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Abstract:

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with an incompletely understood pathophysiology, but includes platelet-clearance in the spleen and liver via T-cells and/or platelet-autoantibodies. Strikingly, thrombopoietin (TPO) levels remain low in ITP. Platelet-glycoprotein (GP)Ib α has been described to be required for hepatic TPO generation, however, the role of GPIb-antibodies in relation to platelet hepatic sequestration and TPO-levels, with consideration of platelet counts, remains to be elucidated. Therefore, we performed a study in which we included 53 chronic and non-splenectomized ITP patients for which we conducted indium labeled autologous platelet scintigraphy, measured platelet-antibody profiles and TPO-levels. Upon stratification towards the severity of thrombocytopenia, no negative association was observed between GPIb/IX-antibodies and TPO levels, suggesting that GPIb/IX-antibodies do not inhibit or block TPO levels. Surprisingly, we observed a positive association between GPIb/IX-antibody levels and TPO levels, and GPIb/IX-antibodies and platelet hepatic sequestration, in patients with severe thrombocytopenia, but not in patients with mild or moderate thrombocytopenia. In addition, platelet hepatic sequestration and TPO levels were positively associated. This collectively indicates that GPIb/IX-antibodies may be associated with an increased platelet hepatic sequestration and elevated TPO levels in severe thrombocytopenic ITP patients, however, further research is warranted to elucidate the pathophysiological mechanisms.

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with an incompletely understood pathophysiology, but includes platelet-clearance in the spleen and liver via T-cells and/or platelet-autoantibodies. Strikingly, thrombopoietin (TPO) levels remain low in ITP. Platelet-glycoprotein (GP)Ib α has been described to be required for hepatic TPO generation, however, the role of GPIb-antibodies in relation to platelet hepatic sequestration and TPO-levels, with consideration of platelet counts, remains to be elucidated. Therefore, we performed a study in which we included 53 chronic and non-splenectomized ITP patients for which we conducted indium labeled autologous platelet scintigraphy, measured platelet-antibody profiles and TPO-levels. Upon stratification towards the severity of thrombocytopenia, no negative association was observed between GPIb/IX-antibodies and TPO levels, suggesting that GPIb/IX-antibodies do not inhibit or block TPO levels. Surprisingly, we observed a positive association between GPIb/IX-antibody levels and TPO levels, and GPIb/IX-antibodies and platelet hepatic sequestration, in patients with severe thrombocytopenia, but not in patients with mild or moderate thrombocytopenia. In addition, platelet hepatic sequestration and TPO levels were positively associated. This collectively indicates that GPIb/IX-antibodies may be associated with an increased platelet hepatic sequestration and elevated TPO levels in severe thrombocytopenic ITP patients, however, further research is warranted to elucidate the pathophysiological mechanisms.

Key Points

- GPIb/IX-antibodies do not appear to inhibit or block TPO production in ITP patients when stratified towards the degree of thrombocytopenia.
- GPIb/IX-antibodies may be associated with higher TPO and increased platelet hepatic sequestration under severe thrombocytopenic conditions.

49 Introduction

50 Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by low platelet
51 counts ($<100 \times 10^9 / L$).¹ The pathophysiological pathways of platelet clearance in ITP are not yet fully
52 unraveled, but involve T cells and/or platelet autoantibodies.² One of the most investigated
53 pathways of platelet clearance include the effects of autoantibodies directed against glycoprotein
54 (GP) complexes.³ Autoantibody binding to GP complexes, present on the platelet membrane, can
55 lead to liver and/or spleen sequestration, phagocytosis and subsequently to thrombocytopenia.² This
56 predominantly occurs via antibody-Fc mediated recognition by Fc γ -receptors on macrophages,
57 resulting in phagocytosis in spleen and/or liver.⁴ There is, however, evidence that Fc-independent
58 mechanisms of ITP also exist, leading to platelet hepatic sequestration.⁵

59 Thrombopoietin (TPO) regulates platelet production via interaction with myeloproliferative
60 leukemia protein receptor (Mpl; CD110) which is present on megakaryocytes and circulating
61 platelets.⁶ TPO plasma levels are mainly derived from the active and continuous TPO production by
62 the liver and to a lesser extent by the spleen, kidney and bone marrow.⁶ In addition, TPO production
63 is induced by the binding of desialylated aged platelets through interaction with the hepatic Ashwell-
64 Morrell receptor (AMR).⁶ Furthermore, it has been shown that certain GPIb α -antibodies trigger
65 platelet desialylation, a process that additionally increases the clearance of these platelets via the
66 hepatic AMR.⁷ In ITP patients, remarkably, TPO levels remain relatively low. GPIb α , independently of
67 platelet desialylation, was demonstrated to be required for hepatic TPO generation in mice.⁸ GPIb α -
68 α mice showed lower TPO levels compared to wildtype mice, and in agreement, patients with
69 Bernard-Soulier Syndrome (BSS), who lack the GPIb-IX-V complex, have low TPO levels with low to
70 moderate platelet counts.⁸ In that respect, it was suggested that GPIb/IX-antibodies may interfere
71 with hepatic TPO production in ITP, possibly explaining the relatively low TPO levels in patients with
72 ITP.⁸ A study by Porcelijn et al, however, did not find an association between GPIb/IX-antibodies and
73 TPO levels in a large cohort of 3490 patients suffering from ITP.⁹ This study did not incorporate data
74 on platelet counts of these patients. The latter could be of importance because in healthy subjects it

is well known that, apart from the via AMR-binding induced TPO production, the total platelet mass negatively influences the unbound and measurable TPO levels.¹⁰ As low platelet counts in ITP patients do not intriguingly trigger high TPO levels, we aimed to shed light on the interplay between GPIb/IX-platelet antibodies, the site of platelet sequestration, and TPO levels, with consideration of platelet counts in a cohort of chronic and non-splenectomized ITP patients.

Methods

In this study we investigated both the association between 1) GPIb/IX-antibodies and the site of platelet sequestration by indium labeled autologous platelet scintigraphy and 2) GPIb/IX antibodies and TPO levels in a cohort of 53 chronic and non-splenectomized ITP patients.¹¹ The included patients had a clinical indication for a scintigraphy scan as indicated by the treating hematologist (indication for a second/third line therapy with splenectomy as one of the therapeutic options), and had a mean age at diagnosis of 36 (SD $\pm$ 18) years. Importantly, we stratified these patients by the degree of thrombocytopenia: mild (platelet counts $>50 \times 10^9 / L$) and moderate/severe ($<50 \times 10^9 / L$). An additional sensitivity analysis was performed for severe thrombocytopenia (platelet count $<25 \times 10^9 / L$). Antibody levels were measured using direct (antibody bound directly on patient platelets) and indirect (antibody binding on donor platelets incubated with serum from the patient) Monoclonal Antibody-specific Immobilization of Platelet Antigen (MAIPA), with a cut-off of 0.130 OD (optical density).¹² TPO levels were measured as described by Folman et al. with a normal range in healthy subjects of 4-32 A.U./ml.¹³ All patients underwent an Indium-111 labeled autologous platelet sequestration scintigraphy; the sequestration outcome was used as a continuous variable ranging 0 to 100% sequestration in liver. In the clinical setting the outcome is categorized in splenic, mixed and platelet hepatic sequestration pattern based on the splenic to liver ratio (S:L ratio).¹⁴ Associations between TPO levels and anti-GP antibody levels were primarily tested using linear regression models, and multivariable models included platelet counts as a confounder. Differences were considered

statistically significant at $p < 0.05$. The study was approved by the Dutch Medical Ethical Review Board, which was conducted in accordance with the Declaration of Helsinki.

Results and discussion

Anti-platelet antibodies were measured in 53 patients. 29 patients had mild thrombocytopenia (platelet count $>50 \times 10^9/L$) and 24 patients had moderate to severe thrombocytopenia (platelet count $<50 \times 10^9/L$). Of the latter group 6 patients had severe thrombocytopenia (plt $<25 \times 10^9/L$). GPIb/IX-antibody OD values were found to be above the detection threshold in 13 of these patients (direct MAIPA, and 11 patients using the indirect MAIPA), of which 9 patients suffered from mild and 4 patients from moderate/severe thrombocytopenia. The presence of other platelet-antibodies, using direct and indirect MAIPA, in these 13 patients is depicted in Table S1. Upon stratification towards the severity of thrombocytopenia, no negative association was observed between GPIb/IX-antibodies (direct and indirect MAIPA) and TPO levels (Table 1). This suggests that GPIb/IX-antibodies levels do not inhibit or block the regulation of TPO levels in ITP patients, indicating that other factors and pathways are responsible for the relatively low levels of TPO in patients with ITP. Surprisingly, a significant positive association was observed between GPIb/IX-antibody levels (direct MAIPA) and TPO levels, and GPIb/IX-antibodies (direct and indirect MAIPA) and platelet hepatic sequestration, in patients with severe thrombocytopenia, but not in patients with mild (direct and indirect MAIPA) or moderate thrombocytopenia (direct MAIPA) (Table 1). In addition, platelet hepatic sequestration and TPO levels were positively associated (Table 1). Collectively, this may suggest that GPIb/IX-antibodies could be associated with an increased platelet hepatic sequestration and elevated TPO levels in severe thrombocytopenic ITP patients (by a direct and/or 2 sequentially indirect pathways as indicated in Figure 1). In contrast, we found no significant associations between GPIIb/IIIa- or GPV-antibodies (direct and indirect MAIPA) and platelet hepatic sequestration or TPO levels in ITP patients with severe thrombocytopenia (Table S2). This indicates that the previously mentioned associations may be specific for GPIb/IX-antibodies under severe thrombocytopenic conditions.

In this study we find that GPIb/IX-antibodies do not appear to inhibit or block TPO production in ITP patients when stratified towards the degree of thrombocytopenia. In addition, we find that GPIb/IX-antibodies may be associated with a stimulated TPO production through the liver, but only under severe thrombocytopenic conditions. In this setting of increased clearance, there may be an additional contribution of specific GPIb/IX-antibodies directed against the ligand-binding domain (LBD) in a Fc-independent manner.¹⁵ This may result in mechanomolecular signaling¹⁵ leading to increased platelet clearance via the liver which may stimulate an increase in TPO levels through a feedback mechanism. Although GPIb α was suggested to be required for hepatic TPO generation independently of platelet desialylation in mice⁸, our human data suggests that under severe thrombocytopenic conditions there may be an additional contribution of GPIb/IX-induced platelet-desialylation and consequently increased hepatic clearance via the AMR, resulting in increased TPO levels. For the first time, our results support a possible link between GPIb/IX-antibodies, platelet hepatic sequestration and increased TPO levels in ITP patients with platelet counts below $25 \times 10^9/L$. Although suggestive, from observational data we cannot conclude on causality. Possible bias may be introduced through indication of scanning relapse and refractory patients. Furthermore, it has been described that different types of GPIb-antibodies can have divergent functions¹⁶, however, in the current study we were unable to differentiate the different types of GPIb-antibodies. Therefore, the mechanistic pathways should be further experimentally explored in vitro and in vivo animal models. Importantly, these results will also need to be validated in larger cohorts. The current findings add to the largely unknown pathophysiology of ITP and could ultimately be applied for the development of new individualized treatment options for patients suffering from ITP.

147 **Authorship contributions:**

148 RK, SNA, VSN and MS conceived and designed the study; SNA and RK performed the study and
149 analyzed data, SNA and RK wrote the manuscript; and VSN, LP, TN, JJZ, MdH and MS critically
150 reviewed and edited the manuscript. All authors interpreted the data and approved the final draft.

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152 **Disclosure of Conflicts of Interest:**

153 All authors declare no conflict of interest.

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Tables

Table 1: Regression models for the association between anti GPIb/IX antibodies, TPO levels and platelet hepatic sequestration, under thrombocytopenic conditions, in a cohort of chronic and non-splenectomized ITP patients. Statistical significance: $p < 0.05$ (*).

	Linear regression ITP patients with platelet count $>50 \times 10^9/L$ (n=29) β (95%-CI), p value	Linear regression ITP patients with platelet count $<50 \times 10^9/L$ (n=24) β (95%-CI), p value	Sensitivity analysis: Linear regression ITP patients with platelet count $<25 \times 10^9/L$ (n=6) β (95%-CI), p value
Association 1: Anti-GPIb/IX & TPO-level	Direct MAIPA: 0.000 (-0.002 – 0.002), $p=0.91$ Indirect MAIPA: 0.000 (-0.0005 – 0.004); $p=0.88$	Direct MAIPA: -0.045 (-0.180 – 0.089); $p=0.489$ Indirect MAIPA: 0.051 (0.004 – 0.097) $p=0.03^*$	Direct MAIPA: 0.092 (0.012-0.172), $p=0.03^*$ Indirect MAIPA: 0.116 (-0.131 – 0.364) $p=0.18$
Association 2: Anti-GPIb/IX & platelet hepatic sequestration	Direct MAIPA: -0.001 (-0.004 – 0.002), $p=0.63$ Indirect MAIPA: -0.002 (-0.004 – 0.001) $p=0.22$	Direct MAIPA: 0.025 (-0.029 – 0.079), $p=0.35$ Indirect MAIPA: 0.020 (0.006 – 0.035) $p=0.008^*$	Direct MAIPA: 0.026 (0.006 – 0.045), $p=0.02^*$ Indirect MAIPA: 0.045 (0.003 – 0.088) $p=0.04^*$
Association 3: TPO-level & platelet hepatic sequestration	-0.076 (-0.793 – 0.641) $p=0.83$	0.262 (0.124 – 0.400) $p=0.001^*$	0.228 (CI 0.126 – 0.331), $p=0.002^*$

217 **Figure legend**

218 Interplay between GPIb/IX antibodies, platelet hepatic sequestration and TPO levels in chronic and
219 non-splenectomized ITP patients with severe thrombocytopenia.

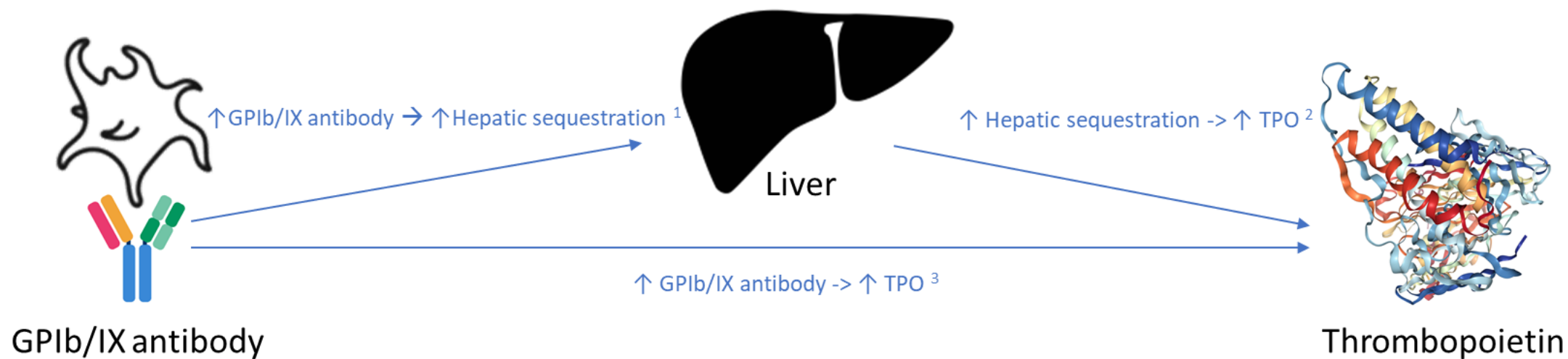
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Figure 1

Figure 1



Footnote:

- ¹ Association found between GPIb/IX antibody levels and increased hepatic sequestration of platelets.
- ² Association found between hepatic sequestration and increased TPO levels.
- ³ Direct association between GPIb/IX antibody levels and increased TPO levels.