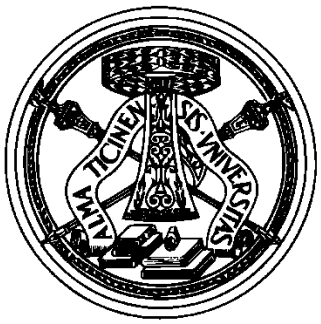

**UNIVERSITA' DEGLI STUDI DI PAVIA**

FACOLTA' DI MEDICINA E CHIRURGIA

DOTTORATO IN MEDICINA SPERIMENTALE, XXXIII CICLO

# THE THROMBOPOIETIN-RECEPTOR AGONIST ELTROMBOPAG FOR THE TREATMENT OF INHERITED THROMBOCYTOPENIAS

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**SUMMARY**

**CONCISE SUMMARY OF THE PhD PROJECT, AND PhD THESIS**

The research stay of my PhD Course of Experimental Medicine has been mainly carried out in the Laboratory of Platelet Physiopathology and in the Internal Medicine Unit of the Department of Internal Medicine of the University of Pavia, under the mentorship of Professor Patrizia Noris. I also performed a one-year research stay at the Institute of Transfusion Medicine of the Department of Immunology and Transfusion Medicine of the University Medicine Greifswald (Germany), under the mentorship of Professor Andreas Greinacher.

My PhD Course project was entitled "Identification of the genetic bases, the clinical characteristics, and new therapeutic options for inherited thrombocytopenias", and addressed the field of inherited thrombocytopenias in a translational way: from the identification of the molecular causes of these disorders, to the implementation of the diagnostic modalities and the clinical characterization of the affected patients, to the investigation of novel therapeutic options.

My PhD thesis is entitled "The thrombopoietin-receptor agonist eltrombopag for the treatment of inherited thrombocytopenias" and focuses on a novel therapeutic possibility for patients affected with inherited thrombocytopenias. The thesis is divided into two parts; each of them has been written according to the typical structure of the scientific article.

In the *Part 1*, the results of a phase 2 clinical trial that investigated the efficacy and the safety of both short- and prolonged administration of the thrombopoietin-receptor agonist eltrombopag in 24 patients affected with 5 forms of inherited thrombocytopenia, are reported and discussed. The outcome of the use of eltrombopag, investigated in a real-world clinical setting in a subgroup of patients with *MYH9*-related disease - who received this medication in preparation for elective surgery at our Institute - is also reported.

In the *Part 2*, the results of a collaboration study with the research group headed by Professor Alessandra Balduini (Department of Molecular Medicine, University of Pavia), which compared the *in vivo* response to eltrombopag obtained in some patients with *MYH9*-related disease and *ANKRD26*-related thrombocytopenia –

some of them enrolled to the clinical trial described in the *Part 1* - with the response to the same drug evaluated *ex vivo* by exploiting a bone marrow tissue model, are reported and discussed.

**ABSTRACT**

Inherited thrombocytopenias are rare disorders characterized by reduced platelet count and variable bleeding diathesis. Patients can require platelet transfusions to raise platelet counts before surgery or invasive procedures. In addition, some subjects suffer from clinically significant spontaneous bleedings impacting their quality of life, and may benefit from a durable improvement of their chronic thrombocytopenia.

The hypothesis that thrombopoietin-mimetic drugs can increase platelet count in patients with in inherited thrombocytopenias is appealing, but evidence is scarce. A first prospective study was conducted in 2010, which enrolled subjects affected with only one form: *MYH9*-related disease. In this trial, a short course of the oral thrombopoietin-receptor agonist eltrombopag was given to 12 patients, and 11 of them showed a significant increase of platelet count. Based on this evidence, a short-term cycle of eltrombopag has been offered to the patients followed at our Institute affected with this disorder, who needed elective surgery and had a platelet count too low for a safe procedure; so far, 11 consecutive procedures (including major surgeries) were carried out in 5 patients after eltrombopag administration, which successfully replaced platelet transfusions in 10 out of 11 cases in the absence of major adverse events.

We have now conducted a second prospective, phase 2 clinical trial to investigate the efficacy and the safety of eltrombopag in more forms of inherited thrombocytopenia. We enrolled 24 patients affected by *MYH9*-related disease, *ANKRD26*-related thrombocytopenia, X-linked thrombocytopenia / Wiskott-Aldrich syndrome, monoallelic Bernard-Soulier syndrome, or *ITGB3*-related thrombocytopenia. The average pre-treatment platelet count was $40.4\times10^9$/L. Patients received a 3- to 6 week course of eltrombopag in a dose-escalated manner. Of 23 patients evaluable for response, 11 (47.8%) achieved a major response (platelet count >100 $\times10^9$/L), 10 (43.5%) a minor response (platelet count at least twice the baseline value), and two patients (8.7%) did not respond. The average increase of platelet count compared to baseline was 64.5 $\times10^9$/L. The extent of platelet response was variable, either among different forms of inherited thrombocytopenia and also among different patients suffering from the same disease. Four patients with clinically significant spontaneous bleeding received

a long-term eltrombopag administration (16 additional weeks): all of them obtained remission of mucosal hemorrhages, which persisted throughout the treatment period. The treatment was globally well tolerated.

By exploiting a silk-based miniaturized 3D bone marrow tissue model, which recapitulates *ex vivo* platelet biogenesis of subjects with different forms of inherited thrombocytopenia, we also investigated the possibility to predict the *in vivo* platelet response to eltrombopag of individual patients affected with two disorders (i.e. *MYH9*-related disease and *ANKRD26*-related) who previously received eltrombopag. The number of platelets recovered in the *ex vivo* model under standardized conditions, including exposure to eltrombopag, significantly correlated with the increase in platelet count that we had observed *in vivo* in the same subjects after eltrombopag treatment.

In conclusion, eltrombopag was safe and effective in increasing platelet count and reducing bleeding symptoms in different forms of inherited thrombocytopenias, which represent, overall, more than 50% of the entire group of these disorders. In particular for *MYH9*-related disease, short-term eltrombopag should be considered as the first-line treatment to prepare subjects with severe thrombocytopenia for elective surgery. Moreover, a long-term assumption of eltrombopag at low dosage could represent a valid strategy to cure a subgroup of patients with inherited thrombocytopenias suffering with chronic, spontaneous bleedings due to severely reduced platelet counts. Despite these encouraging results, caution is recommended when using eltrombopag, particularly in patients with inherited thrombocytopenias predisposing to hematological malignancies.

In the near future, tools that mimic the bone marrow *ex vivo* can be used to predict the individual response to eltrombopag and others drugs stimulating platelet production in the field of inherited thrombocytopenias, thus informing therapeutic choices in a tailored perspective.

**PART 1**

## ELTROMBOPAG FOR THE TREATMENT OF INHERITED THROMBOCYTOPENIAS: A PHASE 2 CLINICAL TRIAL

## INTRODUCTION

Inherited thrombocytopenias are a heterogeneous group of disorders characterized by a reduced number of blood platelets that can result in a bleeding tendency of variable severity. Although inherited thrombocytopenias are rare, recent improvements in the knowledge of these conditions have indicated that, taken together, their prevalence is higher than previously thought. In fact, based on a registry of patients with thrombocytopenia, the prevalence of these disorders in the Italian population is estimated to be 2.7 per 100,000.[1]

Most patients with inherited thrombocytopenias present with mild or no spontaneous bleeding at all: however, even patients who do not have spontaneous hemorrhages often require platelet transfusions prior to surgery or other invasive procedures because their platelet count is below the safe threshold for the specific procedure.[1-5]

However, platelet transfusions have several drawbacks, since they expose patients to the risk of acute reactions, transmission of infectious diseases, allo-immunization with possible consequent refractoriness to subsequent platelet transfusions.[3,6,7] The latter is a particularly critical event in the patients with lifelong thrombocytopenia. Moreover, the availability of platelet units is affected by the scarceness of blood donors.

Conversely, some patients with inherited thrombocytopenias present frequent episodes of spontaneous bleeding that affect their quality of life, expose them to the risk of major hemorrhages, and may require frequent hospitalization and/or transfusions. In these subjects, obtaining an enduring increase of platelet count that is sufficient to stably abolish or reduce spontaneous hemorrhages would be a major achievement.

Thrombopoietin-receptor agonists are targeted agents that can stimulate megakaryopoiesis and platelet production through the activation of the thrombopoietin-receptor, MPL. These drugs are currently approved for the treatment of a few forms of acquired thrombocytopenia.[8] Although the hypothesis that thrombopoietin-

receptor agonists can increase platelet count also in patients with inherited thrombocytopenias is appealing, the evidence on this topic is very scarce, mainly due to the difficulties in carrying out clinical trials in these orphan diseases.

In 2010, a first phase 2 clinical trial had been conducted, which enrolled patients affected with only one form of inherited thrombocytopenia, *MYH9*-related disease.[9] This autosomal-dominant disorder, due to mutations in the gene encoding the non-muscle myosin heavy chain IIA, *MYH9*, represents the most frequent form of inherited thrombocytopenia.[1,7] In this first prospective study, a short course of the oral thrombopoietin-receptor agonist eltrombopag was given to 12 patients with basal platelet counts below 50 $x10^9$/L, and 11 of them showed a significant increase of platelet count without major side effects.[9] Based on these results, short-term eltrombopag was used anecdotally to prepare *MYH9*-related disease patients for major surgery.[10-12] The remaining available clinical information on the effects of thrombopoietin-receptor agonists in inherited thrombocytopenias derives from single case reports,[13-17] and the retrospective investigation of one small case series.[18]

At our own Institution, we proposed since 2010 short-term eltrombopag to all consecutive patients with *MYH9*-related disease who needed elective surgery and had a platelet count too low for a safe procedure according to the current guidelines,[5] and all patients accepted this treatment. In summary, eltrombopag successfully replaced platelet transfusions in preparation for 10 of 11 interventions performed in 5 patients, thus providing a simple and safe option for increasing platelet count, especially in case of refractoriness to platelet concentrates.[19] Patients' platelet counts at baseline ranged from 5 to 25 $x10^9$/L (**Table 1**). All subjects presented with spontaneous muco-cutaneous hemorrhages, and their ISTH Bleeding Assessment Tool[20,21] scores ranged from 3 to 22. Three patients had previously undergone major or minor surgeries: in all cases, the procedures were prepared with prophylactic platelet transfusions. One patient had always been hospitalized to perform dental extractions or periodontal surgery: some of these procedures were complicated by significant bleeding despite prophylactic platelets (Table 1, patient 3). Another patient had developed refractoriness to platelet transfusions. In fact, she had previously been transfused with platelets before surgery (Table 1, patient 2). Later, she needed hysterectomy due to a rapidly growing uterine mass, but the scheduled surgery was shelved because her platelet

count remained around 15 x$10^9$/L despite preoperative transfusion of apheresis platelets. Subsequent investigations detected the presence of allo-antibodies against the platelet glycoprotein Ia-IIa complex. Two patients had previously received eltrombopag within the abovementioned clinical trial.[9]

Eltrombopag was started three weeks before the procedure. We used the dose of 75 mg/day, as this dosage was associated with a better platelet response compared to 50 mg/day within the previous clinical trial, without any significant safety issues.[9] The only exception was a patient who had shown a good response to 50 mg/day within the same trial,[9] and was then treated again with this dose.

In 10 cases (patients 1-4), eltrombopag induced an increase in platelet count that allowed to carry out surgery without the need of platelet transfusions and without any bleeding complication (**Table 2, Figure 1**). Procedures included 5 major surgeries (2 orthopedic, 1 gynecologic, and 2 cochlear implantations), 4 dental surgeries, and a percutaneous kidney biopsy. Preoperative platelet counts ranged from 75 to 180 x$10^9$/L (**Table 2**). In the patients treated more than once, platelet responses were reproducible over time: for instance, patient 3 received 5 eltrombopag courses over a 10-year period with very similar results. The study of *in vitro* platelet aggregation (according to the densitometric method of Born[9]) on the day of surgery showed normal findings, or a slightly reduced response to the lowest ADP concentration in one patient (**Table 3**). All subjects reported the disappearance of bleeding symptoms 7-10 days after starting treatment. Eltrombopag was continued 3 to 7 days after surgery: in all the cases, we obtained a stable platelet count up to 5-7 days after drug discontinuation (i.e. up to 14 days after surgery). Whenever indicated (for instance in major orthopedic and gynecologic surgeries), standard antithrombotic prophylaxis with heparin was administered. Of note, patient 3 no longer required hospitalization for dental surgery. Conversely, eltrombopag induced a very slight increase of platelet count in patient 5 (from 5 to 11 x$10^9$/L). In this case, transfusion of apheresis platelets was deemed necessary before the biopsy of a tonsillar tumor. This patient too experienced remission of spontaneous bleeding from the tonsillar lesion following the mild increase of platelet count during eltrombopag treatment. Of note, this patient presented at baseline with a massive splenomegaly of unknown cause, which could have contributed to the failure to significantly increase platelet count through a mechanism of platelet splenic sequestration.

Concerning adverse events, only one patient referred mild headache at the beginning of two eltrombopag courses, which resolved spontaneously after 2-3 days. Since eltrombopag has been associated with occurrence of cataracts in patients with immune thrombocytopenia, and *MYH9*-related disease is a syndromic disorder predisposing to juvenile cataracts,[7,9] ophthalmological evaluation was carried out at every follow-up visit. None of our patients showed occurrence or worsening of cataracts, not even patients who already presented cataracts at baseline and received repeated eltrombopag courses.

To confirm the results of the first prospective study on patients affected with *MYH9*-related disease, and to investigate the efficacy of eltrombopag in increasing platelet count also in subjects affected with 4 forms of genetic thrombocytopenias, we conducted a second phase 2 clinical trial.[22] The 24 enrolled patients received short-term eltrombopag to test if this treatment can raise platelet count up to safe levels for major surgery. Moreover, in a subgroup of patients presenting with clinically significant spontaneous bleeding, we also tested if a prolonged administration of eltrombopag can induce a persistent remission of the spontaneous hemorrhages.

## METHODS

### Patients

Patients were enrolled at 5 Italian Centres: 1) IRCCS Policlinico San Matteo Foundation, Pavia, Italy (coordinating centre); 2) Azienda Ospedaliera di Perugia, Perugia, Italy; 3) Azienda Ospedaliera di Padova, Padova, Italy; 4) IRCCS Policlinico Agostino Gemelli Foundation, Roma, Italy; 5) Azienda Ospedaliera Universitaria Integrata di Verona, Verona, Italy. The study protocol was approved by the institutional review boards of all Institutes. Patients or their legal guardians signed written informed consent for the study, which was conducted according to the Declaration of Helsinki.

### Inclusion and exclusion criteria

Patients were eligible for the study if they fulfilled all of the following criteria: diagnosis of one of the forms of inherited thrombocytopenia listed in **Table 4,** which were confirmed by molecular analysis; age 16-70 years; basal platelet count <80 x$10^9$/L. The exclusion criteria are also listed in Table 4.

### Study design

This was a phase 2, open-label, dose-escalation trial. The study consisted of two Parts.

*Part 1.* The main aim of Part 1 was to test whether, and in which forms of inherited thrombocytopenia, a short-term course of eltrombopag was effective in increasing platelet count above the safe threshold for all types of surgery (100 x$10^9$/L).[4,5] All patients eligible for the study entered the Part 1.

Patients received eltrombopag 50 mg/day for 3 weeks. Patients who obtained a platelet count >100 x$10^9$/L at day 21 stopped treatment - as they achieved the primary endpoint. In the other cases, patients received eltrombopag 75 mg/day for 3 additional weeks.

*Part 2*. The main aim of Part 2 was to test the efficacy of long-term eltrombopag in achieving an enduring remission of bleeding symptoms in patients presenting with clinically significant spontaneous hemorrhages at baseline.

Criteria for entering the Part 2 were the following: patients with spontaneous bleeding at baseline grade ≥2 according to the World Health Organization (WHO) bleeding scale, who completed Part 1 without severe side effects and obtained a reduction of bleeding symptoms at the end of Part 1.

Part 2 consisted of 16 weeks of treatment. Patients were started on eltrombopag 25 mg/day. Then, they were re-evaluated every 4 weeks, and eltrombopag dose was adjusted based on bleeding tendency and platelet count according to the schedule reported in **Figure 2**.

**Endpoints and outcome measures**

The primary endpoint of Part 1 was the achievement of a platelet count >100 $x10^9$/L, corresponding to the safe level for all types of surgery according to current guidelines.[4,5] Major response was defined as the achievement of the primary endpoint. Minor response was defined as the achievement of a platelet count at least two-fold higher than baseline without reaching the criteria for major response.

The primary endpoint of Part 2 was the stable reduction of spontaneous bleeding manifestations according to the WHO bleeding scale during the last 2 weeks of treatment. Major response was defined as a complete remission of hemorrhages. Minor response was defined as a reduction of bleeding according to the WHO bleeding scale without reaching the criteria for major response.

Secondary endpoints included safety and tolerability of the treatments; dosages of eltrombopag required for achieving the primary endpoints; improvement of health-related quality of life with long-term eltrombopag administration (Part 2 only).

Exploratory endpoints included the effects of treatment on serum thrombopoietin levels and on platelet function investigated by light transmission aggregometry and/or flow cytometry.

**Investigation of patients**

The studies that has been performed to investigate patients at baseline and at each subsequent visit (Part 1: after 3 weeks of treatment and, in some patients, 6 weeks of treatment, and post-treatment assessment at 30 days unless patients entered Part 2. Part 2: after 4, 8, 12, and 16 weeks of treatment and post-treatment assessment at 30 days) are summarized in **Table 5**. Platelet count was measured by both automated cell counters and phase-contrast microscopy in a counting chamber. Since electronic cell counters underestimate platelet count in patients with inherited thrombocytopenia characterized by marked platelet macrocytosis,[23,24] only platelet count measured by microscopy was used for the purposes of this study. Spontaneous bleeding was measured according to the WHO bleeding scale:[25] grade 0, no bleeding; grade 1, cutaneous bleeding only; grade 2, mild blood loss; grade 3, gross blood loss; grade 4, debilitating blood loss.

Adverse events were coded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (CTCAEv4.0).

Patients' health-related quality of life was assessed in patients eligible to Part 2 at baseline and at each subsequent visit as detailed below.

**Measurement of serum thrombopoietin levels**

Measurement of serum thrombopoietin levels was centralized at the IRCCS Policlinico San Matteo, Pavia. Serum thrombopoietin levels were measured using the Quantikine ELISA Human Thrombopoietin Immunoassay Kit (R&D system, Minneapolis, USA) according to the manufacturer's instructions.[26]

***In vitro* platelet aggregation**

*In vitro* platelet aggregation in response to collagen, adenosine diphosphate (ADP) and ristocetin was assessed in patients who obtained platelet count >100 $x10^9$/L, with the densitometric method of Born, as described.[9]

**Flow cytometry investigation of platelet activation**

Platelet activation in response to different agonists was investigated by flow cytometry as reported.[27] Briefly, aliquots of whole blood were incubated with monoclonal antibodies and either ADP 1 µM, ADP 5 µM, TRAP 25 µM, or vehicle HEPES buffer alone, for 10 minutes at 37°C, and then fixed with paraformaldehyde. The following monoclonal antibodies were used: PAC1, which specifically binds to the activated conformation of GPIIb/IIIa (Becton Dickinson, San Josè, CA, USA); CLB-Thromb/6 against P-selectin (CD62P) (Immunotech, Marseille, France); P2 against GPIIb/IIIa (CD41) (Immunotech). Platelets were gated by GPIIb/IIIa (CD41) expression. Platelet activation was expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone. Patients' samples were processed in parallel with those of 25 healthy controls. Data represent the mean ± SD of two independent analyses.

**Assessment of patients' health-related quality of life**

Patients' health-related quality of life was assessed in patients eligible to Part 2 at baseline and at each subsequent visit. Health-related quality of life was measured through the administration of three validated questionnaires with complementary significance.[24, 27-30] The 18-item Functional Assessment of Cancer Therapy-Thrombocytopenia (FACT-Th18) was used to assess the effect of bleeding on health-related quality of life.[28,29] The fatigue subscale of the Functional Assessment of Chronic Illness Therapy (FACIT-F) questionnaire was used to focus on the perception of fatigue.[30] The acute recall version of the short form-36, version 1 (SF-36v1) was used to measure general health-related quality of life.[31]

**Statistical analysis**

Stata 15.1 (StataCorp, College Station, TX) was used for all analyses. The rate of response and its 95% exact binomial confidence interval (95%CI) was computed. We compared baseline to end of Part 1 platelet counts with the Student t test for paired data (after graphically assessing normality of the distribution) and computed the mean change and its 95%CI (normal based).

## RESULTS

### Study population

Twenty-four patients were enrolled between April 2015 and May 2017. They included 9 patients affected with *MYH9*-reladed disease; 9 with *ANKRD26*-related thrombocytopenia;[32] 3 with thrombocytopenia caused by *WAS* mutations (2 with X-linked thrombocytopenia, and 1 with Wiskott-Aldrich syndrome);[33,34] 2 with monoallelic Bernard-Soulier syndrome caused by the Ala156Val mutation of GPIbα;[35] 1 with thrombocytopenia deriving from an *ITGB3* mutation (*ITGB3*-related thrombocytopenia).[36] Patients' mean basal platelet count was 40.4 $x10^9$/L. **Tables 6** and **7** describe the features of the study population at baseline.

### Part 1

Twenty-three patients completed the Part 1 of the study, whereas one patient with *ANKRD26*-related thrombocytopenia discontinued the treatment earlier because of an adverse event.

*Primary endpoint.* Response to Part 1 is summarized in **Table 8** and detailed in **Table 9,** and **10**.

Twenty-one of the 23 evaluable patients (91.3%, 95%CI 72.0-98.9) obtained a response according to the study criteria: 11 patients (47.8%) achieved a major response, and 10 (43.5%) a minor response. Two patients (8.7%) (one with *ANKRD26*-related thrombocytopenia and the patient with *ITGB3*-related thrombocytopenia) did not respond. Most patients with *MYH9*-related disease and the two subjects with monoallelic Bernard-Soulier syndrome obtained a major response, whereas most patients with *ANKRD26*-related thrombocytopenia and the three subjects with X-linked thrombocytopenia/Wiskott-Aldrich syndrome achieved a minor response (**Table 8**). The mean platelet count at the end of Part 1 was 104.9 $x10^9$/L ($p<0.001$ compared to baseline). The mean increase in platelet count with respect to baseline was 64.5 $x10^9$/L (95%CI 43.7-85.3) overall, and 69.5 $x10^9$/L in

the 21 responders. **Table 8** and **Figure 3** report the average increase in platelet count in the responders according to the different forms of inherited thrombocytopenia.

Ten of the 12 patients presenting spontaneous bleeding at baseline (83.3%) obtained complete remission of hemorrhages. In particular, all responders (major or minor response) achieved disappearance of bleeding symptoms if present at baseline. Of the two non-responders, the patient with *ANKRD26*-related thrombocytopenia did not obtain any improvement of bleeding manifestations, whereas the patient with *ITGB3*-related thrombocytopenia experienced a reduction of spontaneous bleeding (WHO grade from 2 to 1) following a mild increase in platelet count (from 62 to 78 $x10^9$/L).

*Eltrombopag dose.* Ten patients (43.5%) achieved a major response with eltrombopag 50 mg/day, and stopped therapy (**Table 11**). These patients were all the individuals with *MYH9*-related disease or monoallelic Bernard-Soulier syndrome who obtained a major response, and one subject with *ANKRD26*-related thrombocytopenia. Thus, 13 patients (56.5%) switched to the dosage of 75 mg/day. Treatment with the higher dose resulted in the achievement of a better response according to the study criteria in 4 of these subjects.

*Exploratory endpoints. In vitro* platelet aggregation in response to collagen, ADP, and ristocetin was studied at the end of treatment in the 11 patients who achieved platelet counts >100 $x10^9$/L. Platelet aggregation was normal in all the cases, except for two patients with *MYH9*-related disease and one with monoallelic Bernard-Soulier syndrome who presented slightly reduced responses to the lowest ADP dose (**Table 12**). In 12 patients, platelet activation in response to ADP and TRAP was also assessed through flow cytometry as the induction of surface expression of P-selectin and of the activated form of GPIIb/IIIa.[37] In these subjects, platelet activation at baseline was not significantly different compared to healthy controls. Overall, platelet responsiveness did not significantly change after eltrombopag treatment with respect to baseline in the investigated patients (**Figure 4**).

Mean serum thrombopoietin level at baseline was 177.8 pg/mL (SD 125). Thrombopoietin levels were unchanged at the end of treatment both considering patients overall and stratifying them according to the different disorders or response to treatment (**Table 13**).

*Safety.* We recorded 7 adverse events in 5 patients (21%) (**Table 14**): all the adverse events were grade 1 (mild) according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0. Four patients reported mild and transient headache and/or diffuse bone pain during the first 2-3 days of treatment. One patient with *ANKRD26*-related thrombocytopenia presented with increased plasma creatinine at the assessment after 3 weeks of treatment with eltrombopag 50 mg/day. Although the adverse event was grade 1 (creatinine 1.6x above baseline), the treatment was discontinued according to the study protocol. Further investigations showed that kidney dysfunction was due to urinary retention because of pre-existing benign prostatic hypertrophy, and suggested that a causal relationship between eltrombopag administration and the adverse event is unlikely (see Table 14 for details). Results of ophthalmic assessment at the end of therapy were unchanged in all cases, including the 3 patients with *MYH9*-related disease presenting cataracts at baseline.

**Part 2**

Six patients had the criteria for enrollment to the Part 2 of the study. Two of them did not consent to long-term treatment for logistic reasons, as they were not available to undergo the repeated visits planned by the study protocol. Thus, 4 patients entered the Part 2 (2 with *MYH9*-related disease, 1 with Wiskott-Aldrich syndrome, 1 with *ITGB3*-related thrombocytopenia). All of them presented at baseline with spontaneous mucosal hemorrhages WHO grade 2 or 3 (epistaxis, gum bleeding, menorrhagia, and/or hematochezia) (**Table 15**).

*Primary endpoint.* The outcome of Part 2 is summarized in **Table 15,** and **Figure 5**.

Three patients completed the 16 weeks of therapy. All of them obtained a stable remission of mucosal bleeding throughout the treatment period. During eltrombopag administration, they experienced only very mild and occasional easy bruising (WHO grade 1), resulting in a minor response according to the study criteria. Concerning the patient with Wiskott-Aldrich syndrome, treatment was discontinued after 8 weeks because of exacerbation of cutaneous eczema (see below). During treatment, he obtained a complete remission of bleeding (WHO grade 0).

*Eltrombopag dose, and health-related quality of life.* Two patients achieved the response with eltrombopag 25 mg/day, whereas two patients required 50 mg/day (**Table 15,** and **Figure 5**).

The reduction of bleeding symptoms was associated with an overall increase in the scores obtained with the FACT-TH18 and FACIT-F questionnaires for the evaluation of health-related quality of life (**Table 16**). The increase was evident in the two *MYH9*-related disease patients presenting the highest degree of bleeding tendency at baseline (WHO grade 3), whereas the two other patients obtained mild or no improvements.

*Exploratory endpoints.* Thrombopoietin levels of the 4 patients did not significantly change during Part 2. Platelet response to ADP and TRAP was assessed by flow cytometry in the two *MYH9*-related disease patients and the Wiskott-Aldrich syndrome patient, and did not show any significant changes with long-term eltrombopag (data not shown).

*Safety.* The patient with Wiskott-Aldrich syndrome referred exacerbation of a pre-existing cutaneous eczema, which is a typical manifestation of this genetic disease. For this reason, eltrombopag was discontinued after 8 weeks of Part 2. The adverse effect was grade 2 according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0. The patient referred similar exacerbations of the eczema before enrollment to this study, which occurred without any apparent causes. However, the eczema improved some

weeks after eltrombopag discontinuation, supporting a possible, causal relationship with the treatment. No additional adverse events were observed during Part 2. In particular, no occurrence or worsening of cataracts was observed, including the two patients with *MYH9*-related disease presenting cataracts at baseline.

**Post-treatment assessments**

Twenty patients were re-evaluated 30 days after the end of Parts 1 or 2 (3 patients refused the visit). Post-treatment assessments did not identify any adverse events. Mean platelet count was 47.0 $x10^9$/L (SD 26), similar to that at baseline in the same patients (40.9 $x10^9$/L, SD 23). Bleeding tendency returned to that recorded at baseline in all the cases.

## DISCUSSION

Thrombopoietin-receptor agonists represent an appealing treatment hypothesis for the majority of patients with thrombocytopenias of genetic origin. In fact, in most forms of inherited thrombocytopenia, the megakaryocyte response to thrombopoietin is totally or partially preserved:[38,39] therefore, thrombopoietin-receptor agonists can potentially increase platelet production in many of these disorders. Moreover, in most patients affected with inherited thrombocytopenias, platelet function is normal or only partially impaired, so that increasing platelet count is expected to effectively improve the hemostasis.[38,40] Patients with inherited thrombocytopenias may benefit from short-term courses of thrombopoietin-receptor agonists as well as a prolonged treatment.

Short-term courses may be given in preparation for elective surgery or other invasive procedures whenever platelet count is below the safe threshold for the specific procedure. In this context, thrombopoietin-receptor agonists can replace perioperative platelet transfusions, thus preventing allo-immunization and the other risks of blood derivatives, and provide an option to increase platelet count even in patients refractory to platelet transfusions.[10-12,14,15] On the other hand, patients presenting with clinically significant spontaneous bleeding may benefit from a long-term thrombopoietin-receptor agonists administration to achieve an enduring remission of bleeding symptoms and reduce the risk of major hemorrhages. Despite these premises, clinical evidence on the efficacy and safety of thrombopoietin-receptor agonists in inherited thrombocytopenias is very scarce.[41]

The only previous prospective study has tested short-term eltrombopag in patients with *MYH9*-related disease, and showed that most of them responded to treatment without major side effects.[9] The present trial investigated the response to short-term eltrombopag in a wider range of thrombocytopenias of genetic origin, and provides information on the effects of a prolonged treatment in those patients presenting with clinically significant spontaneous hemorrhages at baseline.

We gave short-term eltrombopag to patients affected with 5 different disorders: the large majority of them (91.3%) responded to the drug, and the mean platelet count at the end of therapy was increased by 64.5 $x10^9$/L compared to baseline ($p<0.001$). However, we observed some differences in the degree of platelet response

between the different forms of inherited thrombocytopenias. Eltrombopag was highly effective in *MYH9*-related disease, thus confirming and extending the results of the previous trial.[9] All the patients affected with this specific form responded, most of them (78%) reached a platelet count >100 x$10^9$/L, and the mean increase in platelet count compared to baseline was 98.1 x$10^9$/L. The two individuals with monoallelic Bernard-Soulier syndrome also achieved major responses with an increase in platelet count close to that of subjects with *MYH9*-related disease (80.5 x$10^9$/L). Although 7 of the 8 evaluable patients with *ANKRD26*-related thrombocytopenia responded to eltrombopag, the extent of platelet response was globally lower than in *MYH9*-related disease and monoallelic Bernard-Soulier syndrome. In fact, most subjects with *ANKRD26*-related thrombocytopenia obtained minor responses and the mean increase in platelet count in responders was 41.8 x$10^9$/L. In the three patients affected with X-linked thrombocytopenia / Wiskott-Aldrich syndrome, results appeared similar to those of *ANKRD26*-related thrombocytopenia: these patients reached a minor response with an average rise in platelet count of 41.4 x$10^9$/L. In spite of these differences, we believe that in all the above disorders the response to eltrombopag is highly significant for the use of the drug in preparation for surgery in clinical practice. In fact, current guidelines define a platelet count of 50 x$10^9$/L as the threshold level recommended for the majority of major surgeries, with the exception of neurosurgery and posterior eye surgery that require 100 x$10^9$/L platelets.[4,5] In this view, while the response observed in *MYH9*-related disease and monoallelic Bernard-Soulier syndrome appears a very good result, even the extent of the increase in platelet count obtained in *ANKRD26*-related thrombocytopenia and X-linked thrombocytopenia / Wiskott-Aldrich syndrome appears sufficient to avoid the use of platelet transfusions to prepare most patients for most surgical procedures.

Finally, we treated only one patient with *ITGB3*-related thrombocytopenia, who failed to achieve a platelet response according to the study criteria.

All the patients who responded to Part 1, even achieving a minor response, showed a complete remission of bleeding symptoms whenever these were present at baseline. Even one of the two patients classified as non-responders according to the study criteria, experienced the remission of mucosal bleeding following a mild increase in platelet count. Remission of bleeding is consistent with the results of platelet function studies during

eltrombopag treatment: since platelet response to different agonists was normal or only slightly impaired, increasing platelet count was effective in improving hemostasis. Consistent with previous findings in patients affected with X-linked thrombocytopenia / Wiskott-Aldrich syndrome,[18] flow cytometry showed that platelet responsiveness to ADP and TRAP does not significantly change with eltrombopag administration.

Concerning the dosage of eltrombopag, 10 of the 11 patients achieving a major response obtained this result with 50 mg/day, while one patient required the dose of 75 mg/day. Overall, 10 of the 13 patients who were switched from 50 to 75 mg/day obtained a further increase of platelet count with the higher dose: 4 subjects reached a better response according to the study criteria, while 6 patients achieved only slightly higher platelet counts. All the patients with *ANKRD26*-related thrombocytopenia or X-linked thrombocytopenia / Wiskott-Aldrich syndrome, but one, required the switch to the higher dose that resulted in a higher platelet count in most cases. These data suggest that 75 mg/day is the most reasonable starting dose for preoperative eltrombopag in these two disorders.

Effects of long-term eltrombopag administration were investigated in 4 patients presenting with frequent episodes of spontaneous mucosal bleeding. All of them achieved a stable remission of mucosal hemorrhages that persisted throughout the treatment period. In two patients, the reduction of spontaneous bleeding was associated with a very mild increase of platelet count (around 10 $x10^9$/L). The same observation was previously made in a patient affected with Wiskott-Aldrich syndrome and treated with long-term eltrombopag because of severe bleeding symptoms.[17] Two patients achieved remission of bleeding with the dosage of 25 mg/day, suggesting that, in some patients with inherited thrombocytopenias, clinical benefit can be maintained with prolonged administration of relatively low doses of eltrombopag. Interestingly, the two patients with the highest degree of bleeding tendency at baseline experienced not only a stable improvement of health-related quality of life related to bleeding, but also an increase of the score measuring the subjective perception of fatigue.

The observation that some patients obtained a significant reduction of bleeding tendency following a very mild increase in platelet count may suggest the hypothesis that eltrombopag improves some discrete platelet functions in addition to raise platelet concentration. As mentioned, overall we did not observe any significant

change in platelet GPIIb/IIIa activation or P-selectin expression in response to ADP and TRAP after eltrombopag treatment compared to baseline, in 12 investigated patients. However, we cannot exclude that the drug could have improved some other mechanisms of platelet function in some patients,[42] and further investigations are required to test this hypothesis.

Short-term eltrombopag was globally well tolerated, with 17% of patients reporting mild and transient headache and or bone pain at the beginning of treatment. In one patient with *ANKRD26*-related thrombocytopenia, we observed a mild increase in plasma creatinine; clinical investigation of this subject suggested that a causal relationship between eltrombopag and this adverse event is unlikely. Regarding the long-term treatment, the patient affected with Wiskott-Aldrich syndrome experienced worsening of a pre-existing cutaneous eczema that required eltrombopag discontinuation after 14 weeks. This adverse event has never been described with the previous retrospective reports on Wiskott-Aldrich syndrome patients who received eltrombopag.[17,18] No other adverse events were recorded with long-term therapy.

Eltrombopag has been associated with the occurrence of cataracts in patients with immune thrombocytopenia,[43] and *MYH9*-related disease is a syndromic disorder predisposing to juvenile cataracts.[44] Thus, it is noteworthy that none of our subjects affected with this form showed development or progression of cataracts, not even the two patients who received long-term therapy and already presented signs of cataracts at baseline.

A previous study raised the suspicion that the thrombopoietin-receptor agonist romiplostim favors the progression to myeloid leukemia in patients with myelodysplastic syndromes.[45] Subsequent trials of eltrombopag monotherapy in patients affected with myelodysplastic syndromes did not observe safety issues in this regard:[46-48] however, a trend for an increased risk of disease progression was reported in a study testing eltrombopag in association with azacytidine in intermediate- or high-risk myelodysplastic syndromes.[49] These observations raise a concern on safety of thrombopoietin-receptor agonists in *ANKRD26*-related thrombocytopenia, a condition that increases the risk of myeloid malignancies.[50] In the present study, short-term eltrombopag did not result in any change of blood cells parameters or morphology (with the exception of platelet count) in the subjects with *ANKRD26*-realted thrombocytopenia. However, further clinical data on this topic are needed, and caution should

be used when treating individuals affected with *ANKRD26*-related thrombocytopenia or other forms of inherited thrombocytopenia predisposing to hematological malignancies[51] with thrombopoietin-receptor agonists, especially with long-term administration.

## CONCLUSIONS

In conclusion, this study shows that eltrombopag is effective in increasing platelet count in 4 different forms of inherited thrombocytopenia, which, taken together, affect more than one half of patients with genetic thrombocytopenias.[40] In most patients, short-term eltrombopag increased platelet count above the threshold for major surgery recommended by current guidelines,[4,5] indicating that the drug can efficiently replace perioperative platelet transfusions in preparation for surgery or other invasive procedures.

Although only 4 patients received long-term treatment, the results indicate that a prolonged eltrombopag therapy can induce a persistent remission of spontaneous bleeding.

Both short- and long-term treatments with eltrombopag were globally well tolerated. Although it is certainly required a greater amount of clinical data on the use of thrombopoietin-receptor agonists in inherited thrombocytopenias, our results suggest that eltrombopag will probably have a central role in the treatment of thrombocytopenias of genetic origin in the next future.

## TABLES AND FIGURES

**Table 1.** Main clinical features at presentation of the 5 patients with *MYH9*-related disease treated with eltrombopag prior to surgery at our Institution.

| Patient ID | Gender /Age[1] | MYH9 variant | Platelet count [x10$^9$/L][2] | WHO bleeding score[3,5]/ ISTH BAT[4,5] | Previous surgery | | | Other features of the *MYH9*-RD[7] |
|---|---|---|---|---|---|---|---|---|
| | | | | | Type of surgery (number)[6] | Hemostatic preparation | Bleeding complications | |
| 1 | F/40 | p.D1424H | 19 | 3/11 | Kidney Transplantation<br>Dental extraction (2) | Platelet transfusions in all the cases | None | Nephropathy<br>Deafness<br>Cataracts |
| 2 | F/52 | p.R1165L | 15 | 2/7 | Inguinal hernia repair<br>Caesarean section (2) | Platelet transfusions in all the cases | None | Deafness<br>Cataracts |
| 3 | F/44 | p.N93K | 7 | 4/22 | Splenectomy<br>Hysterectomy<br>Appendectomy<br>Dental extraction (5)<br>Periodontal surgery (3)<br>Nasal polyps removal | Platelet transfusions in all the cases | Severe bleeding after hysterectomy treated with re-intervention, platelet and RBC transfusions.<br>Bleeding after 3 dental extractions and 2 periodontal surgeries treated with resuturing and platelet transfusions | Nephropathy<br>Deafness<br>Cataracts |
| 4 | M/47 | p.D1447V | 25 | 2/3 | None | - | - | Deafness |
| 5 | M/49 | p.K74del | 5 | 4/13 | None | - | - | Deafness |

**Notes:** [1] = age at the start of the first eltrombopag course. [2] = as determined through phase-contrast microscopy in a counting chamber. [3] = severity of bleeding manifestations according to the World Health Organization (WHO) bleeding scale.[25] [4] = the International Society for Thrombosis and Haemostasis (ISTH) Bleeding Assessment Tool (BAT) was assessed as previously reported.[20,21] [5] = WHO bleeding score and ISTH BAT score were calculated by considering the whole patients' medical history. [6] = number is indicated whenever an intervention was performed more than once. [7] = non-hematological manifestations of the *MYH9*-related disease at the start of the first eltrombopag course[9]. **Abbreviations:** RBC = red blood cells.

**Table 2.** Preoperative eltrombopag courses and the 11 surgical procedures in 5 patients with severe *MYH9*-related thrombocytopenia followed at our Institution.

| Patient ID[1]/ Gender | Age[2] | Platelet count at baseline[2,3] [x10$^9$/L] | Spontaneous bleeding at baseline[2] | Eltrombopag dose | Day of Surgery[4] | Platelet count at day of surgery [x10$^9$/L][3] | Spontaneous bleeding at day of surgery | Type of surgery | Peri-operative platelet transfusion | Adverse events |
|---|---|---|---|---|---|---|---|---|---|---|
| 1/F | 40 | 19 | Easy bruising<br>Gum bleeding<br>Menorrhagia | 50 mg/day | 20 | 180 | None | Correction of severe right bunion with osteotomy of the first and second metatarsal and placement of intramedullary *K*-wire[5] | No | None |
| | 41 | 20 | Easy bruising<br>Gum bleeding<br>Menorrhagia | 50 mg/day | 20 | 172 | None | Correction of severe left bunion<br>(see above) | No | None |
| | 43 | 23 | Easy bruising<br>Gum bleeding<br>Menorrhagia | 50 mg/day | 21 | 161 | None | Percutaneous biopsy of transplanted kidney | No | None |
| 2/F | 52 | 15 | Easy bruising<br>Menorrhagia | 75 mg/day | 21 | 75 | None | Laparoscopic hysterectomy and bilateral annessiectomy | No | None |
| | 54 | 17 | Easy bruising | 75 mg/day | 22 | 78 | None | Right cochlear implantation | No | None |
| 3/F | 44 | 7 | Easy bruising<br>Petechiae<br>Gum bleeding<br>Epistaxis | 75 mg/day | 21 | 100 | None | Dental extraction | No | Headache[6] |
| | 47 | 9 | Easy bruising<br>Petechiae<br>Gum bleeding<br>Epistaxis | 75 mg/day | 21 | 120 | None | Periodontal surgery | No | Headache[6] |
| | 50 | 10 | Easy bruising<br>Petechiae<br>Gum bleeding<br>Epistaxis | 75 mg/day | 21 | 95 | None | Dental extraction | No | None |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 53 | 10 | Easy bruising<br>Petechiae<br>Gum bleeding<br>Epistaxis | 75 mg/day | 22 | 132 | None | Periodontal surgery | No | None |
| 4/M | 47 | 25 | Easy bruising<br>Gum bleeding | 75 mg/day | 23 | 104 | None | Right cochlear implantation | No | None |
| 5/M | 49 | 5 | Easy bruising<br>Bleeding from tonsillar tumor | 75 mg/day | 21 | 11 | Easy bruising | Biopsy of tonsillar tumor mass | Yes | None |

**Notes:** [1] = please see Table 1. [2] = at the start of each eltrombopag course. [3] = as determined with phase-contrast microscopy in a counting chamber. [4] = days after the start of the eltrombopag treatment. [5] = this surgical intervention has been previously reported.[10] [6] = mild headache at the beginning of the eltrombopag course, responsive to low doses of acetaminophen; spontaneous remission after 2 to 3 days.

**Table 3.** *In vitro* platelet aggregation investigated at the day of surgery in the patients 1-4: results are expressed as maximal extents (percentage). Platelet aggregation could not be studied in the patient 5 because of the very low platelet count at the day of surgery.

| Patient ID[1] | Age | Platelet count at day of surgery [x10$^9$/L][3] | Type of surgery | Collagen, 4 µg/mL | Collagen, 20 µg/mL | ADP, 5 µM | ADP, 20 µM | Ristocetin, 1.5 mg/mL |
|---|---|---|---|---|---|---|---|---|
| 1 | 40 | 180 | Correction of severe right bunion with osteotomy of the first and second metatarsal and placement of intramedullary *K*-wire | 90% | nd | 80% | nd | 95% |
| | 41 | 172 | Correction of severe left bunion (see above) | 87% | nd | 76% | nd | 100% |
| | 43 | 161 | Percutaneous biopsy of transplanted kidney | 90% | nd | 70% | nd | 100% |
| 2 | 52 | 75 | Laparoscopic hysterectomy and bilateral annessiectomy | 89% | nd | 78% | nd | 100% |
| | 54 | 78 | Right cochlear implantation | 87% | nd | 75% | nd | 100% |
| 3 | 44 | 100 | Dental extraction | 88% | nd | 35% | 95% | 98% |
| | 47 | 120 | Periodontal surgery | 89% | nd | 80% | nd | 96% |
| | 50 | 95 | Dental extraction | 90% | nd | 35% | 90% | 100% |
| | 53 | 132 | Periodontal surgery | 88% | nd | 80% | nd | 100% |
| 4 | 47 | 104 | Right cochlear implantation | 88% | nd | 77% | nd | 95% |
| **Normal values of platelet aggregation (range)** | | | | 66-90% | 78-100% | 43-80% | 68-95% | 67-100% |

**Notes:** [1] = please see Table 1. **Abbreviations:** nd = not done, since platelet aggregation resulted normal with the lower dose of the same agonist.

**Table 4.** Inclusion and exclusion criteria of the study.

**Inclusion criteria**

Patients were considered for enrollment if they fulfilled all of the following 4 criteria.

1 - Diagnosis of one of the following forms of inherited thrombocytopenia confirmed by molecular analysis:
- *MYH9*-related disease (OMIM 155100, 605249, 153640, 153650)
- Bernard-Soulier Syndrome deriving from monoallelic mutations (OMIM 153670)
- Wiskott-Aldrich syndrome (OMIM 301000).
- X-linked thrombocytopenia (OMIM 313900).
- X-linked thrombocytopenia with thalassemia (OMIM 314050).
- Dyserythropoietic anemia with thrombocytopenia (OMIM 300367).
- *ITGA2B*/*ITGB3*-related thrombocytopenia (OMIM 187800).
- *ANKRD26*-related thrombocytopenia (OMIM 188000).
- *TUBB1*-related thrombocytopenia (OMIM 613112)
- *ACTN1*-related thrombocytopenia (OMIM 615193)
- *GFI1B*-related thrombocytopenia (OMIM 187900)
- *CYCS*-related thrombocytopenia (OMIM 612004)
- *SLFN14*-related thrombocytopenia (OMIM not available)

2 - Age ≥ 16 years and ≤ 70 years
3 - Average platelet count at baseline and during the previous year less than 80 $x10^9$/L
4 - Written informed consent

**Exclusion criteria**

Patients were excluded from enrollment if they presented one or more of the following criteria.

- Hypersensitivity to eltrombopag or one of the excipients.
- History of thromboembolic events.
- Treatment with anti-platelet drugs or other drugs affecting platelet function and/or with anticoagulants.
- Concurrent diseases or conditions that significantly increase the risk of thromboembolic events.
- Moderate to severe liver failure (Child-Pugh score ≥ 5).
- Altered renal function as defined by creatinine ≥ 2 mg/dL
- Previous or concurrent clonal disorders of the myeloid series (acute myeloid leukemias and myelodysplastic syndromes).
- Females who are pregnant or nursing (a negative pregnancy test was required before enrolment of fertile women).
- Formal refusal of any recommendations for a safe contraception.
- Alcohol or drug addiction.
- Any other disease or condition that by the advice of the responsible physician would make the treatment dangerous for the patient or would make the patient ineligible for this study, including physical, psychiatric, social and behavioral problems.

**Table 5.** Studies for investigation of the enrolled patients at baseline and at each subsequent on-treatment and post-treatment assessments - unless otherwise specified in notes.

| |
|---|
| - Medical history |
| - Physical examination |
| - Evaluation of bleeding tendency according to WHO bleeding scale during the previous 1 or 2 weeks [1] |
| - Complete blood counts and differential by automated cell counter |
| - Measurement of platelet count by phase-contrast microscopy in a counting chamber |
| - Peripheral blood smear examination |
| - Measurement of plasma aspartate transaminase (AST), alanine transaminase (ALT), total and fractionated bilirubin, and creatinine |
| - Urine analysis |
| - Ophthalmic assessment to monitor for cataracts or other ocular changes[2] |
| - Measurement of serum thrombopoietin level |
| - Assessment of health-related quality of life with the FACT-Th18, FACIT-F, and SF-36v1 questionnaires[3] |
| - Investigation of *in vitro* platelet aggregation in response to collagen (5 and 20 µg/mL), ADP (2 or 5 and 20 µM), and ristocetin (1.5 mg/mL)[4] |

**Notes:**

[1] = previous 1 week at baseline and during Part 1, previous 2 weeks during Part 2.

[2] =performed only at baseline, at the end of Parts 1 and 2, and at the assessment 30 days after the end of Parts 1 and 2.

[3] = performed only at baseline, during the Part 2, and at the assessment 30 days after the end of Part 2.

[4] = performed at the end of the Parts 1 and 2 whenever platelet count was over 100 $x10^9$/L.

**Table 6.** Main features of the study population at baseline.

| | **Overall** | ***MYH9*-RD** | ***ANKRD26*-RT** | **XLT/WAS** | **mBSS** | ***ITGB3*-RT** |
|---|---|---|---|---|---|---|
| **Patients, no.** | 24 | 9 | 9 | 3 | 2 | 1 |
| **M/F, no. of patients** | 14/10 | 2/7 | 7/2 | 3/0 | 2/0 | 0/1 |
| **Age, years** - mean [SD] | 41.1 [13.7] | 42.9 [14.7] | 40.9 [15.1] | 29.3 [6.8] | 50 [5.7] | 45 [-] |
| **Platelet count,[1] x10$^9$/L** - mean [SD] | 40.1 [22.4] | 38.2 [22.7] | 37.4 [22.2] | 26.3 [15.8] | 70 [1.4] | 62 [-] |
| **Spontaneous bleeding, [2] no. of patients** | 13 | 3 | 7 | 1 | 1 | 1 |
| ***WHO grade = 1, no.*** | *7* | *0* | *7* | *0* | *0* | *0* |
| ***WHO grade = 2, no.*** | *4* | *1* | *0* | *1* | *1* | *1* |
| ***WHO grade = 3, no*** | *2* | *2* | *0* | *0* | *0* | *0* |
| **Previous splenectomy,[3] no. of patients** | 2 | 2 | 0 | 0 | 0 | 0 |

**Notes:** [1] = as evaluated by phase-contrast microscopy in a counting chamber. [2] = spontaneous bleeding presented during the week preceding baseline evaluation according to World Health Organization (WHO) bleeding scale. [3] = previous splenectomy because of a mistaken diagnosis of immune thrombocytopenia.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; mBSS = monoallelic Bernard-Soulier syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 7.** Detailed features of the study population at baseline.

| Patient no./ Family no. | Gender / age | Diagnosis | Gene and causative mutation | Automated platelet count,[1] x$10^9$/L | Microscopic platelet count,[2] x$10^9$/L | WHO bleeding score[3] | Bleeding symptoms[4] | Other disease features[5] |
|---|---|---|---|---|---|---|---|---|
| 1/1 | F/49 | *MYH9*-RD | *MYH9* c.279C>G | 4 | 14 | 3 | EB, Pe, GB, Ep | Nephropathy, sensorineural deafness, cataracts |
| 2/2 | M/57 | *ANKRD26*-RT | *ANKRD26* c.-125 T>G | 22 | 17 | 1 | EB | - |
| 3/3 | F/55 | *MYH9*-RD | *MYH9* c.3493C>T | 58 | 69 | 2 | EB, GB | Sensorineural deafness |
| 4/4 | M/63 | *ANKRD26*-RT | *ANKRD26* c.-118C>A | 37 | 33 | 1 | EB | - |
| 5/5 | M/43 | *ANKRD26*-RT | *ANKRD26* c.-125T>G | 13 | 12 | 1 | EB, Pe | - |
| 6/6 | M/46 | mBSS | *GPIBA* c.515C>T | 65 | 69 | 2 | GB, Ep, Hm | - |
| 7/7 | F/24 | *ANKRD26*-RT | *ANKRD26* c.-128G>A | 42 | 37 | 1 | EB | - |
| 8/8 | M/54 | mBSS | *GPIBA* c.515C>T | 67 | 71 | 0 | - | - |
| 9/9 | M/40 | *ANKRD26*-RT | *ANKRD26* c.-116C>T | 67 | 63 | 1 | EB | - |
| 10/2 | M/54 | *ANKRD26*-RT | *ANKRD26* c.-125T>G | 33 | 33 | 1 | EB | - |
| 11/2 | M/19 | *ANKRD26*-RT | *ANKRD26* c.-125T>G | 55 | 53 | 1 | EB | - |
| 12/10 | F/45 | *MYH9*-RD | *MYH9* c.4270G>C | 23 | 38 | 3 | EB, GB, Me | Nephropathy, sensorineural deafness, cataracts |
| 13/11 | M/37 | XLT/WAS | *WAS* c.257G>A | 11 | 30 | 0 | - | - |
| 14/11 | M/27 | XLT/WAS | *WAS* c.257G>A | 52 | 40 | 0 | - | - |
| 15/12 | M/19 | *MYH9*-RD | *MYH9* c.2104C>T | 16 | 12 | 0 | - | Nephropathy, sensorineural deafness |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16/12 | M/46 | *MYH9*-RD | *MYH9* c.2104C>T | 67 | 70 | 0 | - | Nephropathy, sensorineural deafness |
| 17/13 | F/45 | *ITGB3*-RT | *ITGB3* c.2134+1G>C | 55 | 62 | 2 | EB, Me | - |
| 18/7 | F/39 | *ANKRD26*-RT | *ANKRD26* c.-128G>A | 78 | 75 | 0 | - | - |
| 19/14 | F/47 | *MYH9*-RD | *MYH9* c.5797C>T | 61 | 57 | 0 | - | - |
| 20/14 | F/34 | *MYH9*-RD | *MYH9* c.5797C>T | 30 | 27 | 0 | - | - |
| 21/15 | F/25 | *MYH9*-RD | *MYH9* c.3485G>C | 23 | 18 | 0 | - | |
| 22/16 | M/24 | XLT/WAS | WAS c.777+3inst | 10 | 9 | 2 | EB, Ep, Hm | Cutaneous eczema, immunodeficiency |
| 23/17 | F/66 | *MYH9*-RD | *MYH9* c.4270G>A | 37 | 39 | 0 | - | Cataracts |
| 24/18 | M/29 | *ANKRD26*-RT | *ANKRD26* c.-126T>G | 14 | 14 | 0 | - | - |

**Notes:** [1] = as evaluated by standard automated cell counters. [2] = as evaluated by phase-contrast microscopy in a counting chamber. Only platelet count measured with this method was used for the purposes of this study. [3] = spontaneous bleeding presented during the week preceding baseline evaluation according to World Health Organization (WHO) bleeding scale. [4] = EB, easy bruising. Pe, petechiae. GB, gum bleeding. Ep, epistaxis. Me, menorrhagia. Hm, hematochezia. [5] = other disease features in patients with syndromic forms of ITs.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; mBSS = monoallelic Bernard-Soulier syndrome; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 8**. Response to Part 1 (primary endpoint), overall and according to the different forms of inherited thrombocytopenia.

| | **Overall** | ***MYH9*-RD** | ***ANKRD26*-RT** | **XLT/WAS** | **mBSS** | ***ITGB3*-RT** |
|---|---|---|---|---|---|---|
| Evaluable patients, no. | 23 | 9 | 8 | 3 | 2 | 1 |
| **Response**[1] | | | | | | |
| Any response, % [95%CI] | 91.3 [72.0-98.9] | 100.0 [66.4-100.0] | 87.5 [47.3-99.7] | 100.0 [29.2-100.0] | 100.0 [15.8-100.0] | 0 [0-97.5] |
| Major response, % [95%CI] | 47.8 [26.8-69.4] | 77.8 [40.0-97.2] | 25.0 [3.2-65.1] | 0 [0-70.8] | 100.0 [15.8-100.0] | 0 [0-97.5] |
| Minor response, % [95%CI] | 43.5 [23.2-65.5] | 22.2 [2.8-60.0] | 62.5 [24.5-91.5] | 100.0 [29.2-100.0] | 0 [0-84.2]2 | 0 [0-97.5] |
| **Platelet count**[2] | | | | | | |
| Baseline, $x10^9$/L, mean [SD] | 40.4 [22.8] | 38.2 [22.7] | 38.0 [23.7] | 26.3 [15.8] | 70.0 [1.4] | 62.0 [-] |
| End Part 1, $x10^9$/L, mean [SD] | 104.9 [56.7]§ | 136.3 [68.0]# | 75.5 [28.5]§ | 67.7 [38.4] | 150.5 [13.4] | 78.0 [-] |
| Mean increase, $x10^9$/L [95%CI] | 64.5 [43.7-85.3] | 98.1 [53.3-142.9] | 37.5 [24.1-50.8] | 41.4 [22.1-104.8] | 80.5 [27.5-188.5] | 16.0 [-] |
| Mean increase in responders, $x10^9$/L [95%CI] | 69.5 [48.0-91.1] | 98.1 [53.3-142.9] | 41.8 [31.7-52.0] | 41.4 [22.1-104.8] | 80.5 [27.5-188.5] | - |
| **Spontaneous bleeding (SB)**[3] | | | | | | |
| Patients with SB at baseline, no. | 12 | 3 | 6 | 1 | 1 | 1 |
| Complete remission of SB end Part 1, % [95%CI] | 83.3 [51.6-97.9] | 100 [29.2-100] | 83.3 [35.9-99.6] | 100 [2.5-100] | 100 [2.5-100] | 0 [0-97.5] |
| Partial reduction of SB end Part 1, % [95%CI] | 8.3 [0.2-38.5] | 0 [0.0-70.8] | 0 [0.0-45.9] | 0 [0.0-97.5] | 0 [0.0-97.5] | 100 [2.5-100] |

**Notes:** [1] = according to predefined study criteria. [2] = as evaluated by phase-contrast microscopy in a counting chamber. [3] = spontaneous bleeding presented during the week preceding evaluation according to World Health Organization (WHO) bleeding scale.
§ $p<0.001$ with respect to baseline. # $p=0.001$ with respect to baseline.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; mBSS = monoallelic Bernard-Soulier syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 9.** Detailed response to Part 1.

| Patient no./ Family no. | Gender / age | Diagnosis | Maximal eltrombopag dose[1] | Platelet count - baseline,[2] x$10^9$/L | Platelet count - end treatment,[2] x$10^9$/L | WHO bleeding grade - baseline[3] | WHO bleeding grade - end treatment[3] | Response[4] |
|---|---|---|---|---|---|---|---|---|
| 1/1 | F/49 | *MYH9*-RD | 75 mg | 14 | 80 | 3 | 0 | Minor |
| 2/2 | M/57 | *ANKRD26*-RT | 75 mg | 17 | 49 | 1 | 0 | Minor |
| 3/3 | F/55 | *MYH9*-RD | 50 mg | 69 | 300 | 2 | 0 | Major |
| 4/4 | M/63 | *ANKRD26*-RT | 75 mg | 33 | 82 | 1 | 0 | Minor |
| 5/5 | M/43 | *ANKRD26*-RT | 75 mg | 12 | 35 | 1 | 0 | Minor |
| 6/6 | M/46 | mBSS | 50 mg | 69 | 141 | 2 | 0 | Major |
| 7/7 | F/24 | *ANKRD26*-RT | 75 mg | 37 | 91 | 1 | 0 | Minor |
| 8/8 | M/54 | mBSS | 50 mg | 71 | 160 | 0 | 0 | Major |
| 9/9 | M/40 | *ANKRD26*-RT | 75 mg | 63 | 109 | 1 | 0 | Major |
| 10/2 | M/54 | *ANKRD26*-RT | 50 mg | 33 | 35 | 1 | 0 | Not evaluable |
| 11/2 | M/19 | *ANKRD26*-RT | 75 mg | 53 | 60 | 1 | 1 | No response |
| 12/10 | F/45 | *MYH9*-RD | 50 mg | 38 | 120 | 3 | 0 | Major |
| 13/11 | M/37 | XLT/WAS | 75 mg | 30 | 96 | 0 | 0 | Minor |
| 14/11 | M/27 | XLT/WAS | 75 mg | 40 | 83 | 0 | 0 | Minor |
| 15/12 | M/19 | *MYH9*-RD | 75 mg | 12 | 77 | 0 | 0 | Minor |
| 16/12 | M/46 | *MYH9*-RD | 50 mg | 70 | 122 | 0 | 0 | Major |
| 17/13 | F/45 | *ITGB3*-RT | 75 mg | 62 | 78 | 2 | 1 | No response |
| 18/7 | F/39 | *ANKRD26*-RT | 50 mg | 75 | 115 | 0 | 0 | Major |
| 19/14 | F/47 | *MYH9*-RD | 50 mg | 57 | 110 | 0 | 0 | Major |
| 20/14 | F/34 | *MYH9*-RD | 50 mg | 27 | 178 | 0 | 0 | Major |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 21/15 | F/25 | *MYH9*-RD | 50 mg | 18 | 114 | 0 | 0 | Major |
| 22/16 | M/24 | XLT/WAS | 75 mg | 9 | 24 | 2 | 0 | Minor |
| 23/17 | F/66 | *MYH9*-RD | 50 mg | 39 | 126 | 0 | 0 | Major |
| 24/18 | M/29 | *ANKRD26*-RT | 75 mg | 14 | 63 | 0 | 0 | Minor |

**Notes:** [1] = 50mg, 50 mg/day for 3 weeks. 75mg, 50 mg/day for 3 weeks followed by 75 mg/day for 3 additional weeks. [2] = as evaluated by phase-contrast microscopy in a counting chamber. [3] = spontaneous bleeding presented during the week preceding evaluation according to World Health Organization (WHO) bleeding scale. [4] = according to predefined study criteria.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; mBSS = monoallelic Bernard-Soulier syndrome; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 10.** Results of platelet count measurements during the Part 1 of the study.

| | | | Platelet count x$10^9$/L | | | |
|---|---|---|---|---|---|---|
| **Patient no./ Family no.** | **Gender / age** | **Diagnosis** | **Baseline** | **After 3 weeks treatment with eltrombopag 50 mg/day** | **After 3 additional weeks treatment with eltrombopag 75 mg/day** | **30 days after treatment discontinuation** |
| 1/1 | F/49 | *MYH9-RD* | 14 | 60 | 80 | nd[2] |
| 2/2 | M/57 | *ANKRD26-RT* | 17 | 40 | 49 | 28 |
| 3/3 | F/55 | *MYH9-RD* | 69 | 300 | - | 80 |
| 4/4 | M/63 | *ANKRD26-RT* | 33 | 57 | 82 | 30 |
| 5/5 | M/43 | *ANKRD26-RT* | 12 | 23 | 35 | 15 |
| 6/6 | M/46 | mBSS | 69 | 141 | - | 90 |
| 7/7 | F/24 | *ANKRD26-RT* | 37 | 68 | 91 | 46 |
| 8/8 | M/54 | mBSS | 71 | 160 | - | 64 |
| 9/9 | M/40 | *ANKRD26-RT* | 63 | 82 | 109 | nd[3] |
| 10/2 | M/54 | *ANKRD26-RT* | 33 | 35 | - | - |
| 11/2 | M/19 | *ANKRD26-RT* | 53 | 58 | 60 | 56 |
| 12/10 | F/45 | *MYH9-RD* | 38 | 120 | - | nd[2] |
| 13/11 | M/37 | XLT/WAS | 30 | 54 | 96 | 30 |
| 14/11 | M/27 | XLT/WAS | 40 | 56 | 83 | 37 |
| 15/12 | M/19 | *MYH9-RD* | 12 | 64 | 77 | 22 |
| 16/12 | M/46 | *MYH9-RD* | 70 | 122 | - | 110 |
| 17/13 | F/45 | *ITGB3-RT* | 62 | 68 | 78 | nd[2] |
| 18/7 | F/39 | *ANKRD26-RT* | 75 | 115 | - | 69 |
| 19/14 | F/47 | *MYH9-RD* | 57 | 110 | - | 52 |
| 20/14 | F/34 | *MYH9-RD* | 27 | 178 | - | 24 |
| 21/15 | F/25 | *MYH9-RD* | 18 | 114 | - | 22 |
| 22/16 | M/24 | XLT/WAS | 9 | 21 | 24 | nd[2] |
| 23/17 | F/66 | *MYH9-RD* | 39 | 126 | - | nd[3] |
| 24/18 | M/29 | *ANKRD26-RT* | 14 | 47 | 63 | 16 |

**Notes:** [1] = as evaluated by phase-contrast microscopy in a counting chamber. [2] = not determined (nd) as the patient was admitted to the Part 2 of the study (see Figure 2). [3] = not determined (nd) as the patient refused the follow-up visit.
**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; mBSS = monoallelic Bernard-Soulier syndrome; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 11.** Doses of eltrombopag administered during the Part 1 of the study.

| | **Overall** | ***MYH9*-RD** | ***ANKRD26*-RT** | **XLT/WAS** | **mBSS** | ***ITGB3*-RT** |
|---|---|---|---|---|---|---|
| **Evaluable patients, no.** | 23 | 9 | 8 | 3 | 2 | 1 |
| **Major response with 50 mg/day, no. (%)** | 10 (43.5) | 7 (77.8) | 1 (12.5) | 0 | 2 (100) | 0 |
| **Switch to 75 mg/day, no. (%)** | 13 (56.5) | 2 (22.2) | 7 (87.5) | 3 (100) | 0 | 1 |
| **Improvement of response with 75 mg/day[1], no.** | 4 | 0 | 2[§] | 2[#] | 0 | 0 |

**Notes:** [1] = achievement of a better response according to the study criteria with respect to treatment with 50 mg/day. [§] = 1 patient achieved major response, 1 achieved minor response. [#] = both patients achieved minor response.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; XLT/WAS = X-linked thrombocytopenia/Wiskott-Aldrich syndrome; mBSS = monoallelic Bernard-Soulier syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Table 12.** *In vitro* platelet aggregation at the end of Part 1 of the study in the 11 patients who achieved a platelet count above 100 x$10^9$/L, maximal extent (percentage). Patients are reported according to the laboratories that performed the analysis, as the normal ranges of the assay are slightly different according to the laboratories of the different participating Centers.

| | **Patient ID[1]** | **Patient diagnosis** | **Platelet count,[2] x$10^9$/L** | **Collagen, 4 µg/mL** | **Collagen, 20 µg/mL** | **ADP, 2 or 5 µM[3]** | **ADP, 20 µM** | **Ristocetin, 1.5 mg/mL** |
|---|---|---|---|---|---|---|---|---|
| **Pavia laboratory** | 3/3 | *MYH9*-RD | 300 | 87% | nd | 67% | nd | 85% |
| | 6/6 | mBSS | 141 | 96% | nd | 21% | 86% | 96% |
| | 8/8 | mBSS | 160 | 85% | nd | 58% | 84% | 100% |
| | 9/9 | *ANKRD26*-RT | 120 | 74% | nd | 70% | nd | 84% |
| | 12/10 | *MYH9*-RD | 109 | 88% | nd | 85% | nd | 98% |
| **Perugia laboratory** | 16/12 | *MYH9*-RD | 122 | nd | 182% | nd | 104% | nd |
| **Padova laboratory** | 18/7 | *ANKRD26*-RT | 115 | 94% | nd | 98% | nd | 98% |
| | 19/14 | *MYH9*-RD | 110 | 95% | nd | 89% | nd | 96% |
| | 20/14 | *MYH9*-RD | 178 | 89% | nd | 20% | 85% | 99% |
| | 21/15 | *MYH9*-RD | 114 | 92% | nd | 15% | 79% | 104% |
| **Roma laboratory** | 23/17 | *MYH9*-RD | 126 | 74% | 75% | 75% | 73% | 69% |

**Normal values (range):**

Pavia laboratory: collagen 66-88%, ADP 43-76%, ristocetin 67-90%.Perugia laboratory: collagen 57.8-80.2%, ADP 43.2-73.2%, ristocetin 70-90%. Padova laboratory: collagen 44-86%, ADP 57-101%, ristocetin 76-90%. Roma laboratory: collagen 70-130%, ADP 58-90 %, ristocetin >60 %.

**Notes:** [1] = please see Table 4. [2] = platelet count at the end of Part 1. [3] = the lowest dose of adenosine diphosphate (ADP) was 5 µM in the Pavia and Roma laboratories, and 2 µM in the Padova laboratory.

**Abbreviation:** *MYH9*-RD = *MYH9*-related disease; mBSS = monoallelic Bernard-Soulier syndrome; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; nd = not determined.

**Table 13.** Thrombopoietin levels at baseline, at the end of Part 1, and at the post-treatment assessment 30 days after the end of Part 1.

| | **Overall** | ***MYH9*-RD** | ***ANKRD26*-RT** | **XLT/WAS** | **mBSS** | ***ITGB3*-RT** |
|---|---|---|---|---|---|---|
| **Baseline** (n=23 patients)<br>mean (SD), pg/mL | 177.8 (125) | 139.3 (104) | 274.3 (129) | 141.8 (33) | 54.1 (13) | 69 (-) |
| **End treatment** (n=23 patients)<br>mean (SD), pg/mL | 182.1 (209) | 109.6 (117) | 315.8 (287) | 128.5 (33) | 72.0 (20) | 73 (-) |
| **Post-treatment**[1] (n=17 patients)<br>mean (SD), pg/mL | 173.9 (178) | 104.7 (73) | 310.5 (259) | 151.0 (65) | 63.0 (15) | nd |

**Notes:** [1] = 6 patients did not undergo assessment 30 days after the end of Part 1 (2 refused the post-treatment visit and 4 entered the Part 2).

Normal values, as determined in a cohort of 100 consecutive healthy individuals, are 72.7 pg/mL (mean, SD 47.1).

**Abbreviation:** *MYH9*-RD = *MYH9*-related disease; *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; XLT/WAS = X-linked thrombocytopenia / Wiskott-Aldrich syndrome; mBSS = monoallelic Bernard-Soulier syndrome; *ITGB3*-RT; *ITGB3*-related thrombocytopenia; nd = not determined.

**Table 14.** Adverse events recorded during the Part 1 of the study. All the adverse events were grade 1 according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

| Adverse event | No. of adverse events | No. of patients (%) | Description |
|---|---|---|---|
| Any adverse event | 7 | 5 (21%) | |
| Headache | 4 | 4 (17%) | Mild headache that resolved completely after 2 or 3 days. Some patients took low doses of acetaminophen with benefit. |
| Bone pain | 2 | 2 (8%) | Mild diffuse bone pain that resolved completely after 3 or 4 days. Some patients took low doses of acetaminophen with benefit. |
| Increased plasma creatinine | 1 | 1 (4%) | Increase 1.6-fold above baseline at the evaluation after 21 days of treatment. Treatment was stopped according to study protocol. Further investigations disclosed that the increased creatinine level was due to urinary retention because of concomitant benign prostatic hypertrophy. Creatinine level continued to progressively increase for two months after eltrombopag discontinuation and then completely resolved after specific urologic treatment.<br>Worsening of prostatic hypertrophy has never been reported as an adverse reaction of eltrombopag treatment.[1] Based on the clinical course of the disorder and data from literature, the investigators suggest that a causal relationship between eltrombopag administration and the AE is unlikely. |

**Notes:** [1] Eltrombopag (Revolade®) Product information available at https://www.ema.europa.eu/en/medicines/human/

**Table 15.** Response to Part 2 in the 4 enrolled patients.

| **Patient ID (Patient/Family)**[1] | 1/1 | 12/10 | 17/13 | 22/16 |
|---|---|---|---|---|
| **Gender/age** | F/49 | F/45 | F/45 | M/24 |
| **Diagnosis** | *MYH9*-RD | *MYH9*-RD | *ITGB3*-RT | WAS |
| **Treatment duration**, weeks | 16 | 16 | 16 | 8 |
| **WHO bleeding score - baseline** | 3 | 3 | 2 | 2 |
| **Bleeding symptoms - baseline** | Easy bruising<br>Petechiae<br>Gum bleeding<br>Epistaxis | Easy bruising<br>Gum bleeding<br>Menorrhagia | Easy bruising<br>Menorrhagia | Easy bruising<br>Epistaxis<br>Hematochezia |
| **Platelet count - baseline**, $x10^9$/L | 14 | 38 | 62 | 9 |
| **WHO bleeding score - end Part 2**[2] | 1 | 1 | 1 | 0 |
| **Bleeding symptoms - end Part 2** | Easy bruising | Easy bruising | Easy bruising | None |
| **Platelet count - end Part 2**,[3] $x10^9$/L | 75 | 76 | 70 | 19 |
| **Eltrombopag dose - end Part 2**, mg/day | 50 | 25 | 25 | 50 |
| **Response to Part 2**[4] | Minor | Minor | Minor | Major |

**Notes:** [1] = please see Table 4. [2] = spontaneous bleeding presented during the 2 weeks preceding the last on-treatment visit according to WHO bleeding scale.
[3] = as evaluated at the last on-treatment visit by phase-contrast microscopy in a counting chamber. [4] = according to predefined study criteria.

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; *ITGB3*-RT = *ITGB3*-related thrombocytopenia; WAS = Wiskott-Aldrich syndrome.

**Table 16.** Results of the assessment of health-related quality of life in the 4 patients enrolled to the Part 2.

| | Mean (SD) | | Patient 1 (*MYH9*-RD) | | Patient 2 (*MYH9*-RD) | | Patient 3 (WAS) | | Patient 4 (*ITGB3*-RT) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **FACT-TH18 (Trial Outcome Index)** | | | | | | | | | | |
| Baseline | 83.7 (23.9) | | 55.7 | | 72 | | 102 | | 105 | |
| Week 4 | 102.2 (6.6) | | 98.4 | | 96 | | 103.4 | | 111 | |
| Week 8 | 100 (8.6) | | 96.3 | | 92 | | 99.8 | | 112 | |
| Week 12 | 101.4 (8.4) | | 98.3 | | 95 | | nd | | 111 | |
| Week 16 | 102.4 (8.4) | | 96.3 | | 99 | | nd | | 112 | |
| Post-treatment | 81.7 (27.6) | | 53.2 | | 63 | | 102 | | 108.5 | |
| % at week 16 *vs.* baseline[1] | 113% | | 173% | | 137% | | 98% | | 107% | |
| **FACIT-F** | | | | | | | | | | |
| Baseline | 36.0 (12.2) | | 25 | | 26 | | 48 | | 45 | |
| Week 4 | 43.5 (5.3) | | 39 | | 39 | | 49 | | 47 | |
| Week 8 | 44.7 (5.1) | | 38 | | 44 | | 50 | | 47 | |
| Week 12 | 44.3 (4.0) | | 40 | | 45 | | nd | | 48 | |
| Week 16 | 44.7 (5.9) | | 38 | | 49 | | nd | | 47 | |
| Post-treatment | 39.0 (10.5) | | 25 | | 37 | | 48 | | 46 | |
| % at week 16 *vs.* baseline[1] | 124% | | 152% | | 188% | | 104% | | 104% | |
| **SF-36v.1** | | | | | | | | | | |
| | PCS | MCS | PCS | MCS | PCS | MCS | PCS | MCS | PCS | MCS |
| Baseline | 44.9 (9) | 50.7 (5) | 47.3 | 43.3 | 30.9 | 51.1 | 50.7 | 55.9 | 50.8 | 52.7 |
| Week 4 | 50.8 (3) | 48.7 (6) | 46.8 | 48.6 | 50.4 | 40.0 | 51.3 | 53.8 | 54.7 | 52.6 |
| Week 8 | 49.8 (5) | 52.7 (3) | 42.7 | 49.7 | 50.2 | 51.7 | 51.5 | 57.0 | 54.7 | 52.6 |
| Week 12 | 49.6 (7) | 52.7 (3) | 42.9 | 49.7 | 49.2 | 54.4 | nd | nd | 56.6 | 53.9 |
| Week 16 | 50.3 (7) | 54.1 (5) | 43.5 | 49.7 | 50.5 | 59.6 | nd | nd | 57.0 | 53.0 |
| Post-treatment | 46.0 (8) | 45.6 (8) | 47.3 | 43.3 | 37.5 | 38.9 | nd | nd | 53.3 | 54.7 |
| % week 16 *vs.* baseline[1] | 112% | 107% | 92% | 115% | 163% | 117% | 102% | 102% | 112% | 101% |

**Notes:** [1] = percentage variation at week 16 (end of Part 2) with respect to the baseline value. FACT-Th18 = The 18-item Functional Assessment of Cancer Therapy-thrombocytopenia questionnaire, which measures the effect of bleeding on HR-QoL. FACIT-F = The Fatigue subscale of the Functional Assessment of Chronic Illness Therapy questionnaire, which measures the perception of fatigue. SF-36v1 = The acute recall version of the Short Form-36, version 1, which measures the general HR-QoL. Data are reported separately for the Physical Component Summary (PCS) and the Mental Component Summary (MCS).

**Abbreviations**: *MYH9*-RD = *MYH9*-related disease; WAS = Wiskott-Aldrich syndrome; *ITGB3*-RT = *ITGB3*-related thrombocytopenia.

**Figure 1.** The evolution of the platelet counts during and after eltrombopag administration on the occasion of the 11 surgical procedures in the 5 patients with *MYH9*-related disease followed at our Institution.

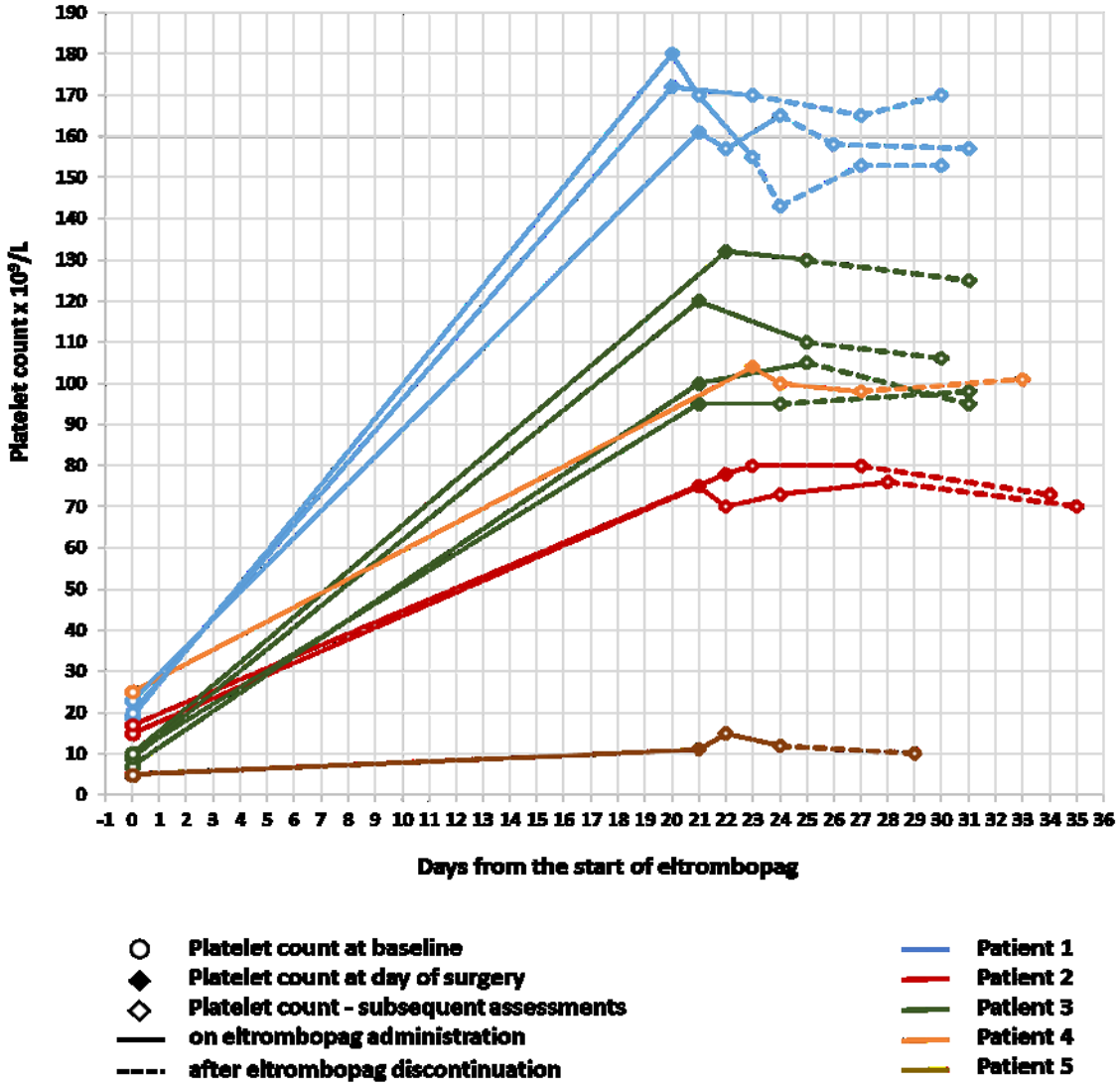


**Figure 2.** Schedule for dose adjustments of eltrombopag during the Part 2 of the study.

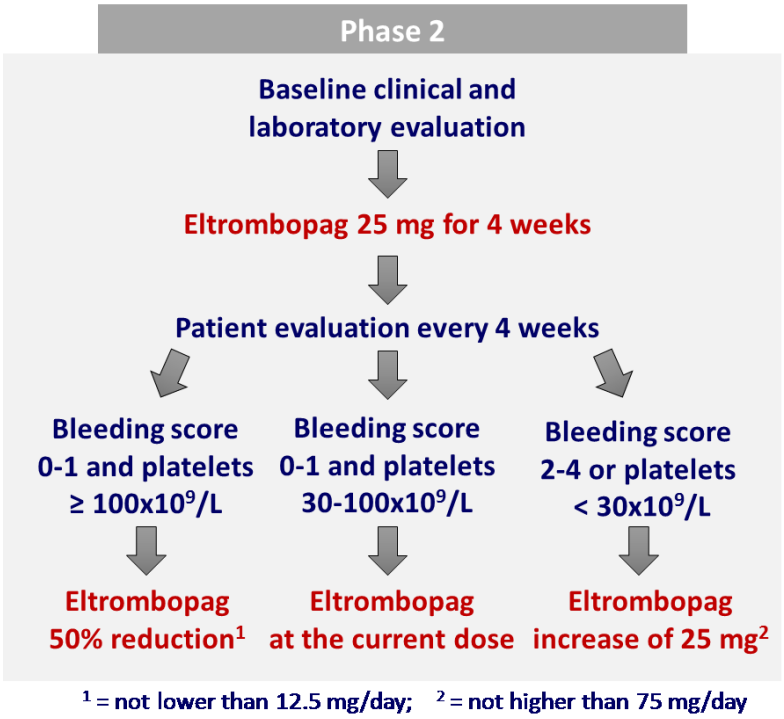
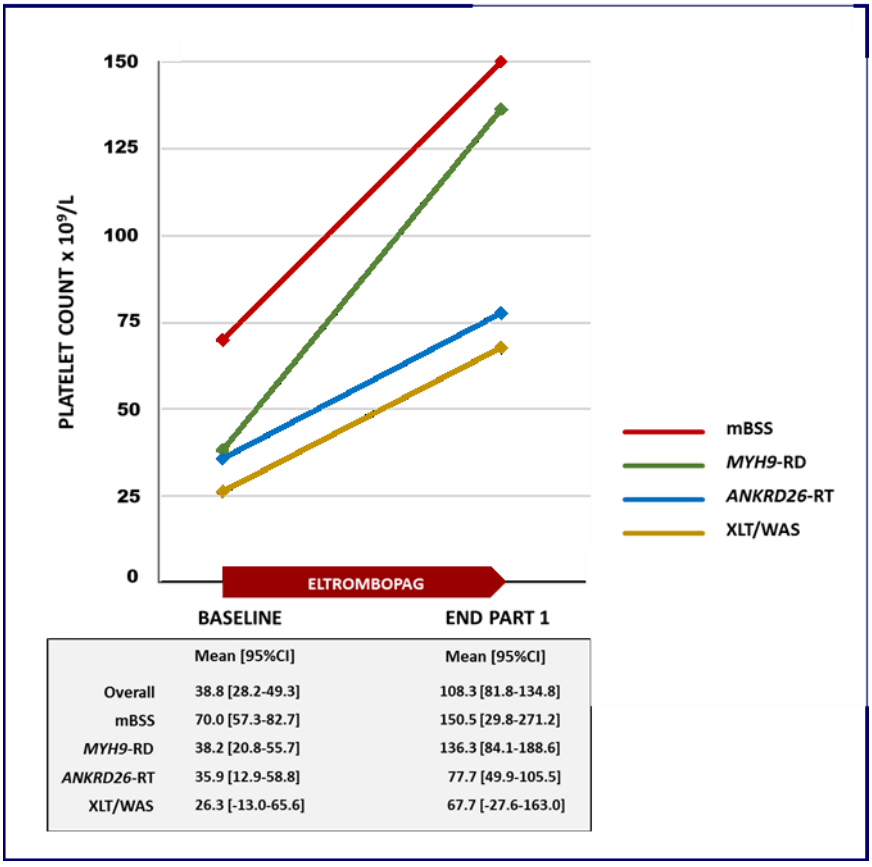


**Figure 3.** Mean increase in platelet count in the responders to the Part 1 of the study.

Patients are categorized according to the diagnosis of the specific form of inherited thrombocytopenia. Mean values of platelet count at baseline and at the end of Part 1 treatment along with their 95% confidence intervals (95%CI) are reported in the grey box.

**Abbreviations:** *MYH9*-RD = *MYH9*-related disease; mBSS = monoallelic Bernard-Soulier syndrome; *ANKRD26*-RT= *ANKRD26*-related thrombocytopenia. XLT/WAS= X-linked thrombocytopenia / Wiskott-Aldrich syndrome.




| | Mean [95%CI] | Mean [95%CI] |
|---|---|---|
| Overall | 38.8 [28.2-49.3] | 108.3 [81.8-134.8] |
| mBSS | 70.0 [57.3-82.7] | 150.5 [29.8-271.2] |
| *MYH9*-RD | 38.2 [20.8-55.7] | 136.3 [84.1-188.6] |
| *ANKRD26*-RT | 35.9 [12.9-58.8] | 77.7 [49.9-105.5] |
| XLT/WAS | 26.3 [-13.0-65.6] | 67.7 [-27.6-163.0] |

**Figure 4**. Platelet responsiveness to ADP and TRAP in 12 patients at baseline and at the end of Part 1.

Flow cytometry study of platelet activation in response to ADP and TRAP was carried out in 12 patients at baseline and at the end of Part 1: investigated patients were 4 subjects with *MYH9*-related disease, 5 with *ANKRD26*-related thrombocytopenia, 2 with monoallelic Bernard-Soulier syndrome, and 1 with Wiskott-Aldrich syndrome. Results obtained in patients were compared with those of 25 healthy controls who were processed in parallel. Platelet surface expression of P-selectin and of the activated form of GPIIb/IIIa (PAC1 antibody binding) was measured after incubation with ADP 1 µM, ADP 5 µM, TRAP 25 µM, or the vehicle HEPES buffer alone. Platelet activation is expressed as the ratio between the mean fluoresce intensity (MFI) measured after stimulation with each agonist and the MFI measured after incubation with the buffer alone (resting platelets). The boxes extend from the 25th percentile to the 75th percentile, the solid middle lines indicate the median values, and the whiskers indicate the range of values. Platelet response to ADP and TRAP was not significantly different in patients at baseline compared to controls. Platelet response to the agonists did not significantly change at the end of Part 1 treatment with respect to the baseline.

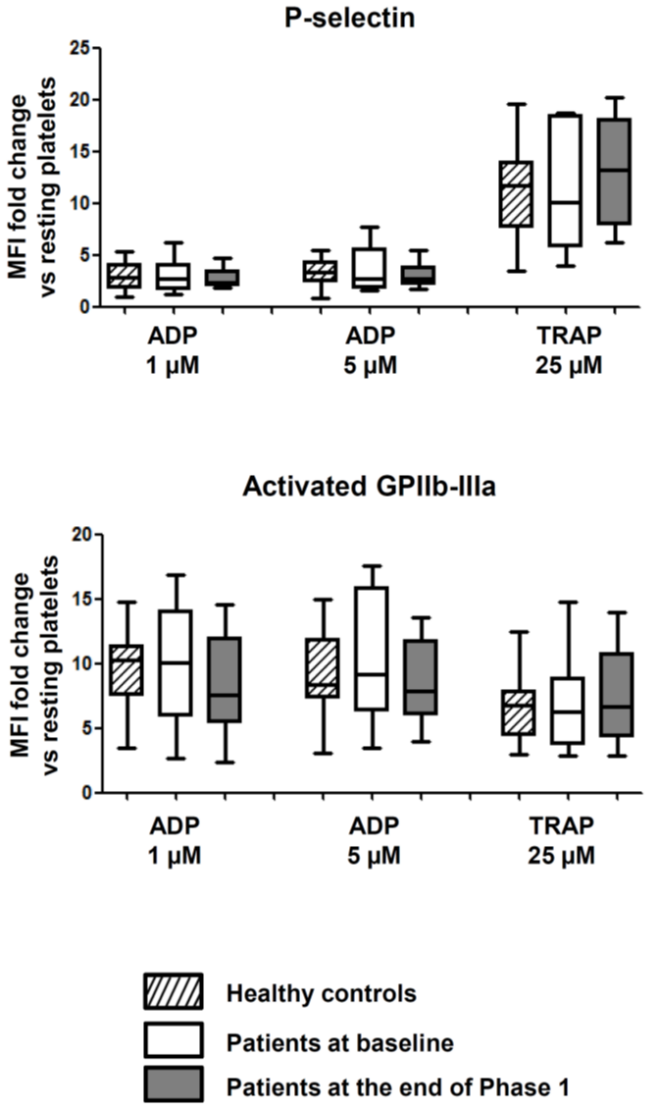
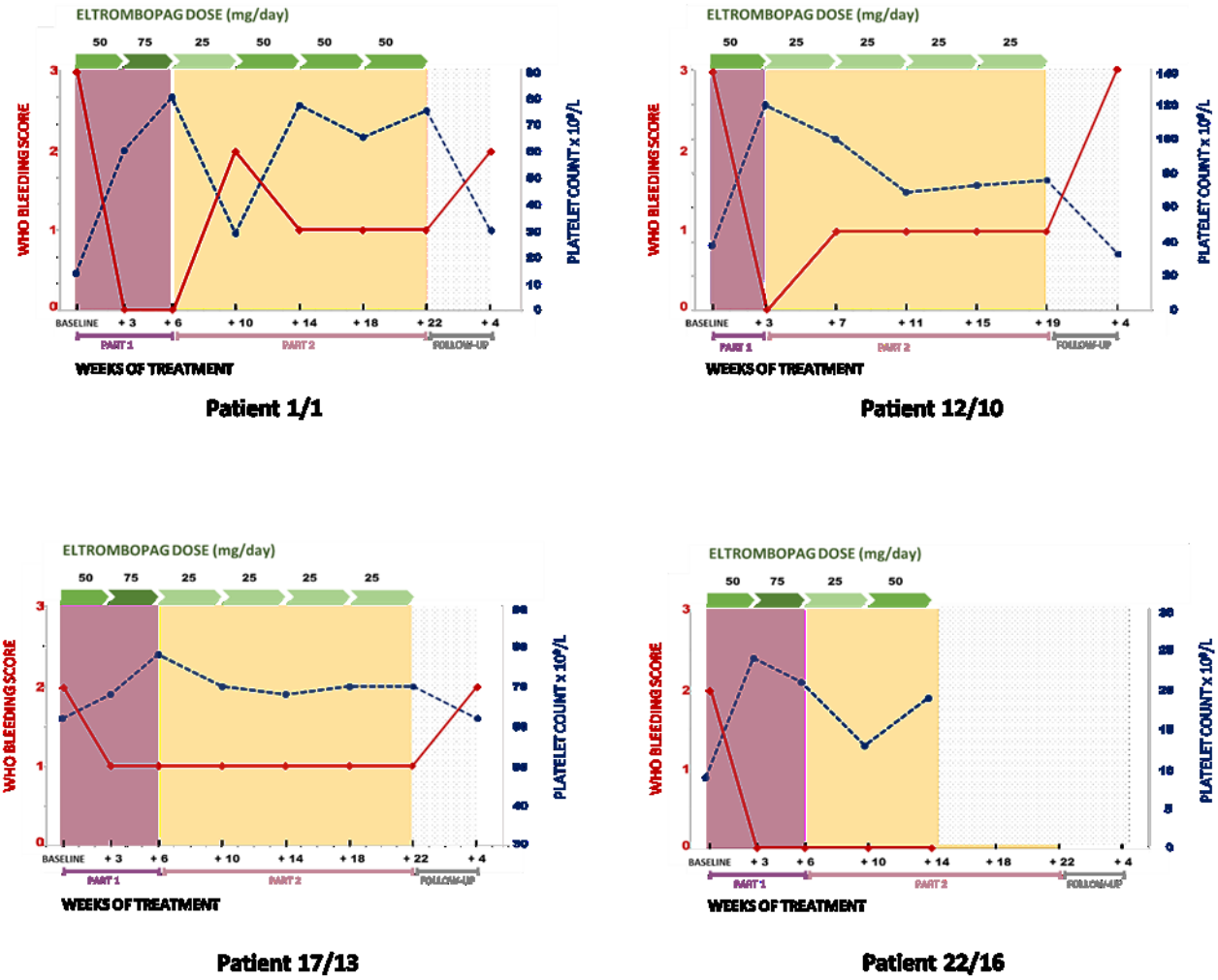


**Figure 5.** Effect of Part 1 and Part 2 treatment in the 4 individuals who received long-term eltrombopag.

The figure summarizes the effects of eltrombopag administration on bleeding symptoms according to the World Health Organization (WHO) bleeding scale and on platelet count. Patients 1/1 and 12/10 were affected with *MYH9*-related disease, patient 17/13 with *ITGB3*-related thrombocytopenia, and patient 22/16 with Wiskott-Aldrich syndrome (see also Table 7).



**PART 2**

## PREDICTION OF THE *IN VIVO* CLINICAL RESPONSE TO ELTROMBOPAG IN INDIVIDUAL PATIENTS USING A MINIATURIZED 3D BONE MARROW TISSUE *EX VIVO* MODEL

### INTRODUCTION

Bone marrow megakaryocytes are responsible for continuous production of platelets in the blood, driven by thrombopoietin through the interaction with its receptor, MPL.[52,53] In vivo, megakaryocytes associate with bone marrow microvasculature, where they extend proplatelets that protrude through the vascular endothelium into the lumen and release platelets into the bloodstream.[54,55]

Countless human pathologies result in alterations in platelet production; yet, for many of these, pathogenesis, and thus optimal targeted therapies, remain unknown.

Inherited thrombocytopenias are a heterogeneous group of disorders characterized by low platelet count, resulting in impaired hemostasis. While often stable, patients can have hemorrhages and/or excessive bleeding provoked by hemostatic challenges such as trauma or surgery; some hemorrhages appear spontaneously.[56,57] Treatment of inherited thrombocytopenias is still unsatisfactory. For patients affected with the severe forms, which are usually fatal at young ages, the treatment of choice is haematopoietic stem cell transplantation.[58-60] However, for most patients with inherited thrombocytopenias, transplantation is not indicated as the risks outweigh the benefits. In the past, the only effective treatments for these subjects were platelet transfusion to stop or prevent bleeding following trauma or during invasive procedures, anti-fibrinolytic agents, recombinant factor VIIa, or local treatment. A significant advance in the treatment of thrombocytopenias is the coming of drugs that stimulate platelet production by mimicking the effects of thrombopoietin. The thrombopoietin-receptor agonists eltrombopag, romiplostim, and very recently avatrombopag, have been approved for the treatment of several forms of acquired thrombocytopenia.[61-65] Thrombopoietin-receptor agonists were first

explored in the field of inherited thrombocytopenias in 2010 in a phase 2 clinical trial of eltrombopag in 12 patients with thrombocytopenia due to mutations in *MYH9* gene.[9] In 2015, eltrombopag was tested in 8 patients with X-linked thrombocytopenia / Wiskott-Aldrich syndrome, with reported platelet increases primarily in subjects affected with X-linked Thrombocytopenia.[18] More recently, a novel phase 2 clinical trial showed that eltrombopag was safe and effective in increasing platelet count and reducing bleeding symptoms in patients with different forms of inherited thrombocytopenia, including *MYH9*-related disease, *ANKRD26*-related thrombocytopenia, X-linked thrombocytopenia / Wiskott-Aldrich syndrome, monoallelic Bernard-Soulier syndrome and *ITGB3*-related thrombocytopenia.[22] Further, elective surgeries in patients affected with *MYH9*-related disease with severe thrombocytopenia have been performed safely after administration of eltrombopag.[19] Overall, these studies indicated that a sizeable proportion of patients with inherited thrombocytopenia respond to eltrombopag, but that the extent of platelet response is highly variable not only among different forms of genetic thrombocytopenia but also among different patients affected by the same disease.

Tools that recapitulate the function of specific tissues or organs are critical to test drug efficacy, reduce ineffective or suboptimal therapies, and personalize the choice of the best treatment for each specific patient as exemplified by organoids. Reproduction of the bone marrow has been very difficult because of its incompletely understood complexity. Current research is focused on duplicating characteristic features of the physiologic bone marrow microenvironment *ex vivo* using relevant biomaterials and bioreactors, along with appropriate human cell sources.[66-71]

Silk is a naturally-derived protein biomaterial with utility for studying platelet production since its fundamental features include non-thrombogenicity, low-immunogenicity, and non-toxicity.[69-73] A combination of modular flow chambers and vascular silk tubes and sponges was used to record platelet generation by primary human megakaryocytes, in response to variations in surface stiffness, functionalization with extracellular matrix components, and co-culture with endothelial cells.[69-71] These systems were able to support efficient platelet formation and, upon perfusion, recovery of functional platelets, as assessed through multiple activation tests,

including participation in clot formation and thrombus formation under flow conditions.[69-71] We describe an *ex vivo* miniaturized 3D bone marrow tissue model that recapitulates *ex vivo* platelet biogenesis of patients with different forms of inherited thrombocytopenias. This device is a radical improvement of the existing model because minimize the number of cultured cells while allowing multiple, simultaneous cultures. The results, starting from only 15 mL of peripheral blood, showed that the *ex vivo* tissue model could predict the *in vivo* clinical platelet response to eltrombopag in individual patients. The number of platelets recovered in the *ex vivo* model under standardized conditions, including exposure to eltrombopag, was significantly correlated with the increase in platelet count observed *in vivo* after eltrombopag treatment in the same patients. Overall, our data suggest that this tissue model will have substantial applicability for the evaluation of the effects of medications to determine their impact on platelet production at a single patient level.

## PATIENTS, MATHERIALS AND METHODS

### Patients

Human peripheral blood samples were obtained from healthy controls and thrombocytopenic patients after informed consent. All samples were processed following the ethical committee of the IRCCS. Policlinico San Matteo Foundation and the principles of the Helsinki Declaration. The main features of the 24 investigated samples from 20 different patients are reported in **Tables 1**. For 4 patients, the analysis was performed on two different occasions, with very similar results. The diagnosis of *MYH9*-related disease or *ANKRD26*-related thrombocytopenia had been confirmed by genetic analysis in all the cases. All patients provided written informed consent for this study, which was approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation, Pavia, Italy.

A sample of 15 mL of peripheral venous blood anticoagulated with acid-citrate-dextrose was collected for the analysis in the 3D bone marrow system. 13 patients had previously received a short-term course of eltrombopag (3- to 6-weeks) either within a phase 2 clinical trial[22] (n = 11) or in preparation for elective surgery[19] (n = 2). In any case, eltrombopag was given at the dose of 50 or 75 mg/day for 3 or 6 weeks. The *in vivo* clinical response to the drug was expressed as the absolute increase in platelet count at the end of eltrombopag treatment with respect to baseline. Blood samples for this study were collected when patients were out of eltrombopag therapy (minimum of 6 months to a maximum of 48 months washout). According to the fast pharmacokinetics of eltrombopag (plasma elimination half-life approximately 21-32 hours), all patients had platelet count at their baseline levels at one month of follow-up after the discontinuation of the treatment.[9,16] Thus, we do not expect that previous exposure to eltrombopag may have influenced haematopoietic stem and progenitor cell functions after months.

**Materials**

*B. mori* silkworm cocoons were supplied by Tajima Shoji Co., Ltd. (Yokohama, Japan). Pharmed tubing was from Cole-Parmer (Vernon Hills, IL, USA). The immunomagnetic separation system was from Miltenyi Biotech (Bergisch Gladbach, Germany, and Bologna, Italy). Recombinant human thrombopoietin, interleukin-6 (IL-6), interleukin-11 (IL-11), human bone morphogenetic protein 4 (BMP4), human vascular endothelial growth factor (VEGF), human fibroblast growth factor (FGF), human Fms-related tyrosine kinase 3 ligand (Flt3L), human stem cell factor (SCF) were from Peprotech (London, UK). CHiR 99021 was from TOCRIS. TruCount tubes and human fibronectin were from Becton Dickinson (S. Jose, CA, USA). The following antibodies were used: mouse monoclonal anti-CD61, clone SZ21, from Immunotech (Marseille, France); rabbit monoclonal anti-β1-tubulin was a kind gift of Prof. Joseph Italiano (Brigham and Women's Hospital, Boston, USA). Alexa Fluor conjugated secondary antibodies and Hoechst 33258 were from Life Technologies (Monza, Italy).

**Methods**

***Production of the drug-testing device***

The chamber was manufactured using 3D FDM printing technology and a biocompatible silicon molding approach. The modeling of the bioreactor was created using CAD software (OnShape, Fusion360 or Inventor2017) and used to generate 3D negative mold components exported as STL (Standard Triangulation Language) files, sliced with Slic3R PE and export to the FDM 3D printer Prusa i3 MK3S (Prusa Research, Czech Republic). The printing is done using a poly (lactic acid) (PLA) high-temperature filament of 1,75 mm (FormFutura, Netherland) deployed in layers of 100 µm by a 0.25 mm nozzle. After printing, the mold was cured in an oven at 100°C for 20 min to increase mechanical properties. To produce the perfusion channel, 21G needles were disposed in the dedicated holes and sealed with a gel of 25% Pluronic F-127. The molding was performed using a polydimethylsiloxane (PDMS) (Sylgard®184, Dow Corning), mixed in a 10:1 ratio of base material and curing agent. The selected material is stable both at low and high temperatures (45°C to 200°C) and it is resistant to UV,

water, and solvents. The PDMS was poured into the 3D printed molds that were positioned into a vacuum chamber to remove all the air bubbles. The curing of the PDMS was performed in a dried oven at 70°C for 4 hours; the molds were then dissociated of the final silicon models sterilizable by autoclave. The chamber consisted of two wells of 22x10 mm, having a hollow cavity of 15x2 mm enclosed in a block of 30x30 mm and connected to the outside of the chamber through channels of 0.9 mm diameter. The luer adaptors for the inlet and outlet were mounted in the channel and sealed with biocompatible silicone adhesive MD7-4502 (Dow Corning, USA). Then, the modular flow chamber was equipped with a silk fibroin sponge functionalized with fibronectin as described previously.[69,70]

***Preparation of the silk fibroin solution***

Silk fibroin aqueous solution was obtained from *B. mori* silkworm cocoons according to previously published literature.[68] Briefly, dewormed cocoons were boiled for 30 min in 0.02 M $Na_2CO_3$ solution at a weight to volume ratio of 10 g to 4 L. The fibers were rinsed for 20 min three times in ultrapure water and dried overnight. The dried fibers were solubilized for 4 h at 60°C in 9.3 M LiBr at a weight to volume ratio of 3 g/12 mL. The solubilized silk solution was dialyzed against distilled water using a Slide-A-Lyzer cassette (Thermo Scientific, Waltham, MA, USA) with a 3500 MW cutoff for three days and changing the water a total of eight times. The silk solution was centrifuged at maximum speed for 15 min to remove large particulates and stored at 4°C. The concentration of the silk solution was determined by drying a known volume of the solution overnight at 60°C and massing the remaining solids.

***Silk bone marrow fabrication and assembly***

Silk solution (8% w/v)[74] was mixed with 25 µg/mL fibronectin and dispensed into the modular chamber. $Na_2Cl$ particles (approximately 500 µm in diameter) were then sifted into the solution in a ratio of 1 mL to 2 g of $Na_2Cl$ particles. The scaffolds were then placed at room temperature for 48 hours and then soaked in distilled water for 48 hours to leach out the $Na_2Cl$ particles. The scaffolds were sterilized in 70% ethanol and finally rinsed five

times in PBS for over 24 hours. Silk scaffolds were characterized by confocal, as subsequently described. Perfusion of the silk scaffold has been tested at different flow rates (1-50 µL/min) by using a peristaltic pump (ShenChen Flow Rates Peristaltic Pump - LabV1, China). The total volume collected after each test corresponded to that injected in the system by the pump.

***Human megakaryocytes differentiation within the silk bone marrow***

$CD45^+$ haematopoietic progenitor cells from peripheral blood samples were separated by immunomagnetic bead selection kit (Miltenyi Biotec, Bologna, Italy) and cultured for 6 days in a flask in presence in Stem Span media (StemCell Technologies, Canada) supplemented with 1% penicillin-streptomycin, 1% L-glutamine, 10 ng/mL TPO, IL-6, and IL-11 in the presence or not of 500 ng/mL eltrombopag (Novartis) at 37°C in a 5% $CO_2$ fully humidified atmosphere, as previously described.[75,76]

On day 6, $CD61^+$ early megakaryocytic progenitors were sorted by immunomagnetic selection kit (Miltenyi Biotec, Bologna, Italy) and seeded for additional 8 days within the silk bone marrow model in presence of 10 ng/mL TPO supplemented or not with 500 ng/mL eltrombopag.

On day 14 of differentiation, the chamber was sealed, and the outlet ports were connected to the outlet needles. Culture media-filled tubes were connected to the inlet needles. The chamber was placed into the incubator (37°C and 5% $CO_2$), and transfer bags for platelet collection were secured to the outlet ports. The peristaltic pump (ShenChen Flow Rates Peristaltic Pump - LabV1, China) was placed outside the incubator, and media was pumped for 4 hours at a flow rate of 10 µL/min, speed range: 0.18 rpm, perfusion pause: 120 sec, perfusion run: 5 min with a peristaltic pump.

***Evaluation of differentiation and proplatelet formation by ex vivo differentiated megakaryocytes***

Megakaryocyte differentiation and proplatelet yields were evaluated by adhesion on fibronectin at the end of the culture (on day 14), as previously described.[76,77] Briefly, 12 mm glass cover-slips were coated with 25 µg/ml human fibronectin (Merck-Millipore, Milan, Italy), for 24 hours at 4°C. Megakaryocytes were harvested from the

silk bone marrow scaffold by extensive washing and seeded in a 24-well plate at 37°C in a 5% $CO_2$ fully humidified atmosphere. After 16 hours, adhering cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 (Sigma Aldrich, Milan, Italy), and stained for immunofluorescence evaluation with rabbit anti-β1-tubulin primary antibody (1:700) or anti-mouse CD61 (1:100) and Alexa Fluor-conjugated secondary antibodies (1:500) (Invitrogen, Milan, Italy). Nuclei were stained with Hoechst 33258 (1:10,000) (Sigma Aldrich, Milan, Italy). The cover-slips were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy) and imaged by an Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany). Proplatelet-forming megakaryocytes were identified as cells displaying long filamentous structure ending with platelet-sized tips. The results were expressed as a percentage of the total number of cells analyzed.

***Imaging of megakaryocyte cultures within the 3D silk bone marrow model***

For immunofluorescence imaging of megakaryocyte cultures within the silk bone marrow tissue model, samples were fixed in 4% paraformaldehyde (PFA) for 20 minutes and then blocked with 5% bovine serum albumin (BSA, Sigma) for 30 minutes at room temperature. Samples were probed with anti-CD61 (1:100) overnight at 4°C and then immersed in Alexa Fluor secondary antibody (1:500) for 2 hours at room temperature. Nuclei were stained with Hoechst. Samples were imaged by a TCS SP8 confocal laser scanning microscope (Leica, Heidelberg, Germany). For silk fibroin scaffolds imaging, we took advantage of silk auto-fluorescence in UV light. In some experiments, silk fluorescence was brightened by staining with Hoechst.[78] For all immunofluorescence imaging, the acquisition parameters were set on the negative controls. 3D reconstruction and image processing was performed using Leica licensed software or Image J software.

***Evaluation of platelet morphology***

For analysis of peripheral blood- and *ex vivo* collected platelet morphology, different approaches were used. First, megakaryocytes at the end of differentiation and platelets from peripheral blood or perfused media were visualized by light microscopy with an Olympus IX53 (Olympus Deutschland GmbH, Hamburg, Germany). For

analysis of cytoskeleton components, cells were stained as previously described.[79] Briefly, collected platelets were fixed in 4% PFA and centrifuged onto poly-L-lysine coated coverslip while peripheral blood smears were air-dried and then fixed in 4% PFA, permeabilized with 0.1% Triton X-100 for 5 minutes, and blocked with 5% BSA for 30 minutes at room temperature. To visualize microtubule organization, samples were probed with anti-β1-tubulin (1:1000) for 1 hour at room temperature and then immersed in Alexa Fluor secondary antibody (1:500) for 2 hours at room temperature. Samples were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy) and then imaged by an Olympus BX51 fluorescence microscope (Olympus, Deutschland GmbH, Hamburg, Germany). For all immunofluorescence imaging, the acquisition parameters were set on the negative controls, which were routinely performed by omitting the primary antibody.

***Flow cytometry***

Flow cytometry settings for analysis of megakaryocytes and *ex vivo* generated platelets were established as previously described.[80-84] For analysis of the percentage of fully differentiated megakaryocytes at the end of the culture (on day 14), 50 $x10^3$ cells were suspended in phosphate buffer saline (PBS) and stained with a FITC-conjugated antibody against human CD41 and human CD42b (eBioscience, Milan, Italy) at room temperature in the dark for 30 minutes and then analyzed. *Ex vivo* collected platelets were analyzed using the same forward- and side scatters as for those in human peripheral blood, and identified as $CD41^+CD42b^+$ events. Isotype controls were used as negative controls to exclude non-specific background signal. The platelet number was calculated using a TruCount bead standard. A minimum of 10.000 events were acquired. All samples were acquired with a Beckman Coulter Navios flow cytometer (Indianapolis, IN, US). Off-line data analysis was performed using Beckman Coulter Navios software package.

***Statistics***

Values were expressed as mean plus or minus the standard deviation (mean±SD) or mean plus or minus the standard error of the mean (mean±SEM). A two-tailed paired t-test was performed for statistical analysis of data

from samples tested in parallel under different experimental conditions. A two-tailed unpaired t-test was performed for statistical analysis of data from different samples. Statistical analysis was performed with GraphPad Software. A p-value of less than 0.01 was considered statistically significant. All experiments were independently replicated at least three times.

## RESULTS

### Device design, and prototyping

In adults, haematopoietic bone marrow is located in the medullary cavity of flat and long bones,[85] served by blood vessels that branch out into millions of small thin-walled arterioles and sinusoids allowing mature blood cells to enter the bloodstream (**Figure 1A**). To mimic such a structure, a device prototype of rectangular shape with 30x30x14 mm size and hollow cavities of 2x15x3.5 mm was developed. The device was connected to an outside peristaltic electronic pump (**Figure 1A**) through 0.9 mm diameter channels equipped with luer lock adaptors. We used devices with up to 2 reservoirs. Crosstalk between channels inside the device was eliminated by appropriate spatial separation and independent perfusion to allow assessment of patient-specific responses, following simultaneous exposure to thrombopoietin alone and thrombopoietin in combination with the tested drug.

3D printing technology is one emerging option for producing new devices in a customized, fast, and cost-effective manner. The printing process for the negative mold of our device is easily scalable. It can be created in less than 1 hour using a polylactic acid (PLA High temperature, FormFutura® Volcano, Figure 1-figure supplement 2), which allows casting and curing of polydimethylsiloxane (PDMS), a non-toxic polymeric organosilicon. The final shape of the system is optically clear (**Figure 1B-E**). Importantly, the device is reusable and autoclavable to ensure overall sterility to the system.

### Silk biomaterials for bone marrow system assembly and characterization

A silk fibroin structure functionalized with fibronectin was prepared with salt leaching method and inserted into the device to model a spongy scaffold that reproduces bone marrow architecture, composition, and microcirculation (**Figure 2A-C**). A 2-days production process allowed us to obtain a sterile 3D silk-fibronectin scaffold that could be stored in water, at 4°C, up to one month after preparation and used upon experimental needs. The silk scaffold was connected to gas-permeable tubing allowing perfusion of the media with a peristaltic

pump connected to inlet and outlet ports (**Figure 2A**). A cover cap closes the system before starting perfusion. The 3D reconstruction of the silk scaffold revealed the presence of multiple spatially-distinct niches (**Figure 2D and 2E**) and also demonstrated the homogeneous distribution of pores from top to bottom of the scaffold (**Figure 2F**). This arrangement efficiently supported the diffusion of cells (**Figure 2G**) and media outflow without altering the shape and integrity of the silk. Importantly, the total volume collected after perfusion corresponded to that injected in the system by the pump.

**Tuning of the silk bone marrow device for testing haematopoietic progenitor response to drugs**

To ascertain the ability of the device to model physiological and pathological bone marrow, we took advantage of our expertise in culturing human haematopoietic stem and progenitor cells[86-88] from peripheral blood of healthy controls and patients affected by two forms of Inherited Thrombocytopenia: *ANKRD26*-related thrombocytopenia and *MYH9*-related disease.[79,86-88] The bone marrow device was able to support efficient differentiation of mature megakaryocytes from both healthy controls and patients (**Figure 3A** and **3B**). However, patient-derived megakaryocytes displayed a decreased percentage of proplatelet formation by about 80%, accompanied by less branching of proplatelet shafts due to a significantly lower number of bifurcations (Healthy Control: 9±2; *ANKRD26*-related thrombocytopenia: 1.9±0.7; *MYH9*-related disease 1.8±0.9) as compared to healthy controls (**Figure 3C-E**).

**Validation of the miniaturized bone marrow device for testing haematopoietic progenitor response to drugs**

To validate the predictive value of the miniaturized bone marrow in response to drugs specifically targeting haematopoiesis, we chose eltrombopag as the model compound since it represents to date the only tested drug shown to increase platelet count of patients with inherited thrombocytopenias. First, we verified the *ex vivo* efficacy of eltrombopag on human adult megakaryocytic progenitors from healthy controls, and demonstrated the ability of the drug to induce increased megakaryocyte output and proplatelet formation with respect to the untreated counterpart (**Figure 3**). Then, we tested the sensitivity of 24 pathological samples from patients

affected with *ANKRD26*-related thrombocytopenia and *MYH9*-related disease (**Table 1**). This cohort included 11 patients previously treated with Eltrombopag in a recent phase 2 clinical trial[22] and 2 patients previously treated in preparation for elective surgery.[19] Blood samples for this study were collected when patients were out of eltrombopag therapy and had platelet count at their baseline levels. Equal numbers of megakaryocytic progenitors were divided between each channel for the *ex vivo* culture.

Patients with inherited thrombocytopenias have normal or slightly increased serum levels of thrombopoietin,[22] thus, *in vivo* haematopoietic progenitors are exposed to stimuli from endogenous thrombopoietin simultaneously with eltrombopag treatment. To better mimic this condition, all the samples were cultured in the presence of 10 ng/mL recombinant human thrombopoietin alone or in combination with 500 ng/mL eltrombopag (**Figure 4A**). Insights into the efficacy of eltrombopag effects *ex vivo* were gained by simultaneously analyzing megakaryocyte differentiation at day 14 for each disorder. Specifically, cells were washed out of the device and analyzed. We observed comparable megakaryocyte maturation in terms of cell size (**Figure 4B**), ploidy profile (**Figure 4C**), and expression of lineage-specific markers (**Figure 4D** and **4E**), with and without eltrombopag. However, the combination of thrombopoietin and eltrombopag resulted in a significant two-fold increase in the output of mature megakaryocytes with respect to thrombopoietin alone for subjects with either *ANKRD26*-related thrombocytopenia and *MYH9*-related disease (**Figure 4F**).

**Assessment of proplatelet formation to inform on mechanisms of action**

Confocal microscopy analysis of 3D scaffolds revealed a homogeneous distribution of $CD61^+$ megakaryocytes throughout the entire construct in both culture conditions, with more clusters in the presence of eltrombopag, from both *ANKRD26*-related thrombocytopenia and *MYH9*-related disease (**Figure 5Ai-iv**). Further, in the presence of eltrombopag, megakaryocytes underwent characteristic cytoplasmic rearrangements typical of proplatelets (**Figure 5Aii,iv**). β1-tubulin staining of megakaryocytes harvested from the device and seeded onto fibronectin-coated coverslips consistently highlighted that thrombopoietin, in combination with eltrombopag, supported the extension of multiple branched shafts resembling nascent platelets at their terminal ends (**Figure**

**5Av-viii**) and a significant increase in the percentage of proplatelet-forming megakaryocytes in both *ANKRD26*-related thrombocytopenia (thrombopoietin: 3±2.6%; thrombopoietin plus eltrombopag: 7.7±4.4%) and *MYH9*-related disease (thrombopoietin: 1.5±1%; thrombopoietin plus eltrombopag: 4.4±4.3%) (**Figure 5B**).

***Ex vivo* platelet count as a predictor of drug efficacy**

Since the desired ultimate effect of eltrombopag in patients with *ANKRD26*-related thrombocytopenia or *MYH9*-related disease is an increase in platelet count, platelet production was the most pertinent parameter evaluated in our *ex vivo* model. We first tested the possibility to harvest and count *ex vivo* produced platelets by perfusing scaffolds cultured with megakaryocytes from healthy controls in the presence of thrombopoietin alone or thrombopoietin in combination with eltrombopag. On day 15 of culture, each channel of the device was connected to a peristaltic pump at the inlet and a gas-permeable collection bag at the outlet. The number of *ex vivo* produced platelets was evaluated and counted with a bead standard by flow cytometry after 4 hours of perfusion, at 37°C and 5% $CO_2$ (**Figure 6A**). The mean absolute number of collected platelets was $24x10^4$/scaffold (range 18-$35x10^4$) in the presence of thrombopoietin, with a significant 1.6-fold increase in the presence of thrombopoietin in combination with eltrombopag ($p<0.01$).

To test whether our device could predict the patient-specific response to eltrombopag, we performed a systematic study comparing the extent of platelet production *ex vivo* to the platelet response observed *in vivo* in the same patients[19,22] Samples were perfused following the conditions previously standardized by using healthy controls. After perfusion, e*x vivo* collected platelets exhibited the β1-tubulin coil at their periphery, typically present in peripheral blood platelets (**Figure 6B**), further supporting the physiological relevance of the reproduced bone marrow environment for replicating *in vivo* thrombopoiesis. *Ex vivo* collected platelets were double-stained with anti-CD41 and anti-CD42b antibodies and counted by flow cytometry (**Figure 6C**). The number of $CD41^+CD42b^+$ platelets collected per single channel was globally increased under the treatment with thrombopoietin in combination with eltrombopag with respect to thrombopoietin alone, in both *ANKRD26*-related thrombocytopenia and *MYH9*-related disease groups (**Table 2**). However, while all samples from healthy

controls responded to the treatment with eltrombopag, in patients the platelet response was variable, with some samples demonstrating a slight or no increase in *ex vivo* platelet production. The same variability was also shown during the treatment *in vivo.*[19,22] Indeed, when the increase in platelet count obtained *ex vivo* in response to eltrombopag was compared with the increase in platelet count observed *in vivo* after eltrombopag administration in the same patients (**Table 2**), there was a statistically significant correlation (R square = 0.78; $p<0.0001$) (**Figure 6D**). The scientific relevance of this correlation was supported by the evidence that the interpolation of the platelet count obtained *in vivo* after eltrombopag administration with the megakaryocyte output calculated *ex vivo* did not gain a significant correlation (R square = 0.35; $p>0.01$) (**Figure 6E**), thus suggesting that *ex vivo* platelet count is the candidate parameter that is likely to predict better the patients' response.

## DISCUSSION

Allogeneic platelet transfusions are widely used to treat acute bleeding in patients with thrombocytopenia of any origin and are also used to prevent bleeding in subjects who developed short-lasting, severe thrombocytopenia after chemotherapy or in those patients with chronic thrombocytopenia in need of a invasive procedure. However, platelet concentrates are not indicated for the prevention of hemorrhages in chronically thrombocytopenic patients for many reasons: they lose efficacy due to possible allo-immunization, acute reactions may occur, and transmission of infectious diseases is possible. Thus, platelet transfusions are not chronically administered to patients with inherited thrombocytopenia unless their platelet count is extremely low and their risk of bleeding is relatively high. Clearly, there is a need for alternative agents or approaches that could increase platelet count in these and other chronic thrombocytopenic conditions.

Thrombopoietin-receptor agonists stimulate megakaryopoiesis and platelet production. Eltrombopag and/or romiplostim and/or avatrombopag are currently approved for the treatment of primary immune thrombocytopenia at various stages in adults and children,[89] thrombocytopenia related to liver disease as long as a procedure is needed, and severe acquired aplastic anemia.[90] Small clinical trials and case reports have suggested that thrombopoietin-receptor agonists are also effective in increasing platelet counts in patients with certain forms of inherited thrombocytopenia,[41] and that at least eltrombopag could be used to replace platelet transfusions to prepare patients for hemostatic challenges.[19] Indeed, a few patients have successfully received long-term treatment with thrombopoietin-receptor agonists, potentially paving the way for chronic treatment of these previously untreated forms of thrombocytopenia. However, platelet response to these drugs was variable among different patients, and sometimes the drugs were ineffective.[9,18,19,22]

Here we have developed a miniaturized 3D bone marrow tissue model that *ex vivo* reproduces *in vivo* platelet biogenesis in such a way that it allows us to predict the response to drugs on a single patient basis. The major advantages of this device over the existing bone marrow models include the rapid customization and manufacture, easy handling, and the implementation of small silk-based three-dimensional scaffolds to allow the seeding of small amounts of adult megakaryocyte progenitors that are cultured for several days, perfused in

parallel and simultaneously, to compare platelet production under different treatments. As proof of principle, we applied our system to study thrombocytopenic patients affected by *ANKRD26*-related thrombocytopenia and *MYH9*-related disease who were treated with the thrombopoietin-receptor agonist eltrombopag.[19,22] According to our clinical data, the extent of platelet response to eltrombopag in patients with *ANKRD26*-related thrombocytopenia was globally lower than that in *MYH9*-realted disease[19,22] This range of variability was reflected *ex vivo*, clearly demonstrating that our tissue model can efficiently predict both the positive and negative response to eltrombopag in individual patients and allow more personalized patient treatment, reducing the number of non-responders unnecessarily exposed to potential side effects of the treatment and ineffective preparation for procedures. In the future, patients might be able to create their own platelets and thus avoid most if not all of the complications discussed above.

The study has several limitations. First, only patients affected by *ANKRD26*-related thrombocytopenia and *MYH9*-related disease were investigated; however, they are among the most frequent forms of inherited thrombocytopenia worldwide. For the many other forms of inherited thrombocytopenia, we do not know if our *ex vivo* predictive system will be similarly predictive.

Second, since *in vivo* clinical trials for patients with inherited thrombocytopenia have been so far limited to eltrombopag, we chose not to include either romiplostim or avatrombopag[61,91] in our *ex vivo* testing. Third, the model was not tested to assess the effect of drugs that negatively impact platelet production, such as chemotherapy. Nonetheless, these limitations can readily be overcome by an additional study of the various permutations discussed. Viewed from this perspective, the study reported here provide motivation and rationale for extending the model to allow identification of the impact of various molecules on platelet production for each patient. Furthermore, this *ex vivo* approach may be useful to study drugs not only in diseases characterized by thrombocytopenia but also in those with thrombocytosis.

Inherited thrombocytopenias represent a prototype of thrombocytopenias deriving from defective platelet biogenesis within the bone marrow. For many forms, the mechanisms of defective platelet production remain unknown. Understanding the cause of thrombocytopenia in these diseases could define the most suitable

treatment for each disorder and identify both novel potential targets and either novel drugs or novel uses of existing drugs. Current 2D assays for functional assessment of megakaryocytes do not effectively monitor the final stage of maturation, in particular proplatelet spreading, platelet formation, and platelet release.[92]

By re-creating megakaryocyte maturation from stem cells to platelet release, our miniaturized 3D bone marrow model demonstrated the ability to reproduce these key steps of thrombopoiesis, including alterations observed in in these disorders.

Besides its ability to stimulate megakaryopoiesis, eltrombopag has also been well-demonstrated to promote multilineage haematopoiesis in patients with acquired bone marrow failure syndromes.[90] Although the exact mechanisms of its effects on haematopoietic progenitor cells are not completely clear, Kao *et al*. recently demonstrated a stimulatory effect on stem cell self-renewal - independently of the thrombopoietin-receptor - mediated through iron chelation-dependent molecular reprogramming.[93] Our benchmark tests highlight that, besides platelet release, the 3D tissue model allowed us to track the effect of eltrombopag on both progenitor cells and megakaryocyte functions, promising to provide a more comprehensive approach to study the effect of thrombopoietin-receptor agonists on haematopoietic stem- and progenitor cells.

**CONCLUSIONS**

In conclusion, we developed a proof-of-concept system that in two weeks measures the impact of thrombopoietin-receptor agonists on megakaryopoiesis and platelet production of individual patients starting from a small amount of their peripheral blood (**Figure 7**). This silk-based technology, which can be produced and customized in 2 days, reaches the expectation of cost efficiency, time-saving, convenience, and personalization of modern therapeutic approaches. The data demonstrated that the *ex vivo* system could predict *in vivo* clinical response to eltrombopag. The increase in the number of platelets collected in the *ex vivo* model was comparable to the increase of platelet count *in vivo* upon treatment with eltrombopag.

The broader impact of this work is in the design of tools to mimic the bone marrow *ex vivo* that can uncover mechanisms of impaired platelet production and enable testing of candidate drug treatments on platelet production using patient-derived cells. In the near future, our system may serve as the basis for highly integrated approaches that could generate solutions for the *ex vivo* production of all blood cells for transfusion.

The system may also represent a benchmark for pre-clinical testing of new therapeutic applications for inherited thrombocytopenias or other hematologic diseases, and for testing the effects of potentially toxic agents for the whole haematopoietic niche.

## TABLES AND FIGURES

**Table 1.** Main features of the 24 investigated samples from 20* patients

| | ***ANKRD26*-RT** | ***MYH9*-RD** |
|---|---|---|
| **Total samples, no.** | 12 | 12 |
| M/F | 9/3 | 5/7 |
| Age - mean (range), years | 46<br>[22-67] | 48<br>[26-59] |
| Platelet count - mean (range) $x10^9$/L | 32<br>[9-75] | 29<br>[5-69] |

**Notes.** * For 4 patients, the analysis was performed on two different occasions.

**Abbreviations**: *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; *MYH9*-RD = *MYH9*-related disease.

**Table 2.** Main features of the investigated patients treated with eltrombopag *in vivo* and *ex vivo*.

| | ***ANKRD26*-RT** | ***MYH9*-RD** |
|---|---|---|
| **Patients treated with Eltrombopag *in vivo* and *ex vivo*, no.** | 6[¥¥] | 7[¥] |
| M/F | 6/3 | 2/6 |
| Age - mean (range), years | 47 [22-67] | 45 [31-59] |
| | | |
| Platelet count at baseline *IN VIVO* mean (range), $x10^9$/L | 35 [12-75] | 24 [5-69] |
| Increase of platelet count after Eltrombopag treatment *IN VIVO** - mean (range), $x10^9$/L | 34 [7-64] | 88 [5-231] |
| | | |
| Platelet count *EX VIVO* – TPO mean (range), $x10^4$ | 8.3 [6-13] | 7.8 [5-12] |
| Increase of platelet count *EX VIVO*** – TPO+EPAG mean (range), $x10^4$ | 27 [0-55] | 33.5 [1-104] |

[¥] of whom 1 patient repeated two times *ex vivo*

[¥¥] of whom 3 patients repeated two times *ex vivo*

* Increase of platelet count with Eltrombopag with respect to baseline.

** Increase of platelet count with Eltrombopag with respect to the untreated counterpart.

**Abbreviations**: *ANKRD26*-RT = *ANKRD26*-related thrombocytopenia; *MYH9*-RD = *MYH9*-related disease; TPO = thrombopoietin; EPAG = eltrombopag.

**Figure 1.** Design of the bone marrow mimicking device.

(**A**) To mimic the vascularized bone marrow tissue structure *ex vivo* a double-flow chamber device was designed in 2 parts. The core contains 2 separates flow channels dedicated to the perfusion having inlet and outlet ports for connection to a perfusion system. (**B**,**C**) The dimension of the polydimethylsiloxane (PDMS) mold cover top and (**D**,**E**) the core device is expressed in millimeters.

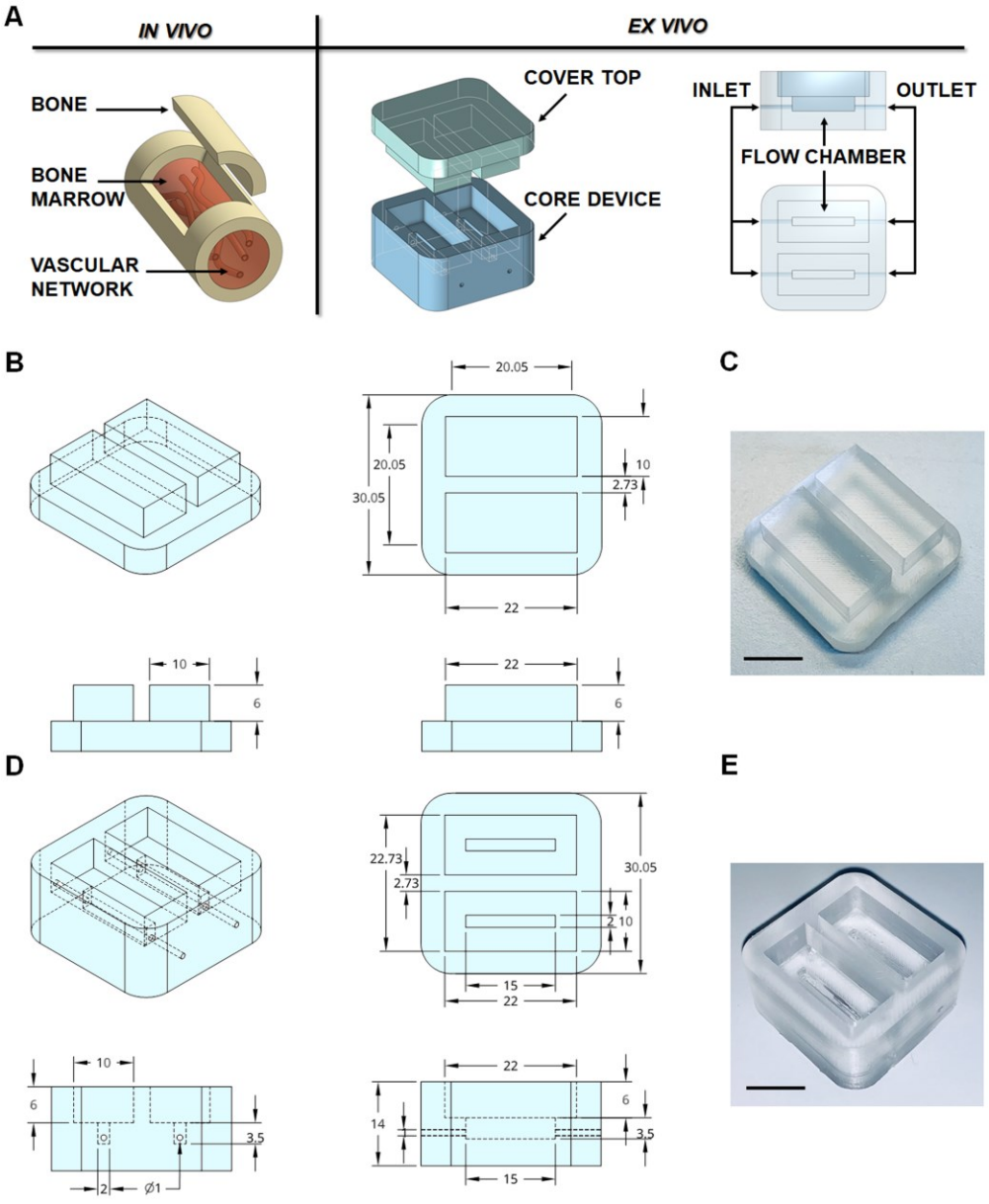


**Figure 2.** Silk sponge bone marrow perfusion system.

(**A-C**) A peristaltic pump drives perfusion of the cell culture medium from a reservoir to the device equipped with a silk fibroin sponge prepared directly inside the chamber by dispensing an aqueous silk solution mixed with salt particles (scale bar B = 1.5 cm; scale bar C = 2 mm). After leaching out the salt, the resulting porous silk sponge can be sterilized. (**D,E**) Confocal microscopy reconstruction of the silk sponge showed the presence of an interconnected alveolar network (scale bar D = 200 µm; scale bar E = 200 µm). (**F**) The analysis of pore diameters measured on the top and bottom of the scaffold demonstrated no significant differences throughout the scaffold. Results are presented as mean±SD (n=150 pore/condition, p=NS). (**G**) Confocal microscopy analysis of $CFSE^{+}$ cells cultured within the silk scaffold (red = CFSE; grey = silk; scale bar = 50 µm).

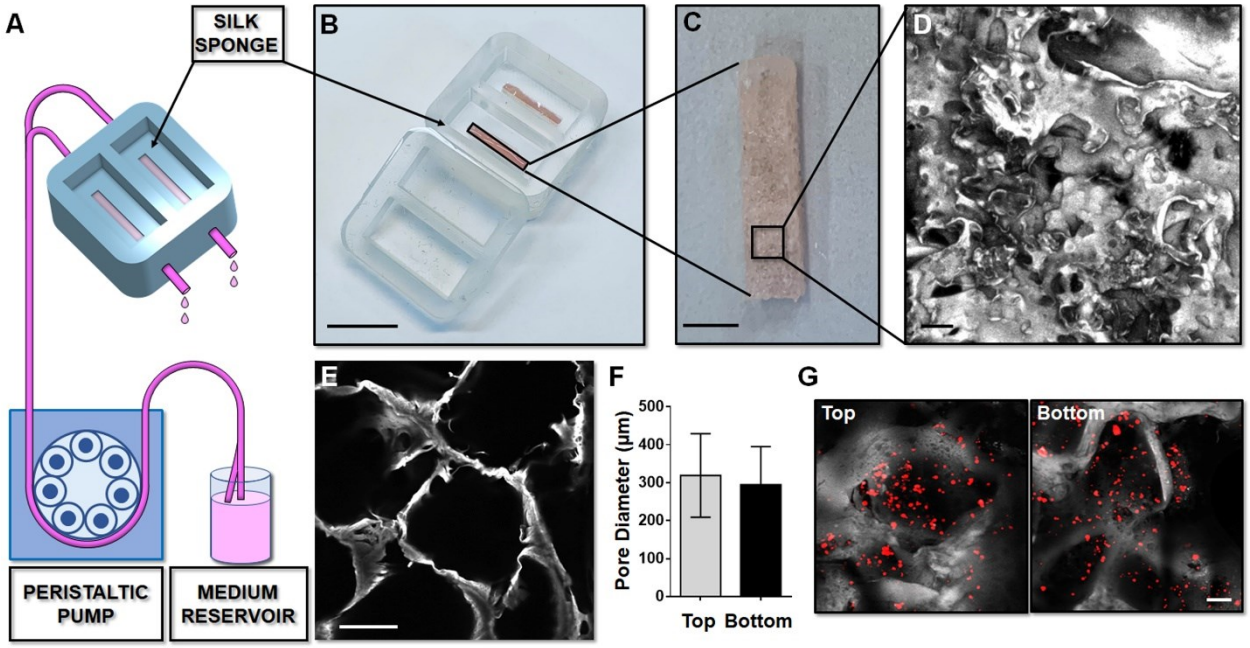


**Figure 3.** Modelling physiological and pathological megakaryopoiesis.

(**A**) Megakaryocytes were differentiated from healthy controls and patients affected by *MYH9*-related disease (*MYH9*-RD) and *ANKRD26*- related thrombocytopenia (*ANKRD26*-RT) patients and cultured into the bone marrow device in presence of 10 ng/mL TPO. (**B**) Output of $CD41^{+}CD42b^{+}$ megakaryocyte at the end of differentiation relative to healthy controls (n=12 Healthy Controls, n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; p=NS) (**C**) Percentage of proplatelet formation relative to healthy controls (n=12 Healthy Controls, n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; *p<0.01). (**D**) The number of proplatelet bifurcation per single megakaryocytes in healthy controls and patients (n=12 Healthy Controls, n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; *p<0.01). (**E**) Representative immunofluorescence staining of proplatelet structure (red=β1-tubulin; blue=nuclei; scale bar=20 µm). All results are presented as mean±SD.

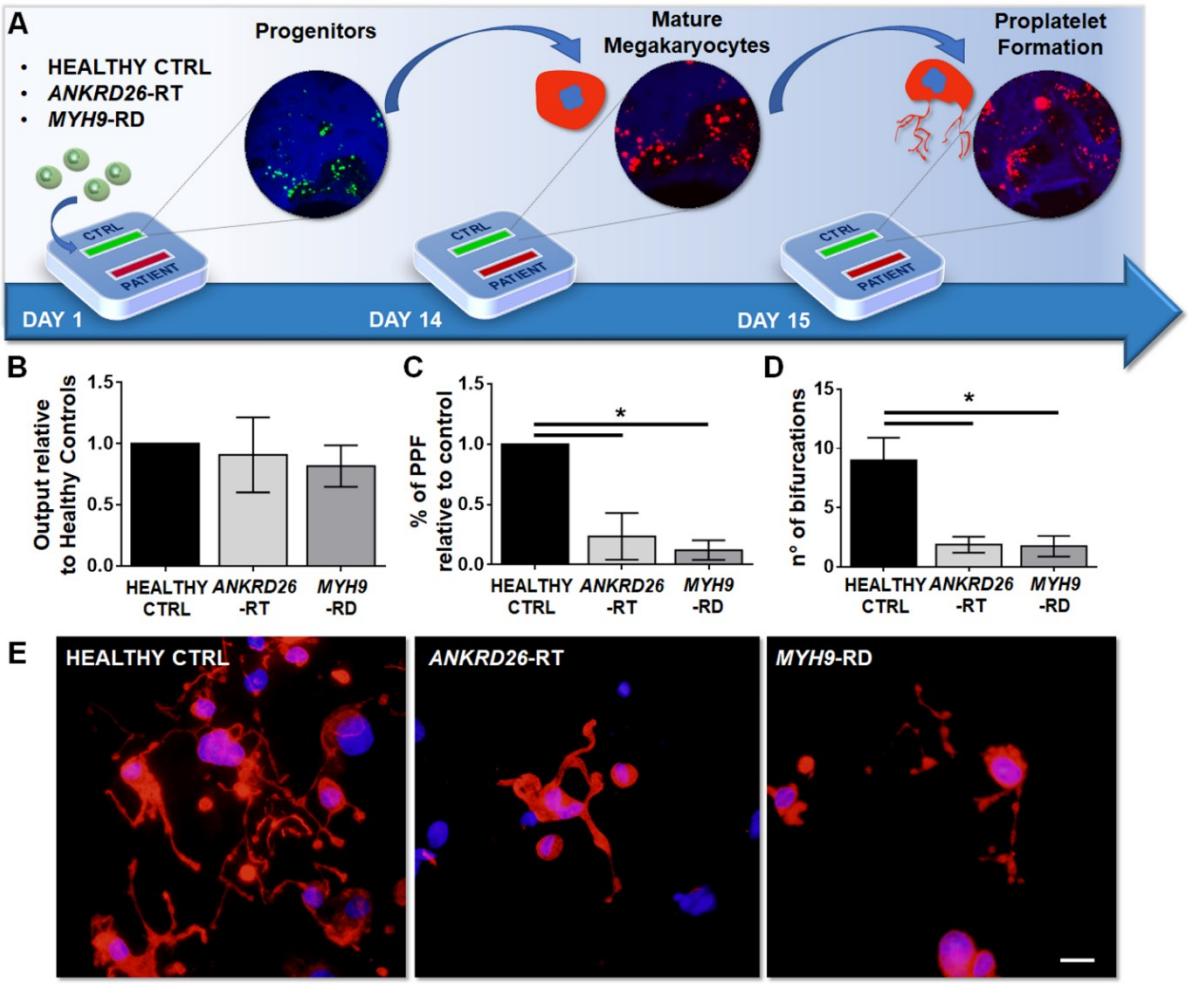


**Figure 4.** Eltrombopag promotes megakaryocyte differentiation *ex vivo*.

(**A**) Megakaryocytes were differentiated from peripheral blood progenitors of patients affected by *MYH9*-related disease (*MYH9*-RD) or *ANKRD26-related thrombocytopenia* (*ANKRD26*-RT), and cultured in the silk bone marrow tissue device in the presence of 10 ng/mL thrombopoietin (TPO) supplemented or not with 500 ng/mL Eltrombopag (EPAG), and analyzed. (**B**) Representative immunofluorescence staining of CD61 (red=CD61; blue=nuclei; scale bar=25 µm) and (**C**) analysis of ploidy levels at the end of the culture (TPO: n=3 *MYH9*-RD; n=3 *ANKRD26*-RT; TPO+EPAG: n=3 *MYH9*-RD; n=3 *ANKRD26*-RT; p=NS). (**D**) Representative flow cytometry analysis of $CD41^{+}CD42b^{+}$ megakaryocytes at the end of the culture and (**E**) statistical analysis of mean fluorescence intensity (MFI) of the markers (TPO: n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; TPO+EPAG: n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; p=NS). (**F**) Output was calculated as the fold increase in the percentage of $CD41^{+}CD42b^{+}$ cells in presence of TPO+EPAG with respect to the percentage of double-positive cells in presence of TPO alone (*ANKRD26*-RT: n=12; *MYH9*-RD: n=12, $p<0.05$). All results are presented as mean±SD.

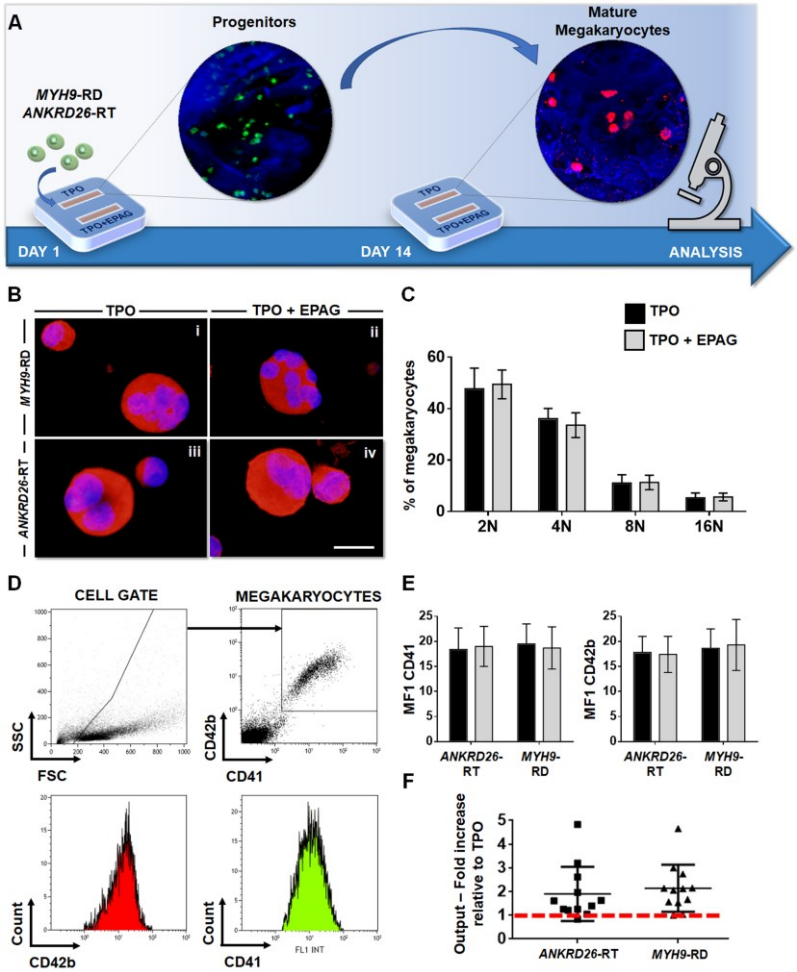


**Figure 5.** Eltrombopag sustains increased proplatelet formation *ex vivo*.

(**A**) Confocal microscopy analysis of 3D megakaryocyte culture imaged at the end of differentiation. Megakaryocytes were elongating proplatelet shafts, which assemble nascent platelets at their terminal ends, within the hollow space of silk pores (red=CD61, blue=silk) (scale bars = 50 µm). (**Av-viii**) Analysis of proplatelet structure was performed by immunofluorescence staining of the megakaryocyte-specific cytoskeleton component β1-tubulin (red=β1-tubulin; blue=nuclei; scale bar=25 µm). In both diseases, the representative pictures show increased elongation and branching of proplatelet shafts in presence of thrombopoietin (TPO) + eltrombopag (EPAG) with respect to TPO alone. (**B**) The percentage of proplatelet-forming megakaryocytes was calculated as the number of cells displaying long filamentous pseudopods with respect to the total number of round megakaryocytes per analyzed field (TPO: n=12 *MYH9*-related disease, *MYH9*-RD; n=12 *ANKRD26*-related thrombocytopenia, *ANKRD26*-RT; TPO+EPAG: n=12 *MYH9*-RD; n=12 *ANKRD26*-RT; **p<0.01; *p<0.05). All results are presented as mean±SD.

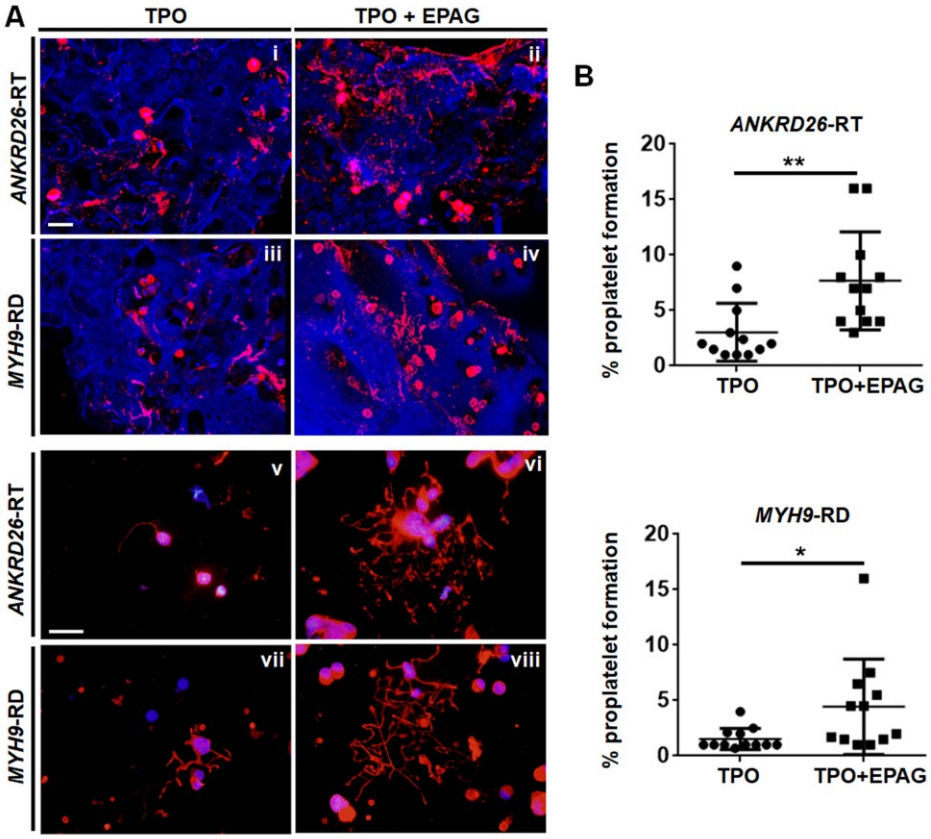


**Figure 6.** *Ex vivo* platelet count for predicting response to treatments.

(**A**) The flow chamber was perfused with culture media and released platelets collected into gas-permeable bags before counting by flow cytometry. (**B**) Light microscopy and immunofluorescent analysis of the collected medium demonstrated the presence of large pre-platelets, dumbbells, and little discoid platelets having the microtubule coil typically present in resting platelets (red=β1-tubulin, scale bars = 10 µm). (**C**) Representative flow cytometry analysis of expression of CD41 and CD42b surface markers. (**D**) Analysis of the correlation between the increase of platelet count analyzed *ex vivo* and the increase of platelet count observed *in vivo* from the same patients. For the *ex vivo* analysis, platelet count was calculated by flow cytometry with counting beads (n=8 *MYH9*-related disease, *MYH9*-RD; n=9 *ANKRD26*-related thrombocytopenia, *ANKRD26*-RT; p<0.0001). (**E**) Analysis of the correlation between *ex vivo* megakaryocyte output and the increase of platelet count observed *in vivo* from the same patients. (n=8 *MYH9*-RD; n=9 *ANKRD26*-RT; p>0.01).

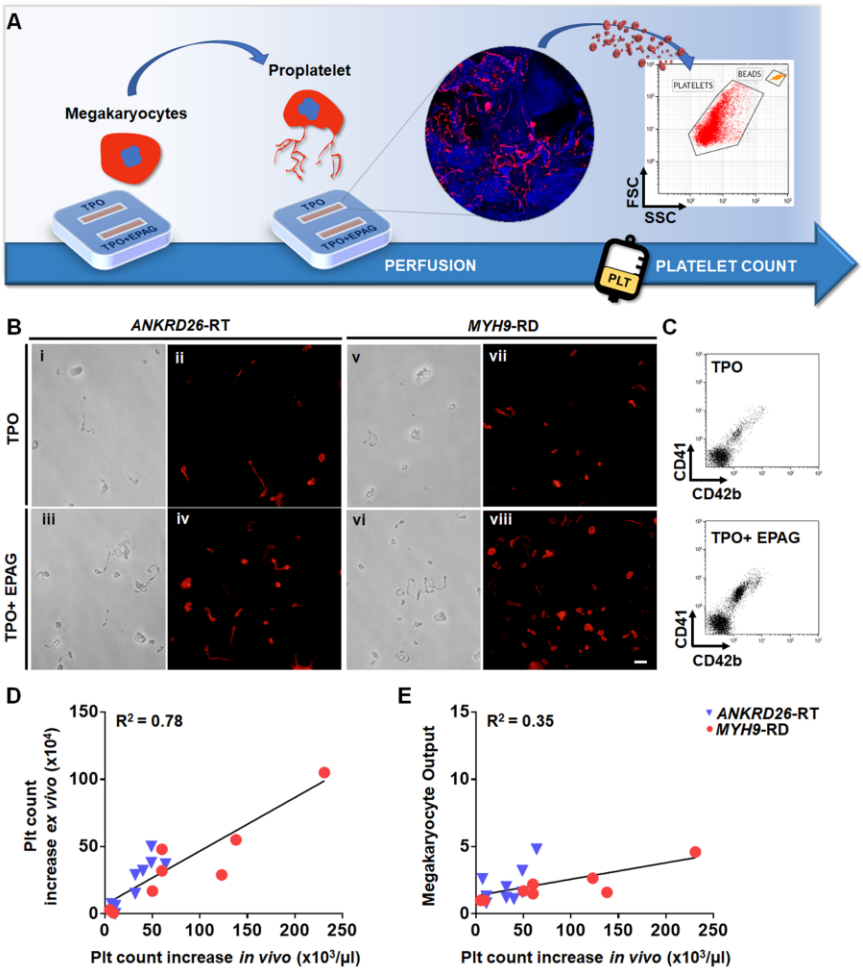
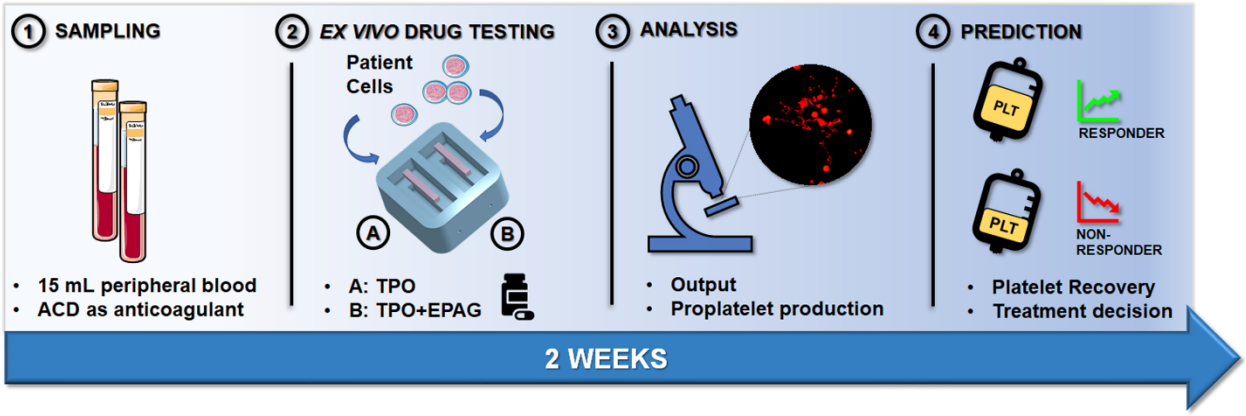


**Figure 7.** Summary of the proposed workflow.

After sampling, the megakaryocytic progenitors are seeded within a 3D bone marrow tissue device and cultured in the presence of the tested drug. After perfusion, platelets are collected and counted in order to assess patient-specific response.



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