharma, MedImmune, Ono, Sobi, Argenx, and Takeda. J.M.L. has received a research grant from Novartis. The remaining authors declare no competing financial interests.

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## References

1. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. *Blood*. 2009;113(26):6511-6521.
2. Neylon AJ, Saunders PW, Howard MR, Proctor SJ, Taylor PR; Northern Region Haematology Group. Clinically significant newly presenting autoimmune thrombocytopenic purpura in adults: a prospective study of a population-based cohort of 245 patients. *Br J Haematol*. 2003;122(6):966-974.
3. Schoonen WM, Kucera G, Coalson J, et al. Epidemiology of immune thrombocytopenic purpura in the general practice research database. *Br J Haematol*. 2009;145(2):235-244.
4. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. *N Engl J Med*. 2002;346(13):995-1008.
5. Stasi R, Evangelista ML, Stipa E, Buccisano F, Venditti A, Amadori S. Idiopathic thrombocytopenic purpura: current concepts in pathophysiology and management. *Thromb Haemost*. 2008;99(1):4-13.
6. Provan D, Arnold DM, Bussel JB, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. 2019;3(22):3780-3817.
7. Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. *Blood Adv*. 2019;3(23):3829-3866.
8. Stasi R, Evangelista ML, Amadori S. Novel thrombopoietic agents: a review of their use in idiopathic thrombocytopenic purpura. *Drugs*. 2008;68(7):901-912.
9. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. *Blood*. 1998;92(1):4-10.

10. Bussel JB, Cheng G, Saleh MN, et al. Eltrombopag for the treatment of chronic idiopathic thrombocytopenic purpura. *N Engl J Med*. 2007;357(22):2237-2247.
11. Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomised, phase 3 study. *Lancet*. 2011;377(9763):393-402.
12. Saleh MN, Bussel JB, Cheng G, et al; EXTEND Study Group. Safety and efficacy of eltrombopag for treatment of chronic immune thrombocytopenia: results of the long-term, open-label EXTEND study. *Blood*. 2013;121(3):537-545.
13. Basciano PA, Bussel J, Hafeez Z, Christos PJ, Giannakakou P. The beta 1 tubulin R307H single nucleotide polymorphism is associated with treatment failures in immune thrombocytopenia (ITP). *Br J Haematol*. 2013;160(2):237-243.
14. Bastida JM, Del Rey M, Lozano ML, et al. Design and application of a 23-gene panel by next-generation sequencing for inherited coagulation bleeding disorders. *Haemophilia*. 2016;22(4):590-597.
15. Wang N, Li GN, Wang XB, Liang T, Hu L. TNF-α promoter single nucleotide polymorphisms and haplotypes associate with susceptibility of immune thrombocytopenia in Chinese adults. *Hum Immunol*. 2014;75(9):980-985.
16. Wu P, Wang Z, Lu S, Zhao X. CD86 +1057G/A polymorphism and risk of chronic immune thrombocytopenia. *Autoimmunity*. 2014;47(7):482-485.
17. Wang D, Hu SL, Cheng XL, Yang JY. FCGR2A rs1801274 polymorphism is associated with risk of childhood-onset idiopathic (immune) thrombocytopenic purpura: evidence from a meta-analysis. *Thromb Res*. 2014;134(6):1323-1327.
18. Xie J, Cui D, Liu Y, et al. Changes in follicular helper T cells in idiopathic thrombocytopenic purpura patients. *Int J Biol Sci*. 2015;11(2):220-229.
19. Despotovic JM, Polfus LM, Flanagan JM, et al. Genes influencing the development and severity of chronic ITP identified through whole exome sequencing. *Blood*. 2015;126(23):73.
20. Schubert D, Bode C, Kenefeck R, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat Med*. 2014;20(12):1410-1416.
21. El-Beblawy NM, Elbarbary NS, Kamal TM, Mahmoud PM. A study of human killer cell immunoglobulin-like receptor and multidrug resistance gene polymorphisms in children with immune thrombocytopenia. *Clin Appl Thromb Hemost*. 2016;22(5):429-440.
22. González-López TJ, Sánchez-González B, Pascual C, et al. Sustained response after discontinuation of short-and medium-term treatment with eltrombopag in patients with immune thrombocytopenia. *Platelets*. 2015;26(1):83-86.
23. González-López TJ, Pascual C, Álvarez-Román MT, et al. Successful discontinuation of eltrombopag after complete remission in patients with primary immune thrombocytopenia. *Am J Hematol*. 2015;90(3):E40-E43.
24. González-Porras JR, Mingot-Castellano ME, Andrade MM, et al. Use of eltrombopag after romiplostim in primary immune thrombocytopenia. *Br J Haematol*. 2015;169(1):111-116.
25. González-López TJ, Alvarez-Román MT, Pascual C, et al. Eltrombopag safety and efficacy for primary chronic immune thrombocytopenia in clinical practice. *Eur J Haematol*. 2016;97(3):297-302.
26. González-López TJ, Fernández-Fuertes F, Hernández-Rivas JA, et al. Efficacy and safety of eltrombopag in persistent and newly diagnosed ITP in clinical practice. *Int J Hematol*. 2017;106(4):508-516.
27. González-López TJ, Alvarez-Román MT, Pascual C, et al. Use of eltrombopag for secondary immune thrombocytopenia in clinical practice. *Br J Haematol*. 2017;178(6):959-970.
28. González-López TJ, Sánchez-González B, Jarque I, et al. Use of eltrombopag for patients 65 years old or older with immune thrombocytopenia. *Eur J Haematol*. 2020;104(3):259-270.
29. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386-2393.
30. Revolade. Summary of product characteristics. Novartis; 2023. Accessed 19 February 2024. https://www.ema.europa.eu/en/medicines/human/EPAR/revolade
31. Moulis G, Rueter M, Duvivier A, et al. Difficult-to-treat primary immune thrombocytopenia in adults: prevalence and burden. Results from the CARMEN-France registry. *Br J Haematol*. 2024;204(4):1476-1482.
32. Al-Samkari H, Schifferli A, Gonzalez-Lopez TJ. The problem of immune thrombocytopenia refractory to both eltrombopag and romiplostim. *Br J Haematol*. 2024;204(4):1143-1145.
33. Miltiadous O, Hou M, Bussel JB. Identifying and treating refractory ITP: difficulty in diagnosis and role of combination treatment. *Blood*. 2020;135(7):472-490.
34. Jiang D, Rosenlind K, Baxter S, et al. Evaluating the prevalence of inborn errors of immunity in adults with chronic immune thrombocytopenia or Evans syndrome. *Blood Adv*. 2023;7(23):7202-7208.
35. Georgi JA, Middeke JM, Bornhäuser M, Matzdorff A, Trautmann-Grill K. Deciphering the genetic basis of immune thrombocytopenia: current evidence for genetic predisposition in adult ITP. *Blood Adv*. 2023;7(14):3710-3724.
36. Zehnder JL, Bussel JB, Cooper N. The role of genetics in refractory immune thrombocytopenia. *Br J Haematol*. 2023;203(1):62-64.
37. Zhang H, Zhang BM, Guo X, et al. Blood transcriptome and clonal T-cell correlates of response and non-response to eltrombopag therapy in a cohort of patients with chronic immune thrombocytopenia. *Haematologica*. 2020;105(3):e129-e132.

38. Zhao YH, Zhou J, Li XX. A study on the relationship between alpha- and beta-isoform of glucocorticoid receptors and glucocorticoid-resistant idiopathic thrombocytopenia purpura. *Zhonghua Nei Ke Za Zhi*. 2005;44(5):363-365.

39. Cao Q, Zhu H, Xu W, et al. Predicting the efficacy of glucocorticoids in pediatric primary immune thrombocytopenia using plasma proteomics. *Front Immunol*. 2023;14:1301227.

40. Gonzalez-Hormazabal P, Musleh M, Bustamante M, et al. Polymorphisms in RAS/RAF/MEK/ERK pathway are associated with gastric cancer. *Genes (Basel)*. 2018;10(1):20.

41. van der Heijden J, Nagelkerke S, Zhao X, et al. Haplotypes of FcγRIIa and FcγRIIIb polymorphic variants influence IgG-mediated responses in neutrophils. *J Immunol*. 2014;192(6):2715-2721.

42. Li C, Gao Z, Li F, et al. Whole exome sequencing identifies frequent somatic mutations in cell-cell adhesion genes in Chinese patients with lung squamous cell carcinoma. *Sci Rep*. 2015;5:14237.

43. Alnouri F, Al-Allaf FA, Athar M, et al. Xanthomas can be misdiagnosed and mistreated in homozygous familial hypercholesterolemia patients: a call for increased awareness among dermatologists and health care practitioners. *Glob Heart*. 2020;15(1):19.

44. Lindberg J, Klevebring D, Liu W, et al. Exome sequencing of prostate cancer supports the hypothesis of independent tumour origins. *Eur Urol*. 2013;63(2):347-353.

45. Vuckovic D, Bao EL, Akbari P, et al. The polygenic and monogenic basis of blood traits and diseases. *Cell*. 2020;182(5):1214-1231.e11.

46. Astle WJ, Elding H, Jiang T, et al. The allelic landscape of human blood cell trait variation and links to common complex disease. *Cell*. 2016;167(5):1415-1429.e19.

47. Oskarsson GR, Oddsson A, Magnusson MK, et al. Predicted loss and gain of function mutations in ACO1 are associated with erythropoiesis. *Commun Biol*. 2020;3(1):189.

48. Sweeney C, Murtaugh MA, Baumgartner KB, et al. Insulin-like growth factor pathway polymorphisms associated with body size in Hispanic and non-Hispanic White women. *Cancer Epidemiol Biomarkers Prev*. 2005;14(7):1802-1809.

49. Diorio C, Brisson J, Bérubé S, Pollak M. Genetic polymorphisms involved in insulin-like growth factor (IGF) pathway in relation to mammographic breast density and IGF levels. *Cancer Epidemiol Biomarkers Prev*. 2008;17(4):880-888.