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ORIGINAL ARTICLE

Platelet aggregation response in immune thrombocytopenia patients treated with romiplostim

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Abstract

The thrombopoietin receptor agonist romiplostim is used for the long-term treatment of chronic immune thrombocytopenia (ITP). ITP patients have an increased thrombotic risk, which could be exacerbated if romiplostim increased platelet hyperreactivity or caused spontaneous platelet aggregation. To investigate this possibility, this study examined platelet function in romiplostim-treated ITP patients and healthy subjects. Light transmission platelet aggregometry utilizing arachidonic acid, collagen, epinephrine, ristocetin, ADP, and saline (to assess spontaneous aggregation) was performed for each subject. In addition, the ADP AC_{50} (ADP concentration that induced half-maximal aggregation) was determined for each patient as a sensitive measurement of altered platelet reactivity. Fifteen ITP patients and 7 healthy subjects entered the study. All ITP patients had active disease and were receiving weekly romiplostim as the sole ITP-directed therapy. Platelet aggregation in response to the strong agonists arachidonic acid, collagen, and ristocetin was not significantly different between ITP patients and healthy subjects ($P = 0.2442$, $P = 0.0548$, and $P = 0.0879$, respectively). Platelet aggregation in response to weak agonists was significantly reduced in ITP patients compared with that in healthy subjects: median (range) aggregation to ADP, 45% (15–84%) versus 89% (70–95%) ($P = 0.0010$), and epinephrine, 21% (1.6–90%) versus 88% (79–94%) ($P = 0.0085$). The median AC_{50} of ADP was threefold higher in ITP patients versus that in healthy subjects (6.3 μM vs 2.1 μM) ($P = 0.0049$). Significant spontaneous aggregation was not observed in any patient. Platelets from romiplostim-treated ITP patients do not show evidence for spontaneous aggregation or hyperreactivity, but instead have a modestly reduced aggregation response to ADP and epinephrine.

Keywords Platelet aggregation · Immune thrombocytopenia · ITP · Romiplostim · Thrombosis · Thrombopoietin receptor agonist · Platelet function testing

Introduction

The thrombopoietin (TPO) receptor agonists are a class of platelet growth factors that have demonstrated effectiveness in the management of immune thrombocytopenia (ITP) [1, 2], severe aplastic anemia [3], thrombocytopenia of chronic liver disease [4, 5], and chronic hepatitis C–associated thrombocytopenia [6], with promise for additional indications [7, 8]. Romiplostim, the only peptide TPO receptor agonist and the most potent [9, 10], is a fusion protein comprised of four TPO receptor–activating peptides conjugated to the human IgG1 heavy chain region [11]. Romiplostim binds to the extracellular domain of the TPO receptor, increasing megakaryocyte growth, differentiation, and platelet production. Romiplostim is currently FDA-approved only for the treatment of chronic immune thrombocytopenia in adults.

Given that platelets express the TPO receptor [12], there has been concern that TPO receptor agonists could be thrombogenic. Initial studies demonstrated that exposure of canine platelets to pharmacologic doses of recombinant human thrombopoietin (rhTPO) did not affect the thrombin responsiveness of platelets [13] and exposure of baboon and human platelets to pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF, a recombinant TPO) did not cause spontaneous platelet aggregation [14, 15]. But subsequent studies showed that healthy

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baboon and human platelets had increased reactivity to agonists such as ADP when exposed to PEG-rHuMGDF ex vivo or when harvested from baboons within the first week of treatment with PEG-rHuMGDF [14, 15].

After development of recombinant human thrombopoietins was abandoned outside of China due to concerns for neutralizing antibody formation, emergence of the second-generation TPO receptor agonists rekindled concern of increased thrombotic risk with these agents due to the possibility of enhanced platelet reactivity. This is of particular concern in treating ITP patients who have an increased risk of both venous and arterial thromboses at baseline [16–18]. While the underlying reason for the enhanced thrombosis rate in ITP patients is unclear, some have proposed that it may be related to increased platelet turnover, increased platelet microparticles [19], or increased baseline platelet function [20], phenomena also thought to contribute to the low bleeding rates in many ITP patients despite severe thrombocytopenia [21]. To date, studies of romiplostim in ITP patients have not demonstrated a significantly increased thrombotic risk when compared with other treatments [1, 2, 22, 23], but two large randomized studies did demonstrate increased rates of venous thromboembolism in chronic liver disease patients treated with eltrombopag [6, 24]. Therefore, the thrombotic risk of TPO receptor agonists in ITP patients receiving these agents remains a concern as there is limited evidence describing platelet function in this population. In particular, no studies have been published assessing the possible impact, if any, of romiplostim treatment on platelet aggregation response to better define a potential heightened thrombotic risk.

This study sought to determine if enhanced platelet production by romiplostim might alter platelet function as assessed by light transmission platelet aggregometry (LTA). Platelets from healthy untreated subjects and ITP patients treated with romiplostim were assessed for spontaneous aggregation as well as their response to standard platelet agonists (arachidonic acid, collagen, epinephrine, adenosine diphosphate (ADP), and ristocetin).

Materials and methods

Patients

Fifteen patients with ITP (diagnosed using criteria from the American Society of Hematology [25]) who received romiplostim (NPlate®, Amgen) including 7 patients with ITP who had participated in a prior phase II dose-finding trial of romiplostim for ITP (Evaluating the Safety and Efficacy of AMG531 in Thrombocytopenic Subjects with Immune Thrombocytopenic Purpura, NCT00111475) and 8 patients with ITP who were treated with commercially available romiplostim along with 7 healthy subjects were recruited to participate in the study. All patients provided written, informed consent for participation in the study before any study procedures were conducted. All procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2008. The Massachusetts General Hospital/Partners Healthcare Institutional Review Board Human Studies Committee provided formal study review and approval (protocol 2007P000590/PHS). All patients and healthy subjects were enrolled at the Massachusetts General Hospital. For ITP patients, the single 20-mL blood sample required for the study was obtained during a routine blood draw performed as part of standard care.

Blood samples and preparation of platelet-rich plasma

Blood samples for platelet aggregation studies were drawn by antecubital venipuncture using a 21-gauge butterfly needle into 2.7-mL buffered sodium citrate vacutainers to produce citrated whole blood samples, 1 part 3.2% (0.108 mol/L) sodium citrate to 9 parts whole blood. For ITP patients, test sample was drawn on the day of romiplostim administration immediately prior to subcutaneous injection of romiplostim (and 7 days from the last romiplostim administration).

To obtain platelet-rich plasma (PRP, platelet count 150–400 × 10^9/L), specimens were centrifuged (Jouan C412 centrifuge) at 150*g* for 5 min. Platelet counts were then performed on PRP samples; any samples > 325 × 10^9/L underwent dilution with platelet poor plasma (obtained via centrifugation of remaining blood sample at 1500*g* for 10 min) to obtain a final platelet count of 300 × 10^9/L ± 25 × 10^9/L.

Platelet aggregation studies

All samples were subjected to standard platelet aggregation studies with arachidonic acid (500 μg/mL), collagen (10 μg/mL), epinephrine (10 μM), ADP (5 μM), and ristocetin (1500 μg/mL). Spontaneous aggregation was studied with the addition of normal saline. In addition, the AC_{50} (agonist concentration that induced half-maximal aggregation) of ADP was determined for each patient as a sensitive indicator of altered platelet reactivity [15]. To determine the ADP AC_{50}, ADP concentrations of 10, 5, 2.5, 1.25, and 0.63 μM were tested with each patient sample.

The platelet aggregation studies were performed on an LTA aggregometer (Helena Laboratories, Beaumont, TX) using 450-μL pre-warmed platelet-rich plasma and 50-μL agonist with a stir bar. Each tracing was recorded for 10 min. Maximum aggregation was recorded by the aggregometer as percent aggregation. Agonists were from Helena Laboratories (arachidonic acid, collagen, ADP, ristocetin) and Biodata Corporation (epinephrine; Horsham, PA).

Statistical analysis

Differences in aggregation responses for each agonist between healthy subjects and ITP patients were assessed using the Wilcoxon rank-sum test. All statistical analyses and figure preparation were performed using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA) and GraphPad Prism 7 (GraphPad, Inc., San Diego, CA).

Data sharing statement

For original data, please contact hal-samkari@mgh.harvard.edu.

Results

Patient characteristics

Baseline characteristics of ITP patients at time of platelet aggregometry are detailed in Table 1. Twelve patients had chronic ITP (> 1 year) and 3 had persistent ITP [26]. The median age was 54 (range 19–73) years and 60% of patients were female. Weekly romiplostim was the sole ITP-directed therapy for all patients, and all patients were responders to romiplostim treatment. No patient had entered remission. The median romiplostim treatment duration was 41 (range 5–236) weeks, and the median dose was 4 (range 1–10) μg/kg. The median platelet count was 223×10^9/L (range $80–377 \times 10^9$/L). The median number of prior ITP treatments was 3 (range 1–7) and 27% of patients were previously splenectomized. No patients were on any medications known to interfere with platelet function.

The median age of healthy subjects was 39 (range 25–50) years and 71% were female. All healthy subjects had a normal platelet count and were taking no medications at the time of the study.

Light transmission platelet aggregometry response in ITP patients and healthy subjects

Figure 1 illustrates results of platelet aggregation testing for healthy subjects and ITP patients (numerical results of all platelet aggregation testing for each subject can be found in the Supplemental Table). Table 2 compares median aggregation response to each agonist for healthy subjects and ITP patients.

Arachidonic acid Aggregation in response to arachidonic acid was similar between healthy subjects (median 91%, range 77–95%) and ITP patients (median 88%, range 12–95%) ($P = 0.2442$). One ITP patient (patient 13) had a significantly reduced response to arachidonic acid (12% aggregation).

Table 1 Baseline characteristics of ITP patients at time of study. *IVIG*, intravenous immunoglobulin

Patient number	Age	Gender	ITP duration (years)	Platelet count ($\times 10^9$/L)	Romiplostim dose (μg/kg)	Romiplostim duration (weeks)	Prior ITP therapies
1	49	F	12.1	377	2	107	Glucocorticoids, IVIG, rituximab, azathioprine, splenectomy
2	22	F	1.2	226	3	23	Glucocorticoids, IVIG, rituximab, anti-D immune globulin
3	48	M	5.7	128	2	19	Glucocorticoids, danazol, romiplostim
4	56	F	17.9	299	5	152	Glucocorticoids, IVIG, danazol, splenectomy
5	73	M	0.8	175	3	11	Glucocorticoids
6	39	M	1.9	223	7	80	Glucocorticoids, anti-D immune globulin
7	54	F	5.8	221	3	91	Glucocorticoids, IVIG, rituximab
8	62	M	0.4	348	5	14	Glucocorticoids, IVIG
9	65	F	1.4	80	7	66	Glucocorticoids
10	47	F	2.5	239	4	35	Glucocorticoids, IVIG, azathioprine, eltrombopag, splenectomy
11	68	M	3.7	182	10	41	Glucocorticoids
12	37	F	7.0	350	3	236	Glucocorticoids, IVIG, splenectomy
13	66	F	4.1	311	1	52	Glucocorticoids, IVIG, rituximab, anti-D immune globulin, azathioprine, eltrombopag, avatrombopag
14	19	M	0.6	184	6	10	Glucocorticoids, IVIG
15	65	F	23.5	114	5	5	Glucocorticoids

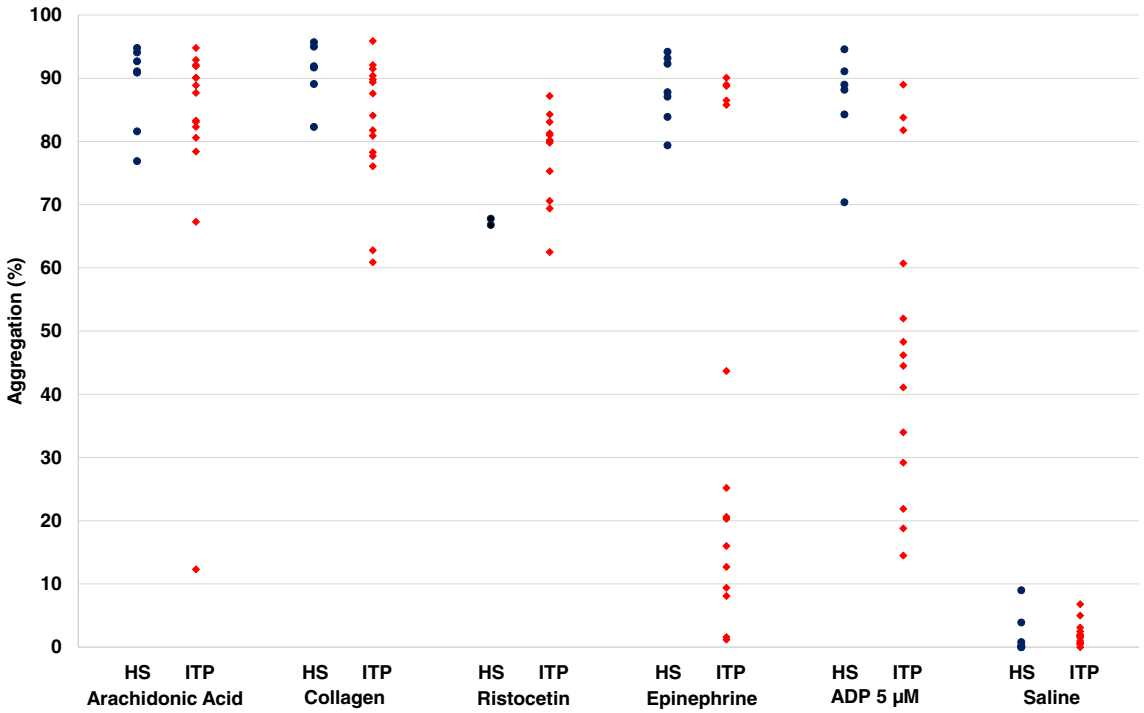

Fig. 1 Light transmission platelet aggregometry results. Results of LTA for healthy subjects (HS, blue) and ITP patients (red) for each agonist. Saline testing was performed as a measure of spontaneous aggregation

Collagen Aggregation in response to collagen was similar between healthy subjects (median 92%, range 82–96%) and ITP patients (median 84%, range 61–96%) ($P = 0.0548$). Two ITP patients (patients 9 and 13) had a moderately reduced response to collagen (61% and 63% aggregation, respectively).

Ristocetin Aggregation in response to ristocetin was similar between healthy subjects (median 67%, range 67–68%) and ITP patients (median 80%, range 71–87%) ($P = 0.0879$), although aggregation testing with ristocetin was performed in only two healthy subjects (due to insufficient specimen quantities in many of the healthy subjects). The normal reference range for ristocetin aggregation response in our special coagulation laboratory is > 68%, consistent with what was seen in our healthy subjects and similar to responses in the ITP patients. All ITP patients had normal aggregation responses to ristocetin.

Saline (spontaneous aggregation) No significant spontaneous aggregation was observed in any of the healthy subjects (median 0.5%, range 0.0–9.0%) or ITP patients (median 1.8%, range 0.0–6.8%), with no significant difference between the two groups ($P = 0.4317$).

Epinephrine Aggregation in response to epinephrine was significantly reduced in ITP patients (median 21%, range 1.6–90%) compared with that in healthy subjects (median 88%, range 79–94%) ($P = 0.0085$). Aggregation in response to epinephrine was nearly absent or greatly reduced in 67% of ITP patients (Supplemental Table).

ADP 5 μM Aggregation in response to standard concentrations of ADP was significantly reduced in ITP patients (median 45%, range 15–84%) as compared with that in healthy subjects (median 89%, range 70–95%) ($P = 0.0010$). Aggregation

Table 2 Comparison of platelet aggregation response for each agonist and ADP AC_{50} between healthy subjects and ITP patients

Measure	Healthy subjects, median (range)	ITP patients, median (range)	*P* value
Arachidonic acid, % aggregation	91.1 (76.9–94.8)	87.7 (12.4–94.8)	0.2442
Collagen, % aggregation	91.8 (82.3–95.7)	84.1 (60.9–95.9)	0.0548
Ristocetin, % aggregation	67.3 (66.8–68.8)	80.2 (70.6–87.2)	0.0879
Epinephrine, % aggregation	87.8 (78.9–94.2)	20.6 (1.6–90.1)	0.0085
ADP, 5 μM, % aggregation	88.6 (70.4–94.6)	45.4 (14.5–83.8)	0.0010
Saline, % aggregation	0.5 (0.0–9.0)	1.8 (0.0–6.8)	0.4317
ADP AC_{50} (μM)	2.1 (1.6–4.2)	6.3 (1.7–25.8)	0.0049

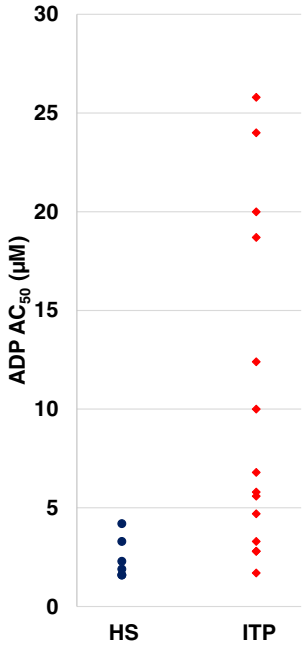

Fig. 2 ADP AC_{50} measurements. AC_{50} (agonist concentration that induced half-maximal aggregation) of ADP for healthy subjects (HS, blue) and ITP patients (red)

in response to 5 μM ADP was moderately or severely reduced in 73% of ITP patients (Supplemental Table).

ADP titration and ADP AC_{50} determination Individual subject results for ADP AC_{50} are illustrated in Fig. 2 and listed in the Supplemental Table (which also contains individual subject results for ADP titration). The median ADP AC_{50} was three-fold higher in ITP patients (6.3, range 1.7–25.8) as compared with that in healthy subjects (2.1, range 1.6–4.2), a significant difference ($P = 0.0049$). Figure 3 illustrates the reduction in aggregation response to ADP over the course of ADP titration of ITP patients as compared with healthy subjects.

Discussion

Platelet function in ITP patients is clinically relevant both in consideration of bleeding risk during profound thrombocytopenic episodes as well as thrombotic risk during therapy (and treatment with thrombopoietin receptor agonists in particular). Platelets in ITP patients may have decreased function secondary to inhibition from antiplatelet antibodies [27, 28]. In contrast, platelets produced by recombinant forms of TPO in healthy non-human primates have increased function [14, 15]. Considering this, the description of platelet function in ITP patients receiving TPO receptor agonists has never been more relevant given the increasing numbers of ITP patients receiving these agents for extended time periods. The goal of this study was to assess the function of platelets produced under romiplostim stimulation in ITP patients. We found no evidence for spontaneous platelet aggregation or hyperreactivity, instead observing normal responses to "strong" agonists (arachidonic acid, collagen, and ristocetin) not

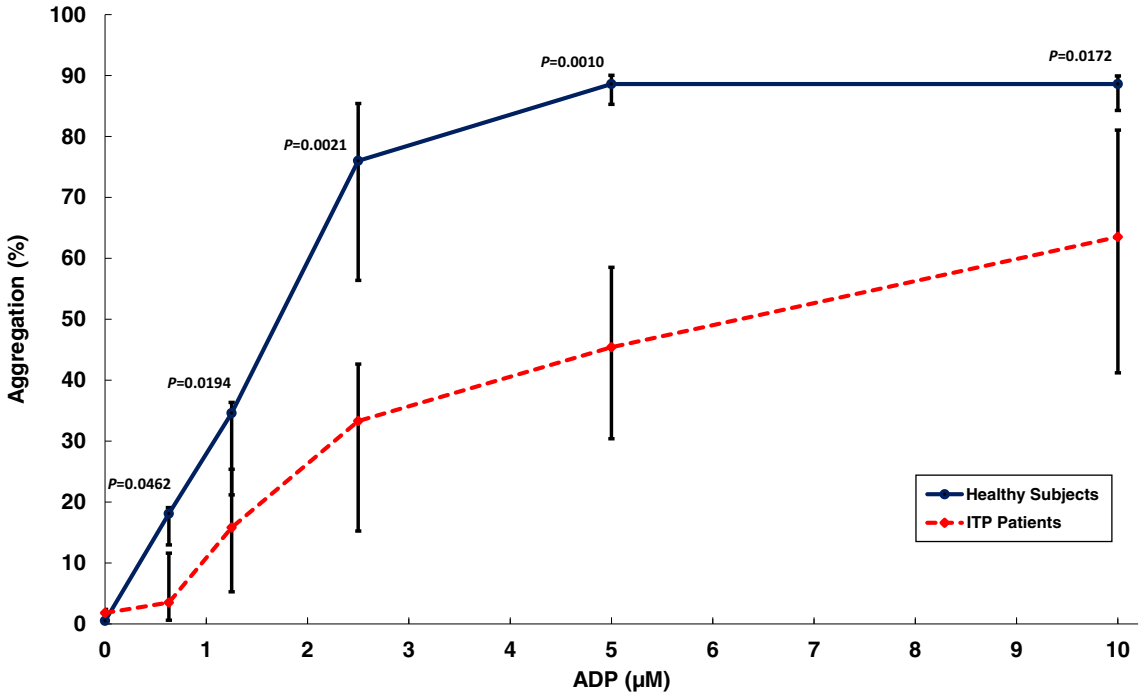

Fig. 3 Results of ADP titration. Median (± interquartile range) percent aggregation plotted for each concentration of ADP for healthy subjects (blue, solid line) and ITP patients (red, dotted line). Results of the Wilcoxon rank-sum testing (*P* values) comparing results of healthy subjects and ITP patients for each concentration are shown

significantly different from those seen in healthy controls and reduced responses to "weak" agonists (ADP and epinephrine). To our knowledge, this is the only study to assess platelet function using LTA in ITP patients receiving the TPO receptor agonist romiplostim.

Light transmission platelet aggregometry, despite lack of standardization and risk of considerable pre-analytic and analytic variability, continues to be the gold standard for assessment of platelet function [29]. Performance of standard LTA is usually not reliable at platelet counts < 100×10^9/L, making it difficult to assess platelet function in patients with active ITP. In this study, platelet counts were increased pharmacologically with romiplostim, thereby allowing for LTA to be performed using techniques in accordance with the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [29]. This is in contrast to prior studies in ITP patients, which did testing in patients in remission [30], utilized indirect techniques (adding sera from ITP patients to donor platelets) [31], or proceeded with platelet aggregometry despite low platelet counts [28, 32] (potentially compromising validity of results [29]). Each of these studies found significant impairment of ADP-induced aggregation, and the two that tested aggregation response to epinephrine [28, 32] found impaired responses to it as well, all in agreement with our findings. We additionally performed LTA at multiple concentrations of ADP to determine the ADP AC_{50} as a sensitive indicator of platelet reactivity. The ADP AC_{50} was significantly higher in patients with ITP than in healthy subjects (median of 6.3 μM vs 2.1 μM, Figs. 2 and 3), confirming a moderate reduction in platelet reactivity of ITP patients versus healthy subjects. Of note, two of the previously described studies (the two utilizing LTA on thrombocytopenic samples) found enhanced platelet aggregation responses to certain agonists in a minority of patients [28, 31]. None of the patients in our study displayed an enhanced platelet aggregation response or spontaneous aggregation to any agonist.

The few prior studies examining platelet aggregation response using LTA in the setting of thrombopoietic agents have all assessed healthy (non-ITP) platelets with mixed findings. Platelets obtained from baboons after treatment with PEG-rHuMGDF demonstrated increased ex vivo platelet aggregation response (measured by LTA) to thrombin receptor–activating peptide (TRAP) and collagen as assessed by a significantly reduced AC_{50} to both agonists [15]. This study also showed that platelets obtained from healthy baboons and humans had an enhanced aggregatory response to ADP when incubated with PEG-rHuMGDF. Another study utilized LTA to measure aggregation response of platelets from healthy human volunteers following addition of either rhTPO or eltrombopag [33]. In this study, eltrombopag did not enhance platelet aggregation at subthreshold concentrations of ADP or collagen, but rhTPO acted synergistically with subthreshold concentrations of ADP and collagen to induce maximal aggregation in response to either agonist.

Other studies examining the possible effect of thrombopoietic agents on platelet function utilized flow cytometric methods to assess for platelet activation. One such study utilized flow cytometric measurement of P-selectin expression to assess platelet activation in rhTPO- and interleukin-6 (IL-6)-treated dogs. This study found increased platelet responsiveness to thrombin after IL-6 treatment (as assessed by a significantly reduced thrombin AC_{50}) but not after TPO treatment (no significant change in thrombin AC_{50}). Two human studies have examined the effect of eltrombopag on healthy volunteers [34] (finding no increased platelet reactivity) and ITP patients [35]. The latter study examined surface expression of activated glycoprotein IIb/IIIa, P-selectin, and glycoprotein Ib in the setting of various concentrations of ADP and TRAP in ITP patients treated with eltrombopag and found that eltrombopag did not cause spontaneous platelet activation or hyperreactivity [35]. Another study utilizing flow cytometry to analyze platelet function included 5 pediatric ITP patients receiving romiplostim, which demonstrated a reduction in baseline platelet activation with romiplostim treatment, as measured by reduced levels of pre-activated integrins and P-selectin externalization. There was no comparison of platelet function in these patients to healthy subjects, and the authors did not comment on spontaneous platelet activation or hyperreactivity [36].

Taken together with the results of these prior studies, our study suggests that romiplostim treatment most likely does not have a discernable impact on platelet aggregation response in patients with ITP. The reduction in aggregation responses in ITP patients thought to be due to antiplatelet antibodies does not appear to be reversed by romiplostim treatment. Even if romiplostim was to induce an enhanced platelet aggregatory response as was seen in the non-ITP studies of rhTPO [33] or PEG-rHuMGDF [15], the inhibitory effect of ITP itself on the aggregation response appears to predominate. Finally, it is possible that romiplostim itself may have an inhibitory effect on platelet aggregation response, which cannot be separated from any effect of antiplatelet antibodies and therefore cannot be excluded. Given that romiplostim has a median elimination half-life of 3.5 days (with a range of 1–34 days) [37], it is expected that all patients in our study had drug present in the circulation. While no studies of romiplostim have been published providing evidence for such an inhibitory effect, a study of eltrombopag that examined this question found no inhibitory effect of the drug on aggregation in response to either ADP or collagen [33].

A limitation in our study is a lack of direct antiplatelet antibody testing in the ITP patients. Identification of such antibodies in patients with diminished aggregation responses (and lack of antibodies in those with preserved responses) could have provided additional evidence as to the specific etiology of the diminished aggregation response. In prior studies, anti-GPIIb/IIIa antibodies have been associated with diminished aggregation response to ADP [28, 31] and anti-GPIb antibodies have been associated with diminished aggregation response to ristocetin [28]. While such correlations in our study cannot be made, the principle findings of the study are not impacted by this in any significant way. Another limitation is the obvious inability to perform LTA on the ITP patients prior to elevating their platelet counts with romiplostim. If possible, this would have allowed separate assessments of the effect of ITP alone and the effect of romiplostim and ITP on the platelet aggregation response.

In conclusion, platelets from romiplostim-treated ITP patients do not show evidence for spontaneous aggregation or increased aggregation responses to strong or weak agonists. Instead, the results of this study suggest that platelets from romiplostim-treated ITP patients have a modestly reduced aggregation response, consistent with the existing literature of platelet function of ITP patients not treated with romiplostim and presumably due to the presence of antiplatelet antibodies. These findings are consistent with the results of large trials of romiplostim in ITP patients, which did not demonstrate an increased risk of thrombotic events [1, 2, 22, 23].

Author contributions H. Al-Samkari analyzed data, created tables and figures, and wrote and revised the manuscript; E. Van Cott analyzed data, wrote a portion of the "Materials and methods" section of the manuscript, and critically revised the manuscript; D. Kuter designed the study, collected and analyzed data, created tables and figures, critically revised the manuscript, and supervised the study.

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Compliance with ethical standards

Conflict of interest H. Al-Samkari has a consultancy with Agios. E. Van Cott declares she has no conflict of interest. D. Kuter has received research funding from the following: Protalex, Bristol-Myers Squibb, Rigel, Bioverativ, Agios, Syntimmune, Principia, Alnylam; consultancy with the following: ONO, Pfizer, 3SBios, Eisai, GlaxoSmithKline, Genzyme, Shire, Amgen, Shionogi, Rigel, Syntimmune, MedImmune, Novartis, Bioverativ, Argenx, Zafgen; and does paid expert testimony for Amgen and CRICO.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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