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Review

Hemostatic changes by thrombopoietin-receptor agonists in immune thrombocytopenia patients

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ABSTRACT

Thrombopoietin receptor agonist (TPO-RA) treatment increases the thrombosis rate in immune thrombocytopenia (ITP). We hypothesize that TPO-RAs influence platelet function, global and secondary hemostasis and/or fibrinolysis. A systematic review was performed. If possible, data were compared between responders (relevant increase in platelet count), and non-responders. Twelve observational studies with 305 patients were included (responders (127/150 (85%))). There were indications that TPO-RA treatment enhanced platelet function, with respect to platelet-monocyte aggregates, soluble P-selectin, GPVI expression, and adhesion under flow. Studies addressing global and secondary hemostasis and fibrinolysis were scarce. Overall, no changes were found during TPO-RA treatment, apart from an accelerated clot formation and conflicting data on levels of plasminogen activator inhibitor (PAI)-1. The parameters that increased have previously been associated with thrombosis in other patient groups, and might contribute to the increased rate of thrombosis observed in TPO-RA-treated ITP patients.

1. Introduction

Immune thrombocytopenia (ITP) is a condition characterized by auto-antibodies causing both peripheral platelet destruction and decreased platelet production by megakaryocytes [1]. Most available treatments target the auto-antibodies by inhibiting the immune system. However, over the past decade, thrombopoietin-receptor agonists (TPO-RAs) have become a major player in the treatment of ITP. Rather than targeting the immune system, these agents increase platelet production by mimicking thrombopoietin (TPO), a glycoprotein that stimulates the megakaryocyte by binding to the thrombopoietin receptor, c-MPL [2].

The two best-known commercially available TPO-RAs are the second-generation agonists eltrombopag and romiplostim (formerly known as AMG531) [2]. A response in platelet count is seen in 50–90% of patients treated with these drugs, and sustained increases in platelet count can be achieved [3]. TPO-RAs are generally effective and well-tolerated, but the occurrence of thrombotic embolic events (TEE) has become a concern in clinical practice. ITP itself is considered a thrombogenic disease [4–8], with a rate of TEE between 0.39 and 1.5 per 100 person-years [5,7,9,10]. However, pooled analysis of the initial and subsequent clinical trials show that this rate is much higher during TPO-RA treatment, for both arterial and venous thrombosis. A TEE rate per 100 patient years of 5.5–7.5 was found for romiplostim [11–13], and 4.0 for eltrombopag [14]. Thrombosis might occur even more frequently in clinical practice, as almost all published clinical trials excluded patients with a medium-high risk for thrombosis, for example patients with a previous venous thrombosis or a combination of risk factors such as smoking, diabetes or treatment for hypertension [15–21]. Relevant protocol numbers from clinicaltrials.gov (if available) are NCT00102323 and NCT00102336 [15–17], NCT00370331 [18], NCT00370331 [19], and NCT00111475 [16,20].

Thus far, the mechanism that leads to thrombosis during TPO-RA treatment is poorly understood [22]. Thrombosis occurs primarily in patients with traditional cardiovascular risk factors, but this provides

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insufficient explanation for the high rate of thrombosis [3]. There is no evidence that occurrence of thrombosis is related to a high platelet count - on the contrary, the platelet count is often below normal at the time of the event [11–13,18,23]. It is also unclear whether the duration of treatment influences the thrombotic risk, although one pooled analysis showed the highest TEE rate within the first 6 months of treatment [12].

We hypothesize that TPO-RA treatment increases the risk of thrombosis by directly affecting platelet function. The TPO-receptor, c-MPL, to which TPO and TPO-RAs bind, is present on platelets [24–26]. Binding of TPO leads to internalization by the platelet, and thus clearance from the circulation. This way, TPO-levels are regulated [2]. However, TPO also stimulates platelet function through this receptor, as was shown in vitro: platelet reactivity, aggregation, adhesion, and degranulation increased [27–36], which in turn is linked to increased thrombin generation [37–46] and the occurrence of thromboembolic events [47–62]. Based on the binding mechanism of eltrombopag and romiplostim to c-MPL, eltrombopag could theoretically have a larger effect on platelet function [63]. Eltrombopag's binding to the transmembrane region allows double stimulation by both eltrombopag and TPO and thus activation of multiple signal transduction pathways, while romiplostim's direct binding to the TPO-receptor is competitive with endogenous TPO [63]. In healthy controls, previous research shows no evidence that eltrombopag increases platelet function in vitro or in vivo, although the exposure was only 1–10 days [64–66]. The effect of romiplostim on platelets from healthy controls was not assessed either in vitro or in vivo. We hypothesize that TPO-RAs affect ITP patients differently than healthy subjects, as platelets are more procoagulant at baseline and the platelet count is lower at the start of treatment [67–73].

TPO-RAs might also directly or indirectly influence the coagulation system or fibrinolysis. Although we consider alterations in platelet function the most likely mechanism to explain an increased rate of thrombosis during TPO-RA treatment, we cannot exclude that regulators of the coagulation system or fibrinolysis are affected. Furthermore, alterations in platelet function can lead to detectable changes in global hemostatic tests, such as thrombin generation, which might also contribute to thrombotic risk [37–46].

A systematic literature review was conducted to assess the effect of TPO-RAs on platelet function, coagulation and fibrinolysis in ITP patients.

2. Methods

For this systematic review we searched the databases Pubmed, Embase and Cochrane up to March 10th 2020, combining key terms for immune thrombocytopenia, thrombopoietin-receptor agonists and hemostasis ("Appendix"). We included all English language articles that assessed hemostasis in vivo in persistent or chronic ITP patients during the use of a TPO-RA. Any hemostatic parameters that could indicate a procoagulant state was considered relevant: markers of platelet function, the coagulation system, global hemostasis or fibrinolysis. Exclusion criteria were underlying malignant disease or pregnancy, and animal studies. Screening, data-extraction and assessment of risk of bias assessment were carried out by two authors independently (W.E.M.D. and O.N.B.); disagreements were resolved through discussion. Data from the included articles were extracted using a standardized form.

The quality of the studies was based on the risk of bias (ROBINS-I Cochrane Collaboration's tool for observational studies), the methodological quality (Newcastle-Ottawa quality assessment scale) and completeness of reporting (STROBE statement: strengthening the reporting of observational studies in epidemiology). These complementary assessments were combined into one overall conclusion, as explicated in Supplementary Table 1.

A meta-analysis could not be performed due to heterogeneity of the data with respect to the use of different methods and assays, and variability in the duration of follow-up. Instead, we have systematically summarized the results according to the different assays and present the data according to treatment duration. Data from case-series with less than five patients are not presented in the tables, because no hypothesis testing could be performed to evaluate whether observed changes were significant.

To deal with the heterogeneity in follow-up duration, and with the uncertainty as to when patients are at risk for thrombosis, we separated the studies into short-term, mid-term and long-term TPO-RA treatment duration:

(1) short-term: hemostatic testing was performed at weekly intervals, at least once in the first 1–2 weeks from start of treatment, and up till 1–3 months of treatment.
(2) mid-term: hemostatic testing was performed at monthly intervals, at least once in the first 1–2 months, and up till 1–6 months.
(3) long-term: hemostatic testing was performed at least once after ≥6 months, but not in the first 3 months.

To assess whether the effect of TPO-RAs on hemostasis was related to the platelet response, we separated the data from responders and non-responders if possible. Responder status was taken from each corresponding study, or, if not provided in the original manuscript, manually assigned according to the following definition: platelet count $\geq 30 \times 10^9$/L and at least a 2-fold increase compared with the baseline count and absence of bleeding [74]. Because insufficient data on bleeding were available, we focused on the laboratory criteria.

3. Results

The search yielded 806 unique articles, of which 12 were included in the final review (Fig. 1). Details of the included studies are reported in Table 1. Eight studies were observational cohorts, two were non-randomized, open-label trials [75,76], and two were case series [77,78]. The quality of 50% of the studies (6/12) was considered sufficient [75,77–81]. The complete critical appraisal is available in Supplementary Table 1.

In total, hemostatic function was tested in 305 ITP patients during TPO-RA treatment. Eltrombopag was used by 122/216 (56%) patients. Most of the included patients were responders (127/150 (85%)). In non-responders, TPO-RA treatment was continued during the entire study.

Four studies had a short-term [75,78–80], four a mid-term [76,82,83] and five a long-term follow-up [77,80,84–86]. One study is included twice in our analysis: the prospective cohort in the short-term

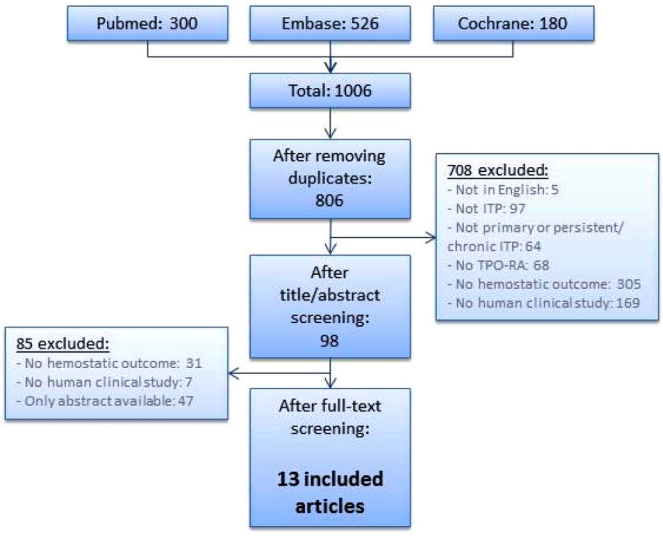

Fig. 1. Flowchart.

Table 1
Included articles.

Study and design	ITP patients treated with TPO-RAs				Control group (N)		Outcome parameters			Critical appraisal (overall score)
	Total (N)	Responders (% of total)	Type of TPO-RA (%)	Treatment duration at hemostatic testing	ITP	HCs	Platelet function		Coagulation and fibrinolysis	
							Flow cytometry	Other		
Short-term usage										
Psaila 2012 [79] *Prosp. cohort*	20	50%	E (100%)	Week 0, 1, 4	–	20	GPIb, P-sel, activated αIIbβ3	–	–	●●●○
Haselboeck 2012 [78] *Case series*	3	100%	E (100%)	Week 0, 1, 2, 3, 4, 9, 11, 12	–	22	P-sel, PMA	Impact-R, MEA	–	●●●○
Haselboeck 2013 [75] *Non-rand. trial*	10	100%	E (100%)	Week 0, 1, 3, 4	12	–	P-sel, PMA	Impact-R	–	●●●●
Garabet 2017 [80] *Prosp. cohort*	26	NR	E (62%) R (38%)	Week 0, 2, 6, 12	–	22	–	sP-selectin	D-dimer, PAI-1, plasma TG, F1+2	●●●○
Mid-term usage										
Alvarez Roman 2014 [82] *Prosp. cohort*	13	100%	E (92%) R (8%)	Week 0 and after response[b]	–	25	PS, (un)activated αIIbβ3	MP activity	Plasma TG	●○○○
Chiou 2015 [76] *Non-rand. trial*	25	48%	E (100%)	Week 0, 4, 8, 26	30	NR	GPIb, GPIX, GPVI	Flow based adhesion test	–	●○○○
Suntsova 2017 [83] *Prosp. pediatric cohort*	3[a]	100%	R (100%)	Week 0, 4, 8, 12, 16	10	10	P-sel, activated αIIbβ3, PS	Mepacrine ratio	–	●○○○
Ignatova 2019 [81]	31	NR	R (100%)	Week 0, 4, 8, 12, 16, 20, 24	–	18	GPIb, P-sel, (un) activated αIIbβ3, PS, FSC-H, SSC-H,	Transmission electron microscopy	Thrombo-elastography	●●○○
Two cohorts	*Cross-sect.*	9	NR	R (100%)	NR	17	mepacrine uptake and ratio	Flow based adhesion test	–	●○○○
Long-term usage										
Gardiner 2010 [77] *Case report*	1	100%	R (100%)	Month 0, 6	–	NR	GPIb, GPVI	LTA	–	●●●○
Ghanima 2012 [84] *Retrospective cohort*	89	NR	R: *5415*[c] E: *5566* Other:[d] *594*	Month 0, 0–4, 4–8, 8–12	–		–	–	D-dimer	●○○○
Garabet 2017 [80] *Cross-sect. cohort*	18	100%	E (50%) R (50%)	Months:[e] 36 (17–47)	*Prosp. cohort*	22	–	sP-sel	D-dimer, PAI-1, plasma TG, F1+2	●●●○
Al-Samkari 2019 [85] *Cross-sect. cohort*	15	100%	R (100%)	Months:[f] 10 (1–55)	–	7	–	LTA	–	●○○○
Sanz 2019 [86] *Cross-sect. cohort*	42	100%	E (64%) R (36%)	Months:[f] 25 (1–75)	40	112	P-sel, activated αIIbβ3, PS, binding of pro-thrombinase complex, CD63	MP activity ROTEM	PAI-1, uPA, tPA, TAFI activity	●○○○

Legend: ●○○○ = insufficient; ●●○○ = medium; ●●●○ = acceptable; ●●●● = good.
Cross-sect.: cross-sectional, E: eltrombopag, F1+2: prothrombin fragments 1+2, FSC-H: forward scatter-height, HCs: healthy controls, LTA: light transmission aggregometry, MEA: multiple electrode aggregometry, non-rand: non-randomized, NR: not reported, MP: microparticle, PC: platelet count, prosp: prospective, P-sel: P-selectin, PS: phosphatidylserine, R: romiplostim, ROTEM: rotational thromboelastometry, sP-sel: soluble P-selectin, SSC-H: sideways scatter-height, TAFI: thrombin activatable fibrinolysis inhibitor, TG: thrombin generation, tPA: tissue plasminogen activator, uPA: urokinase-plasminogenactivator.

[a] Sufficient relevant outcome data was only available in 3 of the 11 included patients.
[b] No definition was given by the article.
[c] The unit is cumulative exposure in weeks.
[d] This group includes AKR-501 and Shionogi-S888 (cumulative exposure 326 and 268 weeks respectively).
[e] The values represent median (interquartile range).
[f] The values represent median (range).

category, and the cross-sectional cohort in the long-term category [80].

The best quality data was available for short-term TPO-RA treatment, including a total of 59 patients (70% responders, 83% eltrombopag). The quality of the mid-term follow-up studies was predominantly insufficient, and included a total of 81 patients (68% responders, 46% eltrombopag). The long-term studies were also predominantly insufficient; data was available for 76 TPO-RA users (100% responders, 48% eltrombopag, median treatment duration between 10 and 36 months; the characteristics are reported in Supplementary Table 2) [77,80,85,86]. One study only assessed a specific subgroup of 89 patients with a suspected thrombosis [84].

3.1. Platelet function

The increased risk of thrombosis during TPO-RA use could be associated with platelet activation in vivo. Several studies addressed this, mostly by flow cytometric measurement of P-selectin expression and the configuration of the αIIbβ3 receptor on platelets, but a broad range of other flow cytometric and plasma parameters were reported as well (Table 2). During short-term follow-up, there was no evidence that TPO-

Table 2
In vivo platelet activation in responders.

TPO-RA treated patients		Hemostatic test				Significant differences?		
Study	N	Test parameter	Method	Antibody	Units	Baseline vs. HCs	TPO-RA vs. baseline	Timing of assessment after start TPO-RA
Short-term treatment								
Psaila 2012 [79]	10	P-selectin	Flow cyt.; WB	CD62p	MFI	↑	↑	Week **1***, 4
		Activated αIIbβ3		PAC1	MFI	=	=	Week 1, 4
Haselboeck 2013 [75]	10	P-selectin	Flow cyt.; PRP[b]	CD62p	MFI	NR	↓	Week 1, **3***, **4***
		PMA	Flow cyt.; WB	CD41 and CD14	%	NR	↑	Week **1***, **3***, **4***
Garabet 2017 [80]	26	Soluble P-selectin	ELISA	–	ng/mL	=	↑†[a]	Week **2, 6, 12**†[a]
Mid-term treatment								
Alvarez-Roman, 2014 [82]	13	Activated αIIbβ3	Flow cyt.; PRP	PAC1	MFI times % of pos. cells	=	=	After response
		Unactivated αIIbβ3		PE-mAb and FITC-mAb	MFI	=	=	
		Phosphatidylserine	Flow cyt.; washed plt.	Annexin A5	%	↑	=	
		Microparticles TF+	Zymuphen kit; PFP	–	nM	=	=	
		PS+				↑	=	
Ignatova 2019 [81]	31	P-selectin	Flow cyt.; PRP	CD62p	arbitrary	↑	=	Week 4, 8, 12, 16, 20, 24
		Activated αIIbβ3		PAC1	units	↑	=	
		FSC-H		-		↑	=‖	
		Phosphatidylserine		Annexin A5	%	↑	↓	Week **4***, 8, 12, 16, 20, 24
	9	P-selectin	Flow cyt.; PRP	CD62p	arbitrary	↑	=	NR (>1 month)
		Activated αIIbβ3		PAC1	units	↑	=	
		Unactivated αIIbβ3		CD61-PE		↑	=	
		FSC-H		–		↑	=	
		SSC-H				↑	=	
		Phosphatidylserine		Annexin A5	%	↑	=	
		Mepacrine uptake	Flow cyt.; PRP loaded with mepacrin (10 μM)	–	arbitrary	↑	=	
		Mepacrine release			units	↑	NR	
		Platelet-associated IgG	Flow cyt.; washed plt.	^{125}I-labeled affinity purified goat antihuman IgG antibodies	% of values in HCs	NR	=	
Long-term treatment								
Sanz 2019 [86]	42	P-selectin	Flow cyt.; PRP	FITC-anti-P-selectin	% pos. cells	↑	=	25 (1–75) months
		Phosphatidylserine	Flow cyt.; washed plt.	Annexin A5	MFI	↑	=	
		Binding prothombinase complex		Anti-FVa and anti-FXa	MFI	↑	=	
		Microparticle activity	ZYMUPHEN MP-Activity kit, PFP	–		↑	↑	
Garabet 2017 [80]	18	Soluble P-selectin	ELISA	–	ng/mL	↑	↑	36 (17–47) months

Legend: ↑: significant increase ($p < 0.05$), ↓: significant decrease ($p < 0.05$), = : no significant change.
ELISA: enzyme-linked immunosorbent assay, flow cyt.: flow cytometry, FSC-H: forward scatter-height, GP: glycoprotein, HCs: healthy controls, mAbs: monoclonal antibodies, IgG: immunoglobulin G, MFI: mean fluorescence intensity, N: number, N/a: not applicable, NR: not reported, PFP: platelet-free plasma, plt.: platelets, PRP: platelet-rich plasma, PS: phosphatidylserine, Sign: significant, SSC-H: sideways scatter-height, TF: tissue factor, WB: whole-blood.
Bold time points are significantly different compared with baseline (P-value <0.05).
* This time point was significantly different compared with baseline (p-value <0.05).
† A significant trend was shown by ANOVA analysis (p-value <0.05).
‖ Week 8 was sign. lower than week 4, week 24 was sign. lower than month 8.
[a] This analysis was corrected for platelet count.
[b] The platelet count was standardized by dilution.

RA treatment led to altered expression or activation of the fibrinogen receptor αIIbβ3, and the reported effect of TPO-RAs on P-selectin expression on unstimulated platelets varied [79,81–83]. Plasma levels of soluble P-selectin increased, independent of the platelet count [80]. Furthermore, one study showed increasing levels of platelet-monocyte aggregates (PMA) during treatment [75]. Mid- and long-term studies reported little evidence of in vivo platelet activation, although one study found raised plasma levels of soluble P-selectin [80].

Rather than leading to in vivo platelet activation, TPO-RAs might increase platelet reactivity towards agonists (Table 3). This was assessed with flow cytometry and platelet aggregation studies. During short-term treatment, stimulation of platelets with thrombin receptor agonist peptide (SFLLRN; TRAP) led to increased P-selectin expression, although this was not observed by other studies [75,78,79]. Other markers of platelet activation in response to TRAP were otherwise uninfluenced by TPO-RAs, although little data was available for the mid-term group [82,86]. Receptor expression in response to adenosine diphosphate (ADP) remained unaltered during short- and mid-term follow-up [75,78,79,81,83]. PMA formation in response to either TRAP or ADP was shown to increase during TPO-RA treatment, in the same study that found increasing PMA formation without stimulation [75]. Another study showed that TPO-RA treatment led to increased expression of GPVI on unstimulated platelets (data not shown) [76]. This receptor activates platelets in response to collagen. No studies used flow cytometry to assess reactivity to collagen. In one case-report, an absent response to collagen with light transmission aggregometry restored after 6 months of TPO-RA treatment [77]. Other studies found that platelet aggregation after stimulation with agonists was unaffected by TPO-RAs

Table 3
Platelet reactivity to agonists in responders.

TPO-RA treated patients		Hemostatic test					Significant differences?		
Study	N	Test parameter	Method	Antibody	Units	Agonist (μM)	Baseline vs. HCs	TPO-RA vs. baseline	Timing of assessment after start TPO-RA
Reactivity to thrombin receptor activating protein (TRAP)									
Short-term treatment									
Psaila 2012 [79]	10	P-selectin	Flow cyt.; WB	CD62p	MFI	1.5	=	=	Week 1, 4
						20	=	=	
		Activated αIIbβ3		PAC1	MFI	1.5	=	=	
						20	=	↑	Week 1, **4***
Haselboeck 2013 [75]	10	P-selectin	Flow cyt.; PRP[a]	CD62p	MFI	5.7	NR	↑	Week **1***, **3***, **4***
		PMA	Flow cyt.; WB	CD41 and CD14	%	7.1	NR	↑	
Mid-term treatment									
Alvarez-Roman, 2014 [82]	13	Activated αIIbβ3	Flow cyt.; PRP	PAC1	MFI × % of pos. cells	100	↓	=	After response
Long-term treatment									
Sanz 2019 [86]	42	P-selectin	Flow cyt.; PRP	FITC-anti-P-selectin	% pos. cells	100	↓	=	25 (1–75) months
		Activated αIIbβ3		FITC-PAC1	% pos. cells		↓	=	
		CD63		FITC anti-CD63 mAb	% pos. cells		↓	=	
Reactivity to adenosine diphosphate (ADP)									
Short-term treatment									
Psaila 2012 [79]	10	P-selectin	Flow cyt.; WB	CD62p	MFI	0.5	=	=	Week 1, 4
						20	=	=	
		Activated αIIbβ3		PAC1	MFI	0.5	=	=	
						20	No	↓	Week 1, **4***
Haselboeck 2013 [75]	10	P-selectin	Flow cyt.; PRP[a]	CD62p	MFI	1.5	NR	=	Week 1, 3, 4
		PMA	Flow cyt.; WB	CD41 and CD14	%	1.5	NR	↑	Week **1***, **3***, **4***
Mid-term treatment									
Ignatova 2019 [81]	9	P-selectin	Flow cyt.; PRP	CD62p	Arbitrary units	5	=	=	NR (>1 month)
		Activated αIIbβ3		PAC1			↑	=	
		Unactivated αIIbβ3		CD61-PE			↑	=	
		FSC-H		–			↑	=	
		SSC-H					↑	=	
		Phosphatidylserine		Annexin A5	%		↑	=	
		Mepacrine uptake	Flow cyt.; PRP loaded with mepacrin (10 μM)	–	Arbitrary units		↑	=	
Reactivity to TRAP + collagen-related peptide (CRP)									
Mid-term treatment									
Ignatova 2019 [81]	31	Mepacrine ratio	Flow cyt.; PRP loaded with mepacrin (10 μM)	–	Ratio resting/stimulated	TRAP: 12.5 CRP: 20 μg/μl	=	=	Month 1, 2, 3, 4, 5, 6

Legend: ↑: significant increase ($p < 0.05$), ↓: significant decrease ($p < 0.05$), = : no significant change.
Flow cyt.: flow cytometry, FSC-H: forward scatter-height, HCs: healthy controls, mAbs: monoclonal antibodies, MFI: mean fluorescence intensity, N: number, N/a: not applicable, NR: not reported, plt.: platelets, PRP: platelet-rich plasma, SSC-H: sideways scatter-height, WB: whole-blood.
Bold time points are significantly different compared with baseline (P-value <0.05).
* This time point was significantly different compared with baseline (p-value <0.05).
[a] The platelet count was standardized by dilution.

[78,85].

Three studies used flow-based assays to simulate the physiological high-shear conditions while testing platelet function. One mid-term study found increased surface coverage during treatment, simultaneous with the increased GPVI expression [76]. Two of these studies (short- and mid-term) showed no evidence for increased adhesion and aggregation under high shear stress [75,81].

Only two studies assessed platelet function in non-responders to TPO-RA treatment, but similar changes were observed as in responders: during short-term treatment P-selectin expression increased [79], and during mid-term treatment, flow cytometric GPVI expression and adhesion under flow increased (Supplementary Table 4) [76]. Pre-treatment, the latter parameters were both impaired compared with healthy controls (Supplementary Table 3) [76].

3.2. Global and secondary hemostasis and fibrinolysis assessment

In addition to platelet function and activation, the effect of TPO-RAs on global and secondary hemostasis was evaluated (Table 4). Several studies assessed plasminogen activator inhibitor (PAI)-1. This inhibitor of fibrinolysis is present in platelets and released upon platelet activation [87]. Increasing levels during TPO-RA treatment could lead to hypofibrinolysis and an increased risk of TEE. PAI-1 levels in TPO-RA users were investigated in two studies, with conflicting results. The

Table 4
Secondary hemostasis and fibrinolysis.

TPO-RA treated patients		Hemostatic test				Significant differences?		
Study	N	Test parameter		Method	Units	Baseline vs. HCs	TPO-RA vs. ITP controls	TPO-RA duration at time of assessment*
Short-term treatment								
Garabet 2017 [80]	26	D-dimer		ELISA	ng/mL	↑	=	Week **2, 6 and 12***†
		PAI-1		ELISA	ng/mL	↑	↑↓†	
		F1+2		ELISA	pmol/L	↑	=	
		Plasma TG	ETP; lag time; peak height; time to peak	Plasma	% [a]	=	=	
Mid-term treatment								
Alvarez-Roman, 2014 [82]	13	Plasma TG	ETP	Plasma	nM/min	↑	=	After response
			Lag time		sec	=	=	
			Peak height		nM	↑	↓	
			Time to peak		min	↓	=	
Ignatova 2019 [81] *Prosp. cohort*	31	Thrombo-elastography	Reaction time (R)	Thrombo-elastograph Analyzer 5000	min	NR	=	Month 1, 2, 3, 4, 5, 6
			Kinetic value (K)		min	NR	↓ month 1 vs 0 month 6 vs 1	
			Maximum amplitude		degrees	NR	↑ month 1 vs 0	
			α value		mm	NR	↑ month 1 vs 0 month 6 vs 1	
Long-term treatment								
Ghanima 2012 [84]	89	D-dimer		ELISA	ng/mL	NR	=	Month 0–4, 4–8, 8–12
Garabet 2017 [80]	18	D-dimer		ELISA	ng/mL	↑	=	36 (17–47) months
		PAI-1		ELISA	ng/mL	↑	=	
		F1+2		ELISA	pmol/L	↑	=	
		Plasma TG	ETP; lag time; peak height; time to peak	Plasma	% [a]	=	=	
Sanz 2019 [86]	42	PAI-1		ELISA	pg/mL	↑	↑	25 (1–75) months
		uPA				=	=	
		tPA				=	=	
		TAFI activity				=	=	
		Thrombo-elastometry	Clotting time	ROTEM, PRP, Adjusted to 25 *10^9 platelets/L	sec	=	=	
			Maximum clot firmness		mm	↑	=	
			Clot lysis after 60 min		%	↑	=	
			α angle		degrees	=	=	

Legend: ↑: significant increase ($p < 0.05$), ↓: significant decrease ($p < 0.05$), = : no significant change
ELISA: enzyme-linked immuno sorbent assay, ETP: endogeneous thrombin potential, F1+2: prothrombin fragments 1+2, HCs: healthy controls, N: number, NR: not reported, PRP: platelet-rich plasma, ROTEM: rotational thromboelastometry, TAFI: thrombin activatable fibrinolysis inhibitor, TG: thrombin generation, tPA: tissue plasminogen activator, uPA: urokinase-plasminogenactivator.
Bold time points are significantly different compared with baseline (P-value <0.05).
* This time point was significantly different compared with baseline (p-value <0.05).
† A significant trend was shown by ANOVA analysis (p-value <0.05): median (IQR) 1.36 (0–2.81), 1.73 (1.02–2.91), 1.09 (0–3.35) and 1.06 (0–1.81) ng/mL for week 0, 2, 6 and 12 respectively, p = 0.005.
[a] The percentage represents the patient's value against pooled normal plasma.

first study included a short-term treated cohort, in which PAI-1 levels changed significantly, but no post-hoc analysis was performed to identify the direction of change. Visually, there seems to be a peak after 2 weeks of treatment followed by a decrease to below baseline (a trend also visible in D-dimer levels, although this change was nonsignificant) [80]. In long-term TPO-RA users, the same study found PAI-1 levels to be similar to ITP controls [80], while another long-term study, using a different assay, found PAI-1 levels to be significantly higher in the TPO-RA-treated group [86]. In the latter cohort, the levels of other regulators of fibrinolysis were similar to ITP controls, but these markers was not assessed by other studies [86]. Clot formation measured with thromboelastography was also similar to the controls [86]. One other study that assessed clot formation with the same methodology reported accelerated clot formation within the first 6 months of treatment in both responders and non-responders to treatment (Supplementary Table 4) [81].

Lastly, a few global assessments of hemostasis were used by some of the studies. There were no indications that TPO-RAs increased global coagulation potential, as plasma thrombin generation parameters were normal in TPO-RA users [80,82]. Furthermore, TPO-RA treatment was not associated with active low grade coagulation processes in vivo, as d-dimer and prothrombin fragments 1+2 levels were normal [80]. One retrospective study found no association between d-dimer levels and treatment duration in TPO-RA users who presented at the hospital with a suspected thrombosis, although the clinical relevance of these results is questionable, and the results cannot be extrapolated to TPO-RA users in general [84].

4. Discussion

TPO-RA treatment in ITP is associated with an increased risk for thrombosis, which we hypothesized to be caused by altered platelet function or activation, coagulation and/or fibrinolysis due to these agents. Within the limits of the available evidence, this systematic review showed that TPO-RAs induce several hemostatic changes, particularly in the beginning of the treatment course. Within weeks, TPO-RAs

seemed to alter platelet activation, with respect to levels of platelet-monocyte aggregates, soluble P-selectin, as well as possibly GPVI expression, and adhesion under flow. At least some TPO-RA-induced hemostatic changes seemed independent of an increase in platelet count. For global and secondary hemostasis and fibrinolysis, the evidence was limited, particularly during short-term treatment. However, both pathways seemed largely unaffected, except for an accelerated clot formation and possibly increasing plasma PAI-1 levels. The increasing hemostatic parameters have previously been associated with occurrence of thrombosis in other patient groups, and could therefore contribute to the increased rate of thrombosis observed in TPO-RA-treated ITP patients. However, a causal relationship remains to be established.

4.1. Strengths and limitations

This is the first paper to systematically review the effect of TPO-RAs on platelet function, coagulation and fibrinolysis in ITP patients. A strength of the review was the extensive search, which makes it probable that all relevant trials were identified. Bias was minimized by the independent screening and data-extraction by two authors, as well as the application of predefined in- and exclusion criteria and a standardized data-extraction form. An important limitation of the review is the low to moderate quality of many studies. The heterogeneity of the results made a meta-analysis impossible and complicated the interpretation of the results.

4.2. Platelet function

The studies included in our review show evidence that TPO-RAs could enhance platelet activation in vivo in ITP patients, based on increases in PMA formation, soluble P-selectin, GPVI expression, and adhesion under flow. Although soluble P-selectin might originate from endothelial cells rather than activated platelets, a recent study found no evidence that TPO-RA treatment activated the endothelium [88]. Previous research on the effect of TPO-RAs on platelet function showed contradictory results for healthy controls and ITP patients [64–66,76,80,89]. In ITP platelets (in vitro) and patients (in vivo) data support an effect of TPO-RAs on platelet activation: in vitro, eltrombopag increased GPVI expression and adhesion under flow [76], and upregulated expression of the P-selectin gene [80]. In vivo, TPO-RAs induced overexpression of several genes involved in platelet aggregation, degranulation and activation, although this observation could be due to the increase in platelet production [89]. In healthy subjects, however, data do not support an effect of TPO-RAs on platelet activation: eltrombopag failed to independently activate platelets either in vitro or in vivo [64–66], even though endogenous TPO directly influences platelet activation, aggregation and adhesion [27–33,64,90–94]. The different observations between healthy and ITP subjects might be due to ITP platelets being more activated at baseline [5,10,22,54].

Several changes in platelet function seemed to occur independent of a change in platelet count. During TPO-RA treatment the platelet count fluctuates, which affects many of the platelet function assays. However, the upregulation of GPVI on platelets is uninfluenced by platelet count [76,89], and the increased flow-based adhesion was also observed in non-responders (without a platelet response) [76]. Lastly, the observed increase in soluble P-selectin levels, although influenced by platelet count, was still statistically significant after correction for platelet count by the authors [80]. However, for PMA formation, the increase during TPO-RA treatment may only reflect the platelet response to TPO-RAs, as low levels of PMA can be expected as long as the platelet count is low [75,80].

Whether or not an effect of TPO-RAs on platelet function was observed seemed to differ for assays performed in whole blood versus platelet rich plasma. Several platelet (re)activity assays, and PMA levels especially, can be influenced by pre-analytical variables, such as blood collection and sample preparation [55]. Both procedures can activate platelets, which in turn can lead to either higher markers of platelet activation, or contradictory lower markers of platelet activation when the platelets' capacity is exhausted [67]. In our review, the studies that used whole blood for their flow cytometric assessments showed an effect of TPO-RA treatment, including increasing P-selectin expression [79], and PMA formation [75], opposed to studies that used platelet rich plasma [75,78,86]. The study that found increased PMA formation (in whole blood) used platelet rich plasma for the other flow cytometric assays. They found no increased P-selectin expression on unstimulated platelets, while the two are closely related as PMA formation depends on interaction with P-selectin [75]. Because the use of whole blood limits artificial activation of platelets, these assessments, that all showed increasing platelet activation, might be most reliable [79].

Some of the observed changes might indeed indicate that TPO-RA treatment induces a hypercoagulable state. This is particularly true for the increased levels of (soluble) P-selectin: the levels during TPO-RA treatment likely became pathologically high, as the pre-treatment (soluble) P-selectin levels in ITP patients were already increased or similar to levels in healthy controls before TPO-RA treatment [79,80], and high levels have been associated with the occurrence of thrombosis [47,95,96]. For PMA formation and GPVI expression, it is unsure if the observed increase indeed indicates a prothrombotic state. PMAs are associated with the occurrence of thrombosis [48,55,78,97], but since no comparison was made with healthy controls, it is unsure if the levels because pathologically high [98]. GPVI has also been shown to play a role in the occurrence of thrombosis [56,99]. GPVI mediates platelet activation by collagen, which is present in the subendothelium of the vessel wall [100], so this mechanism might be explanatory for the occurrence of thrombosis in the presence of endothelial damage, for example in patients with predisposing factors. However, the pre-treatment levels in the ITP cohort were impaired compared with healthy controls, thus this increase might merely reflect normalization.

4.3. Global and secondary hemostasis and fibrinolysis

In the studies included in our review, most assays assessing global and secondary hemostasis and fibrinolysis were uninfluenced by TPO-RA treatment. A possible change was only observed for thromboelastography (assessing clot formation and fibrinolysis), and PAI-1 (an inhibitor of fibrinolysis that is synthesized in part by activated platelets). D-dimer and plasma thrombin generation seemed unaffected by TPO-RA treatment, although both are associated with thromboembolic disease [49–54,62,101–103]. The accelerated clot formation during TPO-RA treatment was observed in both responders and non-responders [81]. This change could be related to the observed changes in platelet function or PAI-1 levels in this review, although it is known that moderate changes in platelet function are not detected by thromboelastography [104,105]. Accelerated clot formation and fibrinolysis as assessed by thromboelastography have been associated with a hypercoagulable state [105,106]. PAI-1 levels during TPO-RA treatment were assessed by three studies included in this review, but the data remain inconclusive. One included study found a change in PAI-1 levels, but they regrettably failed to report the direction of change [80]. Two long-term TPO-RA-treated cohorts showed contradictory results: one found increased levels, and the other no difference in comparison to ITP controls [80,86]. This might be due to the use of a different kit, or differences in baseline characteristics between the cohorts, including the proportion of splenectomized patients in the latter cohort (61% vs 12%). Overall, the results on PAI-1 levels are hard to interpret. Previous research does indicate that TPO-RAs affect PAI-1 levels in vitro [86], but eltrombopag did not affect the genetic expression for PAI-1 in ITP patients in vivo [80]. Although PAI-1 has been previously associated with the occurrence of thrombosis [56,111], more research is needed to conclude if the effect of TPO-RAs on PAI-1 is true and in a prothrombotic direction.

4.4. Duration of TPO-RA treatment

Our data is most reliable for the short-term changes in hemostatic parameters caused by TPO-RAs. Changes in platelet activation were seen as soon as within 2 weeks of treatment [75,79,80]. Indeed, pooled analysis of the clinical studies showed the highest TEE rate early (within 6 months) in the treatment course [12]. An explanation could be that a potential increased thrombotic tendency subsides with prolonged treatment, although there is no evidence available to back up this hypothesis. Alternatively, susceptible patients could develop a thrombotic event early during treatment and drop out of the study, leaving a low-risk population in the extended trials. In our review, a few independent observations suggest increased platelet activation during long-term treatment (>6 months), but the long-term data are less reliable. As in the clinical studies, these cohorts likely represent a low-risk population. Furthermore, these studies are cross-sectional with a separate control group, so any hemostatic changes cannot be directly linked to the TPO-RA treatment.

5. Future considerations

Several articles included in this review suggest potentially prothrombotic changes during TPO-RA treatment. Because ITP itself is associated with a prothrombotic state [5,10,54], even small changes might lead to a clinically relevant increase in thrombosis risk. To assess whether the observed hemostatic changes are truly prothrombotic, patients are ideally prospectively followed until a thromboembolic event occurs. However, such a study would be very challenging due to the low incidence of TEE in ITP. A case-control study comparing risk factors and possible pathophysiological mechanisms in ITP patients with and without thrombosis could be considered. Furthermore, several relevant aspects of hemostasis remain unassessed in ITP patients using a TPO-RA, for instance platelet desialylation and whole blood thrombin generation. Preferably, methods that are used to assess platelet function should be uninfluenced by platelet count and need minimal sample preparation to avoid platelet activation. Lastly, we did not review the effect of TPO-RAs on apoptosis and platelet turnover or endothelial function/activation.

Practice points

- The use of thrombopoietin-receptor agonists (TPO-RAs) in immune thrombocytopenia is associated with an increased rate of thromboembolic events. The mechanism that leads to thrombosis is incompletely understood.
- TPO-RAs seem to induce several hemostatic changes, with respect to platelet activation in vivo, clot formation and possibly altered levels of plasminogen activator inhibitor (PAI)-1.
- At least some TPO-RA-induced hemostatic changes seemed independent of an increase in platelet count.
- The increasing hemostatic parameters are associated with occurrence of thrombosis in other patient groups, and might therefore -partly- explain the increased rate of thrombosis observed in TPO-RA-treated ITP patients. However, a causal relationship remains to be established.

Research agenda

- The effect of thrombopoietin-receptor agonists on platelet apoptosis and desialylation, and endothelial function.
- The relationship between hemostatic changes induced by thrombopoietin-receptor agonists and the occurrence of thrombosis.
- Identification of the patients at risk of developing thrombosis prior to treatment with thrombopoietin-receptor agonists.

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Declaration of competing interest

The authors have no conflict of interest to declare.

Appendix

Search

Format

(ITP) AND (Platelet function OR hemostasis) AND (TPO-RA)

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AND
(‘platelet function’:ti,ab OR ‘platelet reactivity’:ti,ab OR ‘thrombocyte reactivity’:ti,ab OR ‘platelet activity’:ti,ab OR ‘thrombocyte activity’:ti,ab OR ‘platelet adhesion’:ti,ab OR ‘thrombocyte adhesion’:ti,ab OR ‘platelet response’:ti,ab OR ‘thrombocyte response’:ti,ab OR hemostasis:ti,ab OR ‘platelet function test’:ti,ab OR ‘thrombocyte function test’:ti,ab OR ‘clot retraction’:ti,ab OR ‘mean platelet volume’:ti,ab OR ‘mean thrombocyte volume’:ti,ab OR ‘bleeding times’:ti,ab OR ‘bleeding time’:ti,ab OR ‘duke method’:ti,ab OR ‘aspirin tolerance test’:ti,ab OR ‘aspirin tolerance tests’:ti,ab OR ‘ivy method’:ti,ab OR ‘platelet number’:ti,ab OR ‘thrombocyte number’:ti,ab OR ‘platelet numbers’:ti,ab OR ‘thrombocyte numbers’:ti,ab OR ‘platelet activation’:ti,ab OR ‘thrombocyte activation’:ti,ab OR ‘platelet activations’:ti,ab OR ‘thrombocyte activations’:ti,ab OR ‘clot retraction’:ti,ab OR ‘platelet adhesiveness’:ti,ab OR ‘thrombocyte adhesiveness’:ti,ab OR ‘platelet aggregation’:ti,ab OR ‘thrombocyte aggregation’:ti,ab OR Coagulation:ti,ab OR Fibrinolysis:ti,ab OR fibrin*:ti,ab OR ‘blood clotting’:ti,ab OR ‘blood clottings’:ti,ab OR clot*:ti,ab OR ‘blood coagulation test’:ti,ab OR ‘blood coagulation tests’:ti,ab OR ‘prothrombin time’:ti,ab OR ‘prothrombin times’:ti,ab OR ‘viper venom time’:ti,ab OR thrombotest:ti,ab OR ‘quick test’:ti,ab OR ‘thrombin time’:ti,ab OR ‘thrombin times’:ti,ab OR ‘reptilase time’:ti,ab OR ‘reptilase times’:ti,ab OR Thrombelastography:ti,ab OR tromboelastography:ti,ab OR thromboelastometry:ti,ab OR ‘hematologic tests’:ti,ab OR ‘hematologic test’:ti,ab OR ‘blood test’:ti,ab OR ‘blood physiological phenomena’:ti,ab OR ‘blood physiological phenomenas’:ti,ab OR ‘mean platelet volume’:ti,ab OR ‘mean thrombocyte volume’:ti,ab OR ‘platelet distribution width’:ti,ab OR ‘thrombocyte distribution width’:ti,ab OR ‘platelet large cell ratio’:ti,ab OR ‘thrombocyte large cell ratio’:ti,ab OR ‘light transmission aggregometry’:ti,ab OR ‘whole blood aggregometry’:ti,ab OR ‘platelet degranulation’:ti,ab OR ‘thrombocyte degranulation’:ti,ab OR ‘platelet nucleotides’:ti,ab OR ‘thrombocyte nucleotides’:ti,ab OR ‘high performance liquid chromatography’:ti,ab OR ‘flow cytometry’:ti,ab OR ‘flow cytometric’:ti,ab OR ‘Thromboxane A_2’:ti,ab OR ‘P-selectin’:ti,ab OR ‘platelet function analyzer’:ti,ab OR ‘thrombocyte function analyzer’:ti,ab OR ‘cone and platelet analyzer’:ti,ab OR ‘cone and thrombocyte analyzer’:ti,ab OR verifynow:ti,ab OR ‘immature platelet’:ti,ab OR ‘immature thrombocyte:ti,ab OR ‘immature platelets’:ti,ab OR ‘immature thrombocytes:ti,ab OR ‘reticulated platelet’:ti,ab OR ‘reticulated thrombocyte:ti,ab OR ‘reticulated platelets’:ti,ab OR ‘reticulated thrombocytes:ti,ab OR ‘D-dimer’:ti,ab OR fibrin*:ti,ab OR plasmin:ti,ab)
AND
(‘Thrombopoietin Receptor Agonist’:ti,ab OR ‘Thrombopoietin Receptor Agonists’:ti,ab OR ‘TPO-receptor agonists’:ti,ab OR ‘TPO-receptor agonist’:ti,ab OR ‘TPO-RA’:ti,ab OR ‘TPO-A’:ti,ab OR Romiplostim:ti,ab OR nplate:ti,ab OR Eltrombopag:ti,ab OR revolade:ti,ab OR ‘thrombopoietin receptors’:ti,ab OR ‘thrombopoietin receptor’:ti,ab OR ‘CD110 antigens’:ti,ab OR ‘MPL ligand receptor’:ti,ab OR ‘TPO mimetics’:ti,ab OR ‘TPO mimetic’:ti,ab OR ‘thrombopoietin mimetics’:ti,ab OR ‘thrombopoietin mimetic’:ti,ab OR ‘thrombopoietin receptor mimetics’:ti,ab OR ‘thrombopoietin receptor mimetic’:ti,ab))

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.blre.2020.100774.

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